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Protaetia brevitarsis Extract Attenuates RANKL-Induced Osteoclastogenesis by Inhibiting the JNK/NF- κ B/PLC γ 2 Signaling Pathway

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Abstract: *Protaetia brevitarsis* (PB)-derived bioactive substances have been used as food and medicine in many Asian countries because of their antioxidant, antidiabetic, anti-cancer, and hepatoprotective properties. However, the effect of PB extracts (PBE) on osteoclast differentiation is unclear. In this study, we investigated the effect of PBE on RANKL-induced osteoclastogenesis in mouse bone marrow-derived macrophages (BMMs). To investigate the cytotoxicity of PBE, the viability of BMMs was confirmed via MTT assay. Tartrate-resistant acid phosphatase (TRAP) staining and pit assays were performed to confirm the inhibitory effect of PBE on osteoclast differentiation and bone resorption. The expression levels of osteoclast differentiation-related genes and proteins were evaluated using quantitative real-time PCR and Western blotting. PBE attenuated osteoclastogenesis in BMMs in TRAP and pit assays without cytotoxicity. The expression levels of osteoclast marker genes and proteins induced by RANKL were decreased after PBE treatment. PBE suppressed osteoclastogenesis by inhibiting the RANKL-induced activated JNK/NF- κ B/PLC γ 2 signaling pathway and the expression of NFATc1 and c-Fos. Collectively, these results suggest that PBE could be a potential therapeutic strategy or functional product for osteoclast-related bone disease.

Keywords: *Protaetia brevitarsis*; edible insect; osteoclast differentiation; JNK; NF- κ B; PLC γ 2



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1. Introduction

Bone remodeling requires a balance between bone resorption by osteoclasts and bone formation by osteoblasts [1,2]. Excessive osteoclast differentiation and activation are associated with the development of bone diseases with low bone density and bone destruction, such as postmenopausal osteoporosis and rheumatoid arthritis, due to an imbalance between these cells [3–5]. Therefore, strategies to develop optimal treatments for these diseases should focus on the activation of osteoblasts or inhibition of osteoclasts.

Osteoclasts, known as cells with bone resorption function, are bone-specific multinucleated cells generated by proliferation, differentiation, and fusion of a monocyte/macrophage precursor lineage [1,6]. Osteoclast differentiation is controlled by various factors, including growth factors, cytokines, and hormones. An imbalance in these factors disrupts bone homeostasis and leads to bone pathogenesis [5–8].

Macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL), produced by osteoblasts, play important roles in osteoclast differentiation. M-CSF and RANKL promote the proliferation and differentiation of osteoclast precursor cells, respectively [9,10]. RANKL activates TNF receptor-associated factor 6 (TRAF6) by binding to RANK expressed in osteoclasts, which further activates downstream signaling pathways, including phosphoinositide 3-kinase/serine-threonine protein kinase (PI3K/Akt), mitogen-activated protein kinases (MAPKs), and nuclear factor- κ B (NF- κ B). Eventually, it induces the activation of nuclear factors of activated T cells c1 (NFATc1) and c-Fos, which are key transcription factors involved in osteoclastogenic gene expression [11–13].

Insects have been regarded as functional foods and pharmaceutical resources [14]. Compounds derived from *Bruchidius dorsalis* have antioxidant properties [15]. Bioactive compounds, such as sesquiterpenoids and lactams, found in the edible insect *Aspongopus chinensis* Dallas, were effective against pain, dyspepsia, and kidney diseases [16]. *Scolopendra subspinipes mutilans*-derived small-molecule alkaloids exhibit anticoagulant activity [17]. Additionally, insects are used in traditional medicines to treat various conditions, including anemia, inflammation, hypertension, and asthma [18]. *Protaetia brevitarsis* (PB), belonging to the Cetoniidae family, is a white-spotted flower beetle that is widely distributed in East Asia (China, Japan, Korea, and Taiwan) and Europe [19]. Substances extracted from PB exhibit various bioactivities and are used for medicinal purposes in many Asian countries. In our previous study, PB protected islets from cytotoxicity in alloxan-treated pancreatic islets and db/db mice and exhibited antidiabetic efficacy by lowering plasma glucose levels and improving glucose tolerance, blood lipid parameters, and islet structure [20]. Fatty acids (palmitic acid, (Z)-9-octadecenoic acid and octadecenoic acid) extracted from the larvae of PB suggested anticancer effects by inducing apoptosis through potent cytotoxicity, DNA fragmentation, and caspase-3 activation against colon carcinoma cells [21]. A previous study reported that extracts from PB larvae fed a fermented aloe vera mixed diet against CCl₄-induced hepatic injury in Sprague-Dawley rats showed potent hepatoprotective effects by increasing glutathione levels and inhibiting lipid peroxidation [22]. According to a recent report, oral administration of the PB larval extract to radiation-induced testicular injury male mice demonstrated testicular protective effects through reduction of germ cell apoptosis, improvement of sperm cell morphology and motility, and preservation of sperm count. In addition, it suppressed reactive oxygen species generation and exhibited antioxidative effects [23]. A recent study reported that PB extracts regulated bone remodeling in mice with OVX-induced osteoporosis and decreased RANKL-stimulated osteoclastogenesis by inhibiting p38 MAPK activation and NF- κ B phosphorylation [24]. Also, PB-derived proteins showed antioxidant properties and promoted osteogenic differentiation in human bone marrow-derived mesenchymal stem cells [25]. Although PB exhibits various biological activities, its effect on bone remodeling, especially osteoclast differentiation, is not clearly known. Therefore, this study investigated the anti-osteoclastogenic effects of PB, which may provide a potential therapeutic strategy for bone diseases.

2. Materials and Methods

2.1. Samples and Reagents

Protaetia brevitarsis (PB) larvae were provided by Congmaeul. Ltd. (Imsil, Jeonbuk, Republic of Korea). The preparation and compositional analysis of PB extracts (PBE) were performed as previously described [20]. Briefly, the larvae were collected, washed with distilled water, and then lyophilized at -20°C . The sample was then ground using an experimental pulverizer, and a powder was produced through a 30-mesh sieve. The powder of PB larvae was stored in an airtight container at -70°C until the experiment. In addition, a gas chromatograph (GC 7890 B, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a flame ionization detector was used for composition analysis of the PBE. Recombinant murine soluble RANKL and M-CSF were purchased from PeproTech (Rocky Hill, NJ, USA). JNK inhibitor SP600125 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

Four specific pathogen-free 6- to 8-week-old male C57BL/6J mice (21–22 g) were purchased from Samtako Bio Korea, Inc. (Osan, Gyeonggi-do, Republic of Korea). Four animals were housed in a cage with 12-h light/dark cycles at 22 ± 2 °C and 50–60% humidity for 3 weeks. During the experimental period, general solid food (Purina Lab Rodent Chow #38057, Purina Co., Seoul, Republic of Korea) and filtered drinking water were supplied and changed every day for free intake. The euthanasia was performed under inhalation anesthesia (isoflurane, USP). All experiments were performed with bone marrow-derived macrophages (BMMs) isolated from C57BL/6J mice. All animal experiments were approved by the Animal Care and Use Committee of Wonkwang University (Approval No. WKU21-55).

