Original Article

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.apjtb.org

doi: 10.4103/2221-1691.345518

Impact Factor: 1.55

Dieckol isolated from *Eisenia bicyclis* extract suppresses RANKL-induced osteoclastogenesis in murine RAW 264.7 cells

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ABSTRACT

Objective: To demonstrate the effect of dieckol from *Eisenia bicyclis* on osteoclastogenesis using RAW 264.7 cells.

Methods: Murine macrophage RAW 264.7 cells were subjected to dieckol treatment, followed by treatment with receptor activator of nuclear factor kappa-B ligand (RANKL) to induce osteoclastogenesis. Tartrate-resistant acid phosphatase (TRAP) activity was examined using a TRAP activity kit. Western blotting analysis was conducted to examine the level of osteoclast-related factors, including TRAP and calcitonin receptor (CTR), transcriptional factors, including c-Fos, c-Jun, and nuclear factor of activated T cells cytoplasmic 1 (NFATc1), nuclear factor kappa-B (NF-κB), extracellular signal-regulated kinase (ERK), and c-Jun *N*-terminal kinase (JNK). Immunofluorescence staining was conducted to examine the expression of c-Fos, c-Jun, and NFATc1.

Results: Among the four phlorotannin compounds present in *Eisenia bicyclis*, dieckol significantly hindered osteoclast differentiation and expression of RANKL-induced TRAP and CTR. In addition, dieckol downregulated the expression levels of c-Fos, c-Jun, NFATc1, ERK, and JNK, and suppressed NF-κB signaling.

Conclusions: Dieckol can suppress RANKL-induced osteoclastogenesis. Therefore, it has therapeutic potential in treating osteoclastogenesis-associated diseases.

KEYWORDS: *Eisenia bicyclis*; Dieckol; Osteoclasts; ERK; JNK; NF-κB; RANKL; TRAP; Calcitonin receptor; NFATc1; RAW 264.7 cell

1. Introduction

Osteoporosis is a skeletal disease that induces bone loss and damages bone quality, which increases the risk of fractures and decreases quality of life[1,2]. Normal bone undergoes modeling and remodeling through osteoclasts and osteoblasts[1,3]. However, an imbalance between bone resorption and formation results in the generation of bone disorders[4]. Increased osteoclast differentiation plays a crucial role in bone disorders, such as osteoporosis[1,5]. Therefore, blocking osteoclast differentiation and function is essential to treat osteoporosis. Osteoclasts are multinucleated cells derived from monocyte/macrophage precursor cells that resorb

Significance

Dieckol from *Eisenia bicyclis* was shown to be an effective osteoclastogenesis inhibitor *via* blocking NF-κB, JNK, and ERK pathways in RANKL-stimulated RAW 264.7 cells. Dieckol could be a promising agent for treating osteoporosis.

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How to cite this article: Cho SH, Kwon TH, Jeong H, Kim JS, Kim SR, Jeong MS, et al. Dieckol isolated from *Eisenia bicyclis* extract suppresses RANKL-induced osteoclastogenesis in murine RAW 264.7 cells. Asian Pac J Trop Biomed 2022; 12(6): 262-269.

Article history: Received 15 February 2022; Revision 10 March 2022; Accepted 19 April 2022; Available online 30 May 2022

the bone[6]. The receptor activator of nuclear factor-κB ligand (RANKL) plays a pivotal role in osteoclastogenesis process[7]. RAW 264.7 cells, a mouse monocytic cell line, have been widely used as they express receptor activator of nuclear factor-κB (RANK) and differentiate into functional osteoclasts upon stimulation with RANKL[8,9]. RANKL is an important cytokine of the tumor necrosis factor (TNF) family[9]. The binding of RANKL to RANK begins the TNF receptor-associated factor 6 (TRAF6) recruitment[9,10]. TRAF6 recruitment facilitates the activation of downstream pathways, including mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF-κB), thereby inducing osteoclastogenesis-related factors, including tartrate-resistant acid phosphatase (TRAP), matrix metallopeptidase-9, calcitonin receptor (CTR), and cathepsin K, through the activation of nuclear factor of activated T cells cytoplasmic 1 (NFATc1)[11,12].

Bisphosphonates have been used to reduce osteoporotic fracture risk by suppressing bone resorption[13]. However, they have been reported to cause several adverse effects, such as osteonecrosis of the jaw, suppression of bone turnover, and unusual fractures in the femur (thigh bone) and the shaft (diaphysis or sub-trochanteric region) of bone[14,15]. Natural products have been demonstrated to have specific biological activities and low side effects[16]. Therefore, natural products serve as important sources for new drug discovery.