2.3. Isolation and Differentiation of BMMs

Isolation and differentiation of BMMs were performed as described in a previous study [26]. Briefly, the cells were harvested by washing the bone marrow spaces of the mouse tibia and femur with phosphate-buffered saline (PBS), seeded in culture dishes, and cultured for 1 day. Thereafter, non-adherent cells were collected and incubated for 3 days in α -minimal essential medium (Gibco, Rockford, IL, USA) with 10% fetal bovine serum (Gibco) containing M-CSF (30 ng/mL). Then, the adherent cells were used as osteoclast precursors. To generate osteoclasts, BMMs were incubated with M-CSF (30 ng/mL) and RANKL (100 ng/mL) at 37 °C for 4 days.

2.4. Cell Viability Assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich) assay. BMMs (1×10^4 cells/well) were either seeded in 96-well culture plates with varying concentrations of PBE (0, 10, 25, 50, and 100 μ g/mL) at 37 °C for 1 day or were treated with 50 μ g/mL PBE under M-CSF treatment (30 ng/mL) for 4 days at 37 °C. After treatment, MTT solution (0.5 mg/mL in PBS) was added to each well for 30 min at 37 °C. Following incubation, formazan crystals were dissolved in DMSO (100 μ L/well), and the optical density at 570 nm was measured using a microplate reader (Bio-Rad, Richmond, CA, USA). The MTT assays are performed at least three independent experiments ($n = 3$).

2.5. Tartrate-Resistant Acid Phosphatase (TRAP) Staining and Activity

BMMs were treated with RANKL (100 ng/mL) and M-CSF (30 ng/mL) in the presence of various concentrations of PBE (0, 10, 25, 50, and 100 μ g/mL) for 4 days. They were then fixed in 3.8% paraformaldehyde and stained for 1 h using a TRAP Kit (Sigma-Aldrich). The surface area of TRAP-positive polynucleated (nuclei > 3) cells was measured using the ImageJ software V 1.8.0 (NIH, Bethesda, MD, USA). To determine total TRAP activity, the collected cells were washed, lysed, and centrifuged. *p*-Nitrophenylphosphate (Sigma-Aldrich) was used as a substrate for TRAP, and optical density was measured at 405 nm using a microplate reader (Bio-Rad). The experiments were performed in at least three independent experiments ($n = 3$).

2.6. Pit Assay

BMMs were seeded in an osteo assay 48-well plate (COSMO BIO, Carlsbad, CA, USA) at a density of 3×10^4 cells per well, and the cells were treated with RANKL (100 ng/mL) and M-CSF (30 ng/mL) in the presence of PBE for 4 days. The cells were completely removed, washed with distilled water, and dried at room temperature. The bone resorption area was observed using a light microscope (magnification, $\times 50$) and calculated using the ImageJ software V 1.8.0 (NIH). The experiments were performed in at least three independent experiments ($n = 3$).

2.7. RNA Extraction and Quantitative Real-Time PCR (qRT-PCR) Assay

BMMs treated with or without PBE (50 µg/mL) were cultured in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 4 days. The cultured cells were washed with cold PBS, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) on the indicated days. Reverse transcription of 1 µg of total RNA was performed using a PrimeScript™ RT reagent kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer's instructions. The ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Green polymerase chain reaction Master Mix (Applied Biosystems) were used for qRT-PCR. For CT value analysis, the relative gene expression level was calculated using the $2^{-\Delta\Delta C_t}$ method [27]. The results were normalized to the expression of the GAPDH reference housekeeping gene. The experiments were performed in at least three independent experiments ($n = 3$). The primer sequences used for qRT-PCR are listed in Table 1.

Table 1. Primer sequences used for qRT-PCR.

Gene Names	Primer Sequences
Mouse <i>ACP5</i> (TRAP)	forward: 5'-CTGGAGTGCACGATGCCAGCGACA-3' reverse: 5'-TCCGTGCTCGGCGATGGACCAGA-3'
<i>Oscar</i>	forward: 5'-GGGGTAACGGATCAGCTCCCCAGA-3' reverse: 5'-CCAAGGAGCCAGAACGTCGAAACT-3'
<i>CTSK</i>	forward: 5'-ACGGAGGCATTGACTCTGAAGATG-3' reverse: 5'-GTTGTTCTTATTCCGAGCCAAGAG-3'
<i>TM7SF4</i> (DC-STAMP)	forward: 5'-TGGAAGTTCACCTTGAAACTACGTG-3' reverse: 5'-CTCGGTTTCCCGTCAGCCTCTCTC-3'
<i>ATP6V0D2</i>	forward: 5'-TCAGATCTCTTCAAGGCTGTGCTG-3' reverse: 5'-GTGCCAAATGAGTTCAGAGTGATG-3'
<i>NFATc1</i>	forward: 5'-CTCGAAAGACAGCACTGGAGCAT-3' reverse: 5'-CGGCTGCCTTCCGTCTCATAG-3';
<i>GAPDH</i>	forward: 5'-TGCCAGCCTCGTCCCGTAGAC-3' reverse: 5'-CCTCACCCCATTTGATGTTAG-3'

TRAP, tartrate-resistant acid phosphatase; *Oscar*, osteoclast-associated immunoglobulin-like receptor; *CTSK*, cathepsin K; *DC-STAMP*, dendritic cell-specific transmembrane protein; *ATP6V0D2*, ATPase H⁺ transporting V0 subunit D2; *NFATc1*, nuclear factor of activated T cells 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

2.8. Western Blot Analysis

Briefly, lysates were obtained by lysing BMMs in RIPA lysis buffer (Pierce Biotechnology, Rockford, IL, USA). Protein concentrations were determined using the Bradford assay (Bio-Rad). Each protein sample was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (GE, Buckinghamshire, UK). Each membrane was blocked with 5% skimmed milk or 5% bovine serum albumin for 1 h, probed with primary antibodies, and incubated overnight at 4 °C. The membranes were washed with tris-buffered saline containing Tween 20 and treated with secondary antibodies containing horseradish peroxidase for 1 h. Protein levels were detected using a LAS-4000 image analyzer (FujiFilm, Tokyo, Japan). Primary antibodies against extracellular signal-regulated kinase (ERK), *p*-ERK, p38, *p*-p38, c-Jun-N-terminal kinase (JNK), *p*-JNK, p65, *p*-p65, IκBα, *p*-IκBα, *p*-PLCγ2, CREB, *p*-CREB, and c-Fos were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-PLCγ2 and anti-NFATc1 antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The anti-β-actin antibody was purchased from Sigma-Aldrich.

2.9. Statistical Analysis

Data are expressed as the mean $\pm$ standard deviation of results from at least three independent experiments. Statistical analyses were performed using ANOVA and Duncan's test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of PBE on BMM Viability

To confirm the cytotoxicity of PBE against BMMs, cells were incubated with various concentrations of PBE (0, 10, 25, 50, and 100 $\mu\text{g/mL}$) for 24 h and assessed using the MTT assay. When the PBE concentration was 50 $\mu\text{g/mL}$ or lower, there was no effect on the viability of BMMs (Figure 1A). In addition, cell viability was confirmed to be time-dependent at a PBE concentration of 50 $\mu\text{g/mL}$, and there was no significant difference in the viability of BMMs treated with PBE (Figure 1B). Based on these results, 50 $\mu\text{g/mL}$ of PBE was used for further experiments.

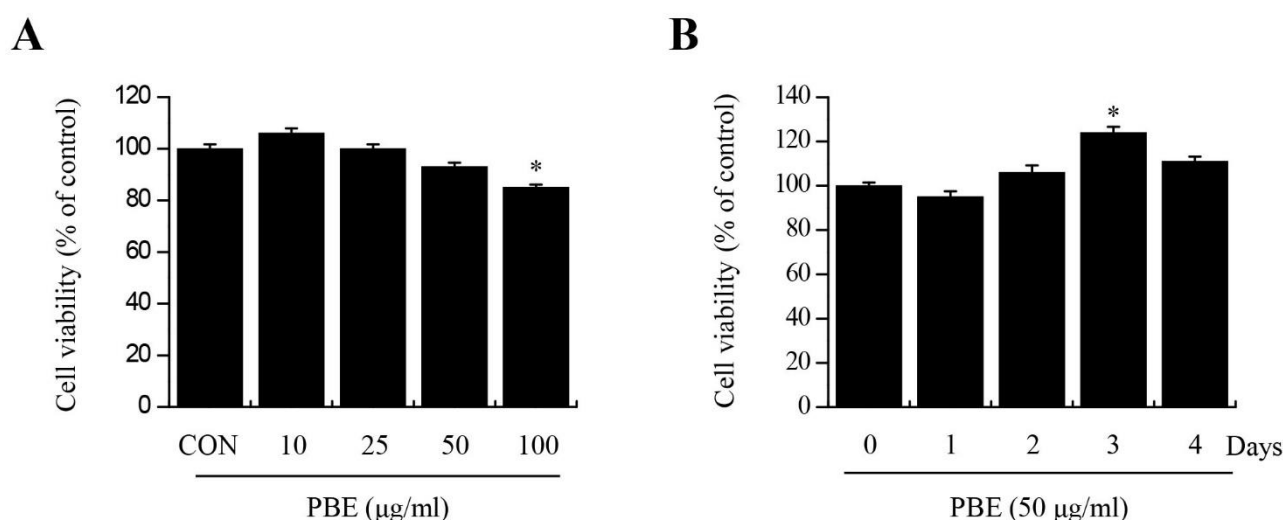


Figure 1. Effects of PBE on BMM viability. (A) BMMs (1×10^4 cells/well) were seeded in 96-well plates, treated with PBE (0, 10, 25, 50, and 100 $\mu\text{g/mL}$), and incubated at 37 $^{\circ}\text{C}$ with 5% CO_2 for 24 h. (B) BMMs were treated with PBE (50 $\mu\text{g/mL}$) and incubated for 0, 1, 2, 3, or 4 days. After incubation, cell viability was measured using the MTT assay. Bars labeled with different superscripts indicate significant differences (* $p < 0.05$ vs. control or vs. 0 days). The results are expressed as the mean $\pm$ standard deviation of at least three independent experiments ($n = 3$).

3.2. PBE Suppressed RANKL-Induced Osteoclast Differentiation and Bone Resorption

To evaluate the inhibitory effects of PBE on osteoclastogenesis, the cells were incubated with RANKL and PBE (0, 25, and 50 $\mu\text{g/mL}$) for 4 days. To confirm osteoclast differentiation, TRAP staining (Figure 2A), TRAP⁺ multinucleated cell (MNC) counting (Figure 2C) and TRAP activity assays (Figure 2D) were performed. In the RANKL-treated group, the formation of TRAP-positive MNCs was observed, in which cells aggregated, bundled, and increased the activation of TRAP, thereby showing that BMMs were fully differentiated into osteoclasts. However, multinucleated osteoclast differentiation and TRAP activity were significantly reduced by PBE in a dose-dependent manner. Osteoclasts have the ability to resorb mineralized matrix and form resorption pits. Therefore, we investigated whether the inhibition of osteoclast formation by PBE affects bone resorption. As shown in Figure 2B,E, RANKL induced bone resorption pits in osteoclasts, and PBE decreased the area of the bone resorption pits in a concentration-dependent manner. These results suggest that PBE inhibited osteoclastic differentiation and bone resorption.

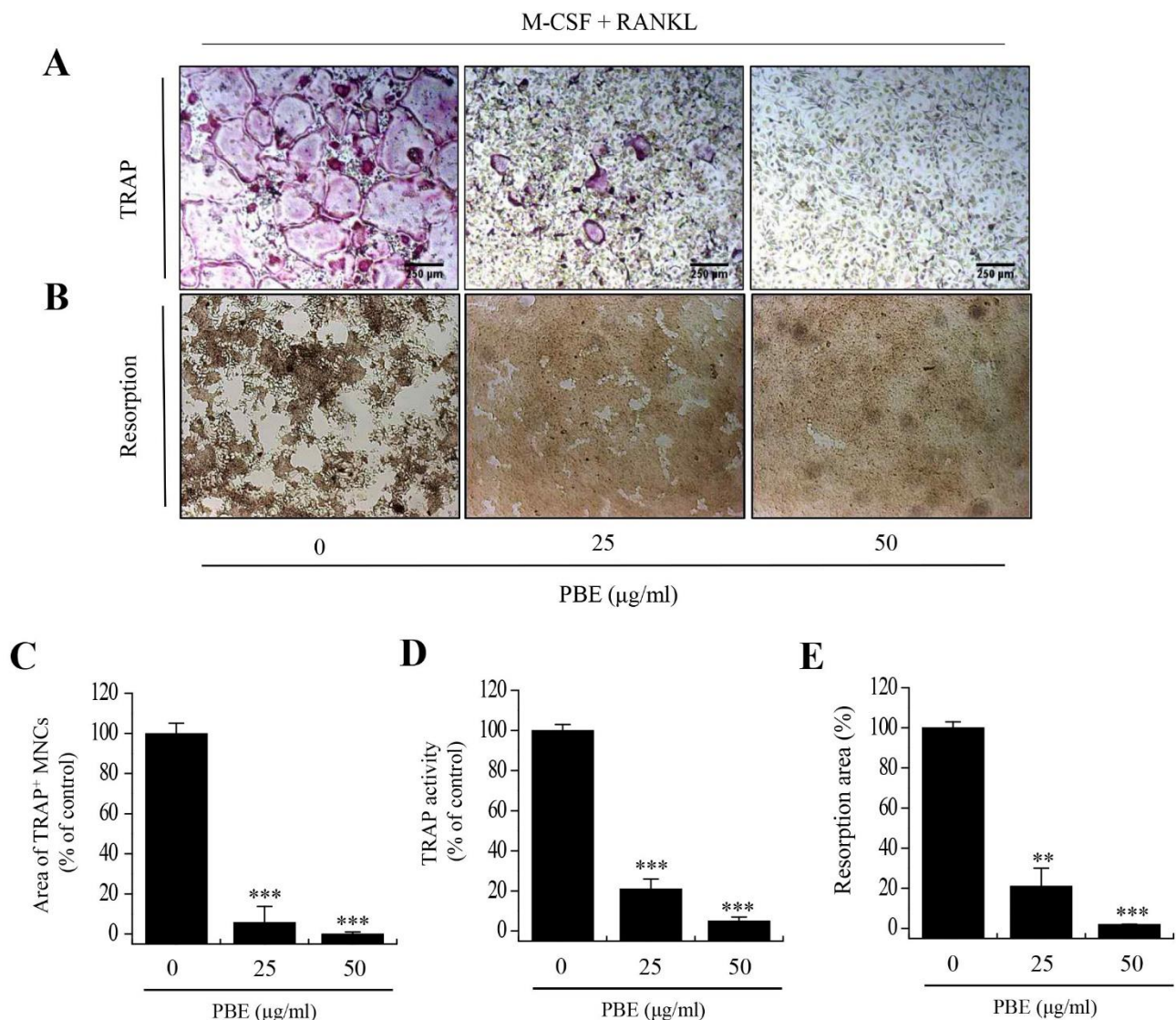


Figure 2. Effects of PBE on RANKL-induced osteoclast differentiation and bone resorption. BMMs were cultured for 4 days in the presence of RANKL (100 ng/mL) and M-CSF (30 ng/mL) with PBE (0, 25, and 50 µg/mL). **(A)** TRAP staining (upper panel) and **(B)** bone resorption (lower panel) were performed to visualize osteoclast differentiation. Scale bar, 250 µm. **(C)** TRAP-positive MNCs (TRAP⁺ MNCs; nuclei > 3) were counted as mature osteoclasts. **(D)** TRAP activity in BMMs was analyzed on day 4 according to the manufacturer's protocol. **(E)** Bone resorption areas were quantified using ImageJ software, and significant differences between the PBE and control groups are indicated (** $p < 0.005$; *** $p < 0.001$). Data are presented as the mean $\pm$ standard deviation and are representative of at least three experiments.

3.3. PBE Down-Regulated the Expression of Osteoclastogenesis-Related Genes

Based on the results of the inhibition of osteoclast differentiation by PBE, the expression of mRNA involved in osteoclast differentiation was evaluated by qRT-PCR. RANKL stimulation increased the mRNA expression of *Acp5* (TRAP), *Oscar*, *CTSK* (cathepsin K), *Tm7sf4* (dendritic cell-specific transmembrane protein, DC-STAMP), *Atp6v0d2*, and *Nfatc1* in BMMs. However, PBE treatment considerably decreased the mRNA expression of these genes (Figure 3). These results suggest that PBE may affect the signaling pathways in the early stages of osteoclastogenesis.

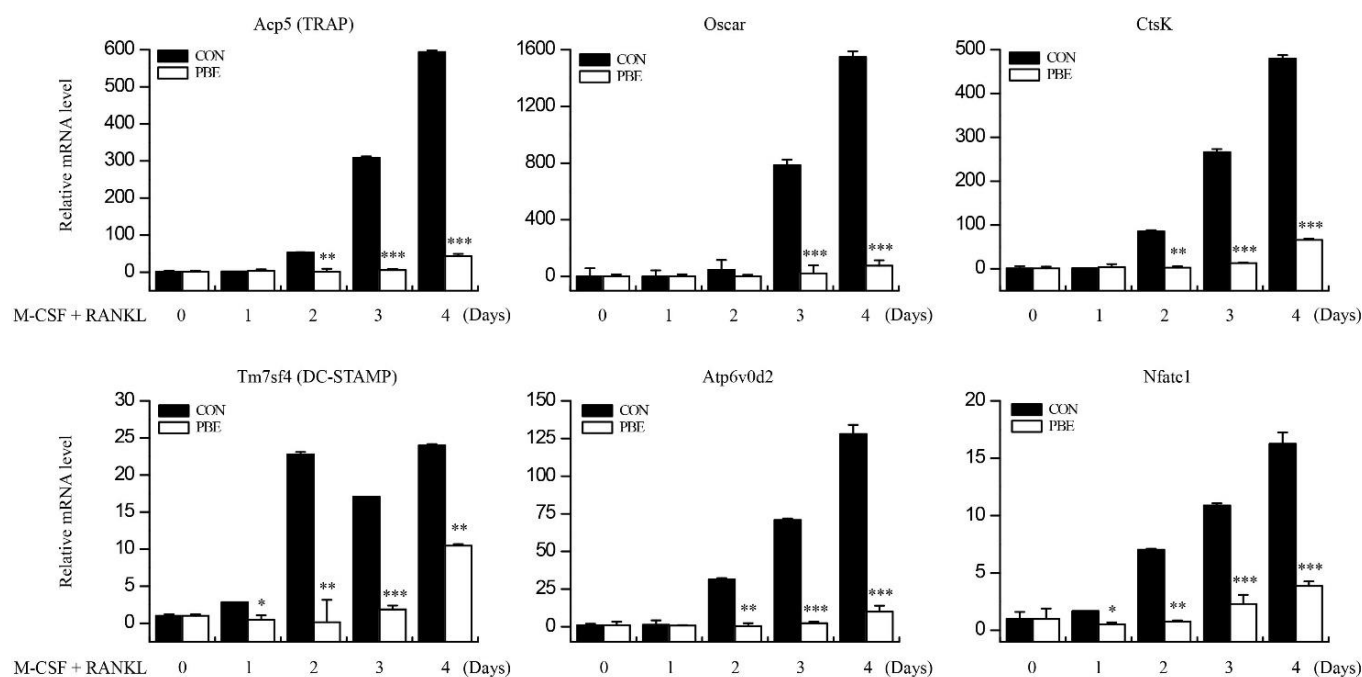


Figure 3. Effect of PBE on the expression of osteoclastogenesis-related genes. BMMs were cultured with RANKL (100 ng/mL) and M-CSF (30 ng/mL) in the absence or presence of PBE (50 µg/mL) for 0, 1, 2, 3, or 4 days. The expression of genes (Acp5 (TRAP), Oscar, CTSK (cathepsin K), Tm7sf4 (dendritic cell-specific transmembrane protein, DC-STAMP), Atp6v0d2, and NFATc1) involved in osteoclast differentiation was evaluated by qRT-PCR. Experiments were performed in triplicate, and the error bars represent the standard deviation. Significant differences between the PBE and control groups are indicated (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$).

3.4. PBE Inhibits MAPK and NF- κ B Activation in BMMs

M-CSF and RANKL induce the MAPK and NF- κ B signaling pathways as early signaling pathways during osteoclast proliferation and differentiation, as well as bone resorption. Therefore, we examined whether PBE could inhibit the activation of MAPK (ERK, p38, and JNK) and NF- κ B in BMMs by Western blot analysis. As shown in Figure 4A, PBE significantly suppressed the phosphorylation of JNK, which was increased by M-CSF and RANKL, but had no significant effect on ERK and p38. In addition, PBE inhibited RANKL-stimulated NF- κ B, p65, and I κ B α phosphorylation (Figure 5A). The expression of each protein was normalized, and the relative expression levels were analyzed (Figures 4B and 5B). These results show that PBE inhibited osteoclast differentiation by downregulating the activation of JNK and NF- κ B signaling pathways.

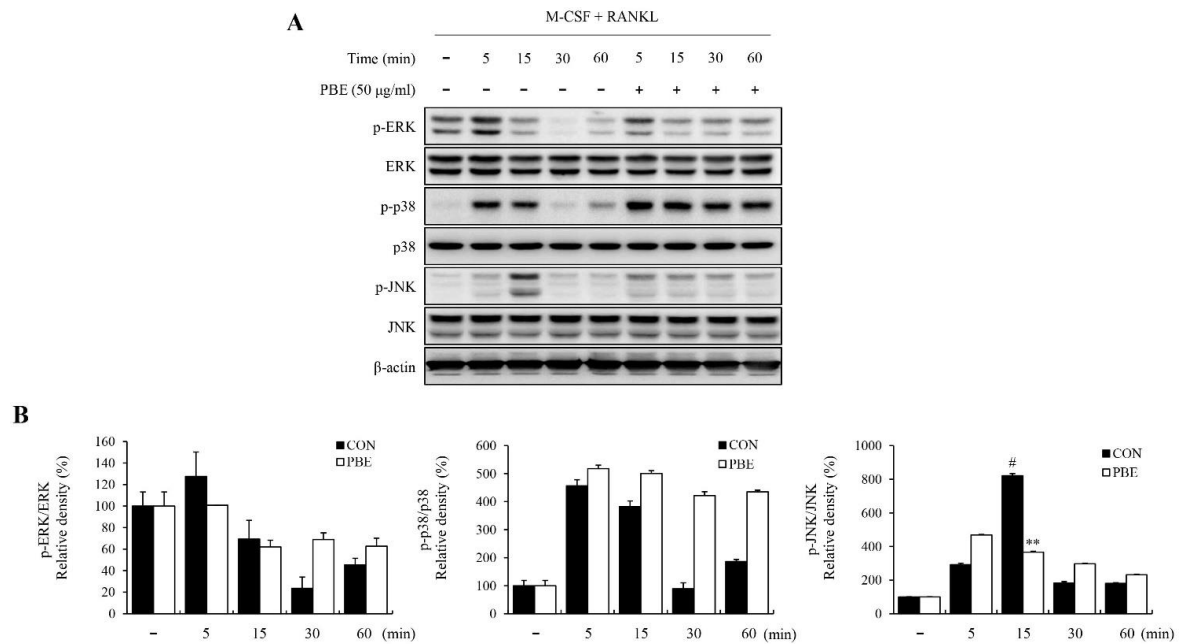


Figure 4. Effect of PBE on MAPK activation in BMMs. BMMs were cultured with RANKL (100 ng/mL) and M-CSF (30 ng/mL), with or without PBE (50 µg/mL), for 5, 15, 30, and 60 min. (A) Protein expression levels were analyzed by Western blotting using anti-ERK, *p*-ERK, p38, *p*-p38, JNK, and *p*-JNK antibodies. (B) Band intensities were quantified using ImageJ software. Bars labeled with different superscripts indicate significant differences (# $p < 0.05$ vs. RANKL-non treated group; ** $p < 0.005$ vs. control). Data are expressed as the mean $\pm$ SD of at least three independent experiments ($n = 3$).

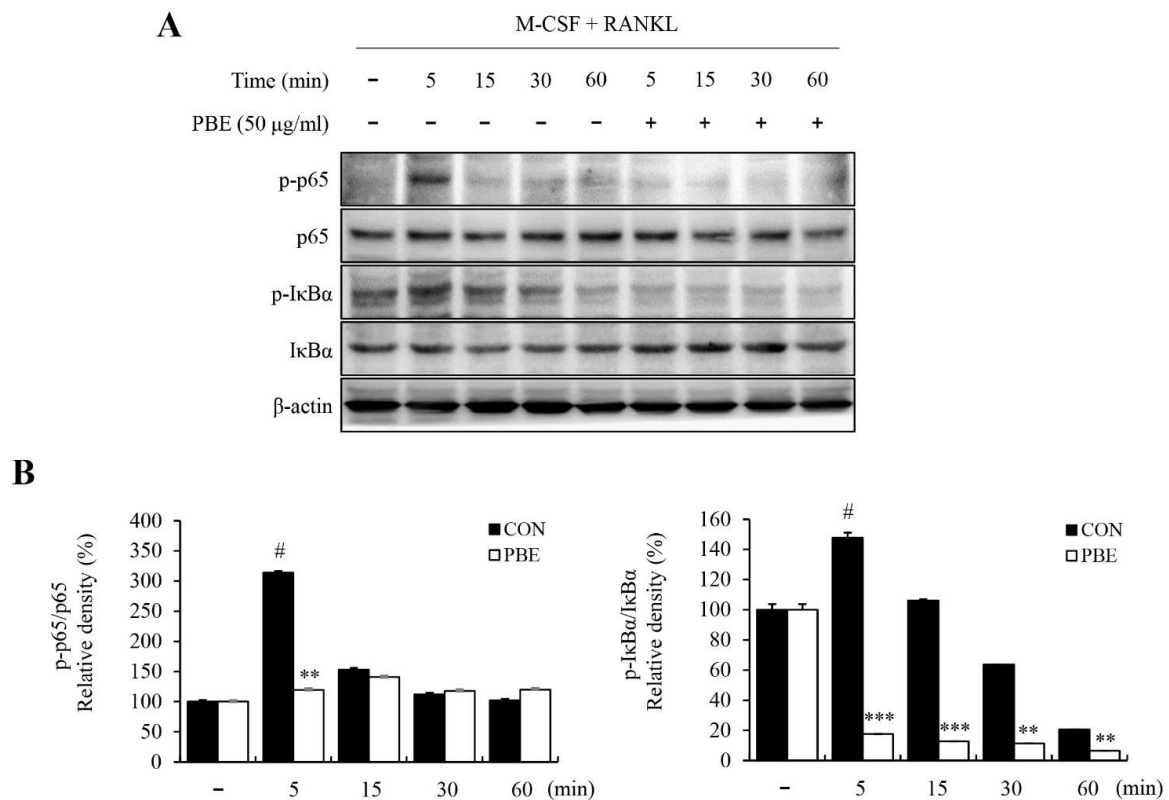


Figure 5. Effect of PBE on NF-κB activation in BMMs. BMMs were cultured with RANKL (100 ng/mL) and M-CSF (30 ng/mL), with or without PBE (50 µg/mL), for 5, 15, 30, and 60 min. (A) Protein expression

levels were analyzed by Western blotting using anti-NF- κ B p65, p-NF- κ B p65, I κ B α , and p-I κ B α antibodies. (B) Image J software was used to quantify the protein bands. Bars labeled with different superscripts indicate significant differences (# $p < 0.05$ vs. RANKL non-treated group; ** $p < 0.005$ vs. control; *** $p < 0.001$ vs. control). Data are presented as the mean $\pm$ SD of at least three independent experiments ($n = 3$).

3.5. PBE Regulates NFATc1 and c-Fos Expression by Inhibiting the JNK/NF- κ B/PLC γ 2 Signaling Pathway

M-CSF and RANKL-induced MAPK, NF- κ B, phospholipase C gamma 2 (PLC γ 2), and CREB activation are known to activate the osteoclast differentiation factors c-Fos and NFATc1. Therefore, we investigated whether PBE could affect the expression of PLC γ 2 and CREB, which are important signaling pathways for RANKL-induced osteoclast differentiation. Our results confirm that PBE significantly reduced the phosphorylation levels of PLC γ 2 and CREB during RANKL-induced osteoclast differentiation (Figure 6A,B). Next, we investigated whether PBE affected the protein expression levels of c-Fos and NFATc1, which are key transcription factors for RANKL-induced osteoclast differentiation. Stimulation with M-CSF and RANKL increased the protein expression levels of c-Fos and NFATc1, and these protein levels, which increased upon treatment with PBE, were significantly reduced (Figure 6C,D). Additionally, to confirm that the inhibitory effect of PBE on osteoclastogenesis is mediated through the JNK signaling pathway, we investigated the phosphorylation level of JNK by co-treating PBE with the JNK inhibitor SP600125. As a result, it was confirmed that the phosphorylation level of JNK increased by M-CSF and RANKL was decreased when treated with PBE, and the phosphorylated level of JNK was lowered more when co-treated with PBE and SP600125. To investigate whether c-Fos and NFATc1, known as the final steps in the signaling pathway of osteoclastogenesis, are regulated by the JNK signaling pathway, we simultaneously treated PBE and SP600125, and examined the protein expression levels of c-Fos and NFATc1. The protein expression levels of c-Fos and NFATc1 were increased by M-CSF and RANKL, but were reduced upon treatment with PBE. Furthermore, the protein expression levels of c-Fos and NFATc1 were more decreased when co-treated with PBE and SP600125 (Figure 7A). The expression of each protein was normalized, and the relative expression levels were analyzed (Figure 7B). Taken together, these results suggest that PBE mediated the inhibition of NFATc1 and c-Fos expression via various signaling pathways, such as the JNK/NF- κ B/PLC γ 2 signaling pathway, during osteoclast differentiation.

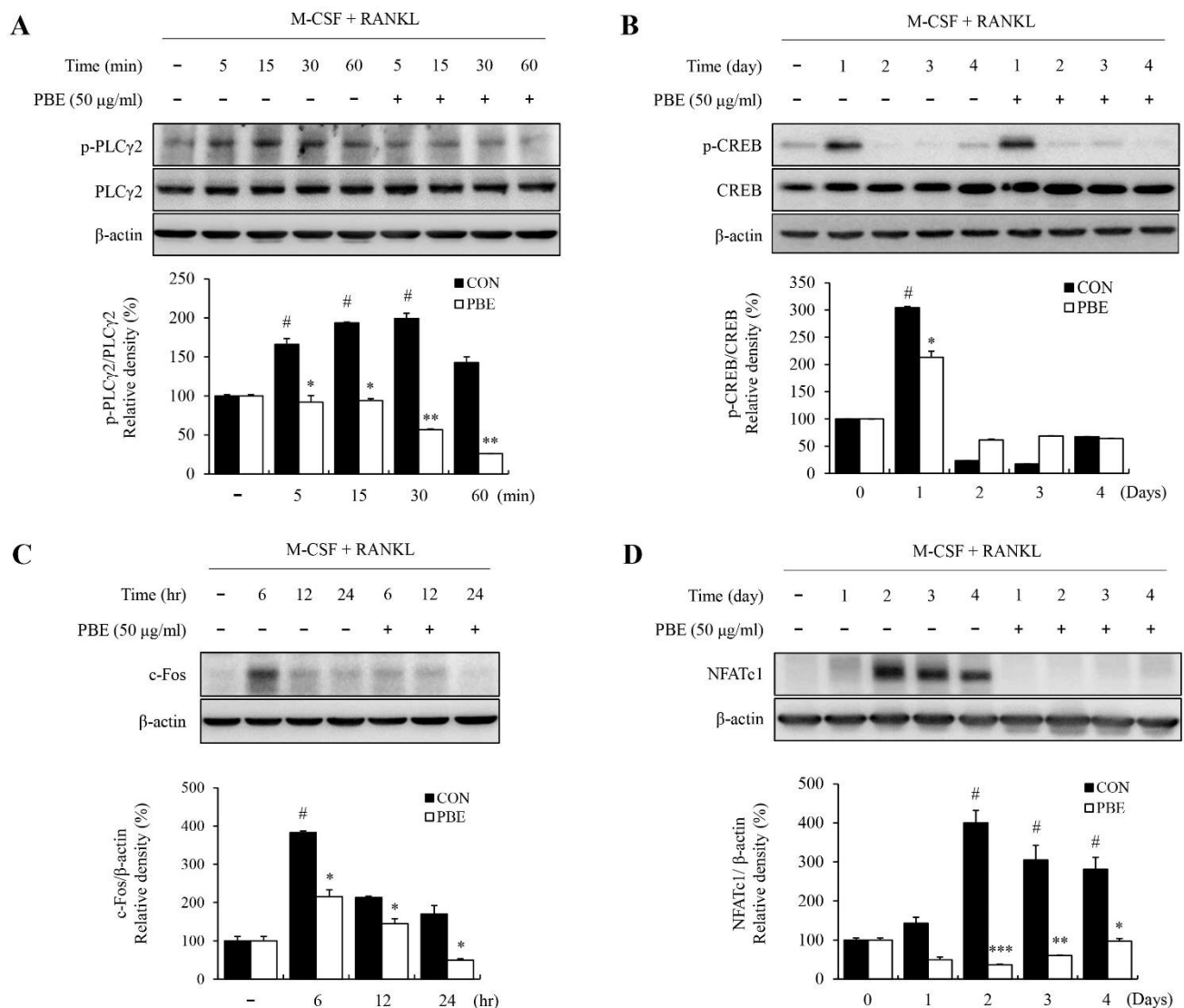


Figure 6. Effect of PBE on RANKL-induced PLCγ2, c-Fos, and NFATc1 expression. BMMs were cultured with RANKL (100 ng/mL) and M-CSF (30 ng/mL), with or without PBE (50 µg/mL), for the indicated time periods. Western blotting was performed to analyze the protein expression levels of (A) PLCγ2, p-PLCγ2, (B) CREB, p-CREB, (C) c-Fos, and (D) NFATc1. Protein expression levels were quantified using ImageJ software, and values are expressed as the mean ± SD ($n = 3$). Bars labeled with different superscripts indicate # $p < 0.05$ vs. RANKL-non treated group; * $p < 0.01$ vs. control; ** $p < 0.005$ vs. control; *** $p < 0.001$ vs. control.

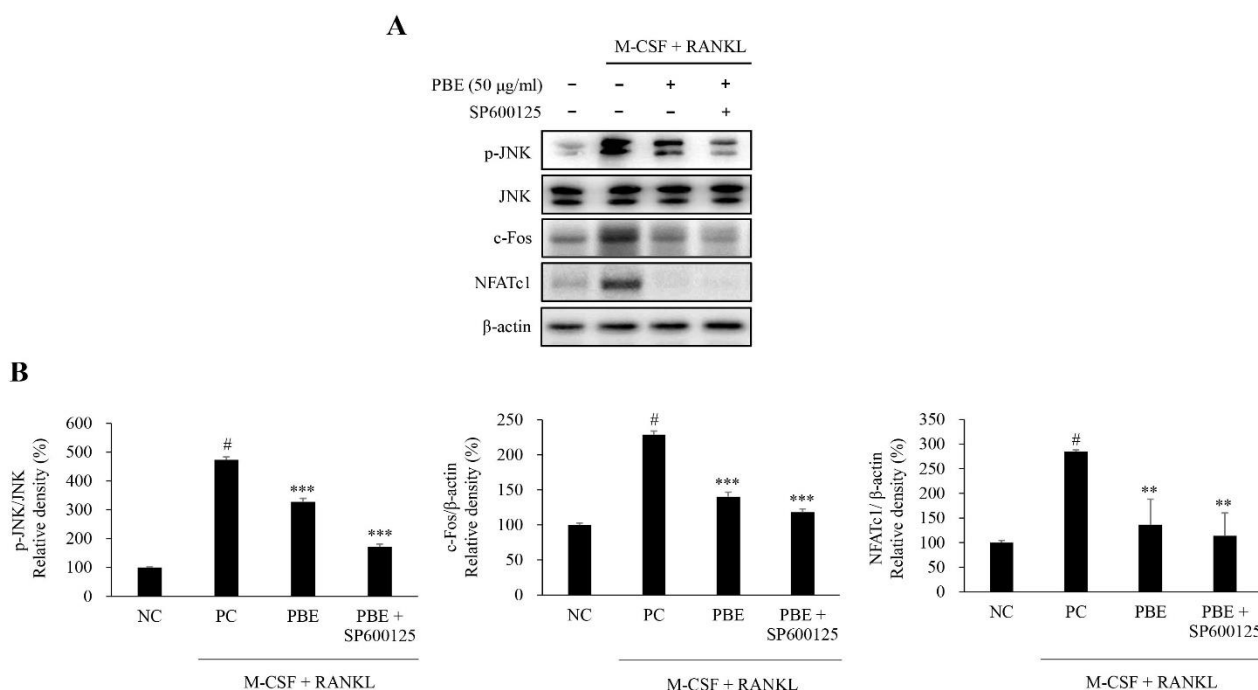


Figure 7. Inhibitory effect of PBE on osteoclastogenesis is mediated through the JNK signaling pathway. BMMs were cultured with RANKL (100 ng/mL) and M-CSF (30 ng/mL) for 4 days. Then, the cells were pretreated with the JNK inhibitor SP600125 (20 µM) for 30 min, followed by stimulation with or without PBE at a concentration of 50 µM. **(A)** Western blotting was performed to analyze the protein expression levels of *p*-JNK, JNK, *c*-Fos, and NFATc1. **(B)** Protein bands were quantified using Image J software. Bars labeled with different subscripts indicate significant differences ([#] $p < 0.001$ vs. RANKL untreated group (NC; normal control), ^{**} $p < 0.005$ vs. RANKL treated group (PC; positive control), ^{***} $p < 0.001$ vs. PC). Data are presented as mean $\pm$ SD from at least three independent experiments ($n = 3$).

4. Discussion

Bone remodeling occurs as a balance between osteoblast and osteoclast differentiation [1,2]. However, when an imbalance occurs, dysregulation of bone homeostasis occurs, which can cause various pathological conditions, such as osteoporosis and rheumatoid arthritis [3–5], primarily resulting from the overexpression of osteoclast activity. Therefore, inhibition of osteoclast activity may be a major therapeutic strategy for these diseases. Recently, various drugs, such as bisphosphonates and estrogens, have been used as anti-osteoporosis drugs, but they have some side effects, such as vaginal bleeding, deep vein embolism, breast cancer, atypical femoral fractures, and necrosis of the jaw [28–31]. Currently, as an alternative treatment strategy to compensate for these side effects, the focus is on discovering insects that are regarded as functional food and pharmaceutical resources.

Many insect-derived bioactive substances are known to be used as antioxidants, anti-coagulants, indigestion, inflammation, and hypertension [15–18]. Among them, bioactive substances derived from PB, an edible insect, have been used as a medicinal and functional food in many Asian countries due to their antioxidant, antidiabetic, anticancer, hepatoprotective, and testicular protective effects [20–23]. As a result of the component analysis of PB larvae in previous studies, oleic acid and volatile compounds were identified, and oleic acid was the most predominant fatty acid [19,20]. The PB used in this study contained a large amount of oleic acid, and it contained 3.402 µg of oleic acid per 50 µg of PB powder (Figure S1). Oleic acid is known to perform various bioactivities [32,33]. In a recent study, oleic acid was reported to be a potential inducer of bone formation by enhancing the differentiation and proliferation of human adipose tissue cells, increasing calcium deposits, and promoting bone formation by improving cell interactions [34]. Previous

studies have reported that dietary supplementation, including dairy drinks rich in oleic acid, fatty acids, calcium, and vitamins, improves some bone metabolism biomarkers in postmenopausal healthy women [35]. Therefore, in this study, we investigated the effect of PBE on osteoclastogenesis.

In this study, we show that PBE can effectively reduce the number of osteoclasts and the bone resorption area, which was confirmed by the downregulation of the expression of marker genes related to osteoclast differentiation. Additionally, PBE suppressed RANKL-induced NFATc1 and c-Fos expression by inhibiting the phosphorylation of JNK and NF- κ B. Collectively, these results suggest that PBE attenuates osteoclastogenesis via the JNK/NF- κ B signaling pathway.

M-CSF and RANKL are important cytokines for osteoclast differentiation and are known to induce osteoclast formation *in vitro* [36–38]. Consistent with previous studies, this study shows that RANKL stimulation induced the formation of osteoclasts and bone resorption pits. However, it reduced RANKL-induced osteoclast formation, as the number of TRAP-positive MNCs, TRAP activity, and bone resorption in BMMs decreased after PBE treatment. These results suggest that PBE inhibits osteoclast differentiation and bone resorption.

In addition, RANKL induces the development of mature osteoclasts by inducing the expression of multiple osteoclast-specific genes [1]. The expression of genes related to osteoclast differentiation, such as Acp5, Oscar, CTSK, Tm7sf4, Atp6v0d2, c-Fos, and NFATc1, is closely related to differentiation and function [1,39,40]. Consistent with these results, the mRNA expression levels of Acp5, Oscar, CTSK, Tm7sf4, Atp6v0d2, and NFATc1 were upregulated by RANKL in our study. However, the increased mRNA expression levels of these genes were reduced by PBE treatment. These results suggest that the inhibitory effect of PBE on osteoclast differentiation and bone resorption occurs through inhibition of the expression of activated osteoclast-specific genes.

The signal transduction pathways in osteoclastogenesis form diverse, complex networks. The MAPK and NF- κ B families are known to be important signaling pathways for osteoclastogenesis. M-CSF and RANKL act through the activation of MAPK, consisting of ERK, p38, and JNK signaling, during osteoclast differentiation and bone resorption [1,41]. Moreover, recent studies have reported that the activation of MAPK by M-CSF or RANKL differs in terms of the extent, duration, and isotype specificity of MAPK phosphorylation [41,42]. In this study, we confirm that PBE or co-treatment with PBE and JNK inhibitors decreases the phosphorylation level of JNK, which was increased by RANKL, but does not affect the phosphorylation of ERK and p38. Previous studies have reported that RANKL stimulates JNK to induce activation of the transcription factors c-Jun, c-Fos, and NFATc1, and blocking the JNK signaling pathway inhibits RANKL-induced osteoclast differentiation [41–44]. These results suggest that PBE may inhibit osteoclast differentiation by regulating the phosphorylation of JNK.

The NF- κ B signaling pathway, which is activated by the interaction between RANKL and RANK receptors, plays an important role in osteoclastogenesis [11,45]. NF- κ B signaling is activated by various stimuli, including RANKL, TNF- α , bacterial endotoxins, toll-like receptor ligands, CD40L, and interleukin-1. These stimuli usually induce complexes via TRAF and activate the I κ B kinase complex. The activated kinase complex phosphorylates the NF- κ B inhibitory protein I κ B α , which undergoes proteasomal degradation, resulting in nuclear translocation and activation of various NF- κ B dimers [11,45]. Previous studies have demonstrated that RANKL induces osteoclast differentiation through the phosphorylation of NF- κ B and proteasomal degradation of I κ B α [46–48]. In addition, activation of NF- κ B p65 by RANKL regulation promotes transcription of osteoclastogenesis-related genes by translocating p65 to the cell nucleus and binding to the NFATc1 promoter [49]. Consistent with previous studies, our results show that the phosphorylation levels of I κ B α and NF- κ B p65 were increased by RANKL. However, the phosphorylation levels of these proteins decreased after PBE treatment. These results suggest that PBE may decrease osteoclastogenesis by inhibiting the NF- κ B pathway.

RANKL-induced MAPK and NF- κ B activation activate the osteoclast differentiation factors c-Fos and NFATc1, which are important for osteoclast differentiation and activity. Also, c-Fos and NFATc1 are known to be the final steps in the MAPKs- and NF- κ B-related osteoclastogenesis signaling pathways [41,45]. NFATc1, a master regulator of osteoclast differentiation, is activated by RANKL and plays an essential role in osteoclastogenesis [50–53]. NFATc1 is a well-known key transcription factor for c-Fos, which plays an essential role in osteoclastogenesis induced by M-CSF and RANKL [52,53]. In this study, we show that PBE or co-treatment with PBE and JNK inhibitors suppressed the expression of NFATc1 and c-Fos, which are key regulators of osteoclasts. Based on previous studies and the present study, we suggest that PBE can inhibit osteoclast differentiation by reducing the expression levels of NFATc1 and c-Fos induced by RANKL. Additionally, the PLC γ 2-calcium ion (Ca^{2+}) signaling pathway also plays an important role in osteoclast differentiation. Previous studies have reported that the RANKL–RANK interaction activates PLC γ 2- Ca^{2+} signaling, which in turn activates CREB or NFATc1 [54–57]. Therefore, we investigated whether PBE affects PLC γ 2 and CREB activation. In this study, PBE reduced the phosphorylation of PLC γ 2 and CREB, which are important for osteoclast differentiation, followed by the repression of c-Fos and NFATc1 expression. In addition, the combined treatment of PBE and SP600125 was found to reduce the expression levels of c-Fos and NFATc1 proteins more than that of PBE alone. These results suggest that PBE regulates osteoclastogenesis by inhibiting RANKL-induced PLC γ 2-CREB signaling pathway activation. However, further studies are needed to clarify the role of PBE in osteoclastogenesis and bone disease. In particular, it is necessary to confirm various clinical data related to bone formation by PBE through animal studies. In this study, in vivo studies were not conducted; however, in vitro experiments may help understand the role of PBE in the bone remodeling process. Taken together, although there are some limitations, this is the first study to demonstrate that PBE suppresses the JNK/NF- κ B/PLC γ 2 signaling pathway and reduces the expression levels of NFATc1 and c-Fos involved in osteoclastogenesis, thereby regulating RANKL-induced osteoclastogenesis.

5. Conclusions

In conclusion, our results demonstrate the inhibitory effect of PBE on osteoclast differentiation and bone resorption. PBE reduced the levels of *p*-JNK, *p*-NF- κ B, and *p*-PLC γ 2 proteins in RANKL-induced BMMs, thus reducing the expression levels of NFATc1 and c-Fos. Therefore, this study suggests that PBE inhibits osteoclastogenesis through the JNK/NF- κ B/PLC γ 2 signaling pathway and could be a potential therapeutic strategy or functional product for osteoclast-related bone diseases.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nu15143193/s1>, Figure S1: Gas chromatography profile of *Protaetia brevitarsis*. Oleic acid was detected.

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