Marine seaweeds are an abundant source of bioactive compounds with biomedical, pharmaceutical, and nutraceutical potential[17,18]. *Eisenia bicyclis* (*E. bicyclis*), a phlorotannin-rich brown alga, is a common kelp inhabiting the middle Pacific coast around Korea and Japan[19,20]. In a previous study, Kim *et al*. demonstrated that *E. bicyclis* extracts inhibit osteoclast activity and promote osteoblast activity *in vitro*[21]. Moreover, it has been demonstrated that *E. bicyclis* hampers the depletion of bone in ovariectomized rats[22]. In a previous study, we obtained four phlorotannin compounds (6,6′-bieckol, 6,8′-bieckol, 8,8′-bieckol, and dieckol) from *E. bicyclis* and demonstrated their effects on adipogenesis and lipogenesis[23]. Dieckol, one of the most potent bioactive compounds, is abundant in brown algae including *Ecklonia cava* (*E. cava*), *E. bicyclis*, *Ecklonia stolonifera*, and *Ecklonia kurome*[24–27]. Dieckol has been proven to have different biological activities, including anti-diabetic, antioxidant, and anti-inflammatory activities[25,28,29]. Previous studies have found that high concentrations of dieckol are non-toxic to several cell lines based on cell viability assays[25]. Therefore, this study aimed to evaluate the effect of dieckol isolated from *E. bicyclis* on RANKL-promoted osteoclast differentiation in RAW 264.7 cells.

2. Materials and methods

2.1. Cell culture

Murine macrophage RAW 264.7 cells were purchased from the Korean Cell Line Bank (KCLB, Korea). Cells were cultured in Dulbecco's Modified Eagle Media (Welgene, Korea) containing 10% fetal bovine serum (Welgene, Korea) and 1% antibiotics (Gibco/BRL, CA) at 37 ℃ in a humidified incubator with 5% CO_2.

2.2. Measurement of cell viability

The four compounds (6,6′-bieckol, 6,8′-bieckol, 8,8′-bieckol, and dieckol; Supplementary Figure 1) had been isolated in previous studies, and their separation methods have been described in detail[23]. The cells were seeded (2×10^4 cells/mL) and treated with 12.5, 25, and 50 μM of the four compounds for 5 d at 37 ℃. The medium was changed to fresh media every 2 d. Cell viability was measured according to the method described by Lee *et al*. with some modifications[30]. Absorbance at 540 nm was measured using a spectrophotometer (Molecular Devices, USA).

2.3. Measurement of TRAP activity

The cells were seeded (2×10^4 cells/mL), pre-treated with 25 μM of the four compounds for 2 h, and incubated for an additional 5 days at 37 ℃ in medium containing RANKL derived from mouse (100 ng/mL; Sigma-Aldrich, USA). The medium was changed to fresh media every 2 d. TRAP activity was analyzed using a TRAP staining kit (Cosmo Bio Co., Ltd., Japan) according to the manufacturer's protocols. TRAP-positive cells were observed and counted under a light microscope (Carl Zeiss, Germany).

2.4. Western blotting analysis

The cells were seeded (2×10^4 cells/mL), pre-treated with various concentrations of dieckol for 2 h, and added with 100 ng/mL RANKL for the indicated times at 37 ℃. Protein expression was examined according to the method described by Ko *et al*. with some modifications[31]. Membranes were left to react overnight using the following primary antibodies: anti-TRAP (ab96372, 1:1 000; Abcam, USA), anti-CTR (ab11042, 1:1 000; Abcam, USA), anti-NFATc1 (556602, 1:1 000; BD Pharmingen™, USA), anti-c-Fos (2250S, 1:1 000; Cell Signaling Technology, USA), anti-c-Jun (9165S, 1:1 000; Cell Signaling Technology, USA), anti-phospho-ERK (9101S, 1:1 000; Cell Signaling Technology, USA), anti-ERK (9102S, 1:1 000; Cell Signaling Technology, USA), anti-phospho-JNK (9251S, 1:1 000; Cell Signaling Technology, USA), anti-JNK (9252S, 1:1 000; Cell Signaling Technology, USA), anti-phospho-IκB (2859S, 1:1 000; Cell Signaling Technology, USA), anti-phospho-p65 (3033S, 1:1 000; Cell Signaling Technology, USA), anti-phospho-p105 (4806S, 1:1 000; Cell Signaling Technology, USA), and anti-β-actin (SC-47778, 1:1 000; Santa Cruz Biotechnology, USA). The membranes were reacted for 2 h using the following secondary antibodies: anti-rabbit and anti-

mouse IgG, HRP-linked antibody (7074S, 1:3 000; 7076S, 1:3 000; Cell Signaling Technology). Protein bands were visualized using a SuperSignal West Femto Trial kit (Thermo Fisher Scientific, USA). Protein band density was measured using ImageJ (NIH, USA).

2.5. Immunofluorescence staining

The cells were seeded (5×10^4 cells/mL), pre-treated with 25 μM dieckol for 2 h, and added with 100 ng/mL RANKL for various time points (NFATc1, c-Fos, and c-Jun: 9 h; p65: 5 min) based on the individual experiment. Immunofluorescence staining was conducted according to the method of Ko *et al*. with some modifications[31]. The cells were incubated overnight at 4 ℃ with the following primary antibodies: anti-NFATc1 (556602, 1:100; BD Pharmingen™, USA), anti-c-Fos (2250S, 1:100; Cell Signaling Technology, USA), anti-c-Jun (9165S, 1:100; Cell Signaling Technology, USA), and anti-p65 (8242S, 1:100; Cell Signaling Technology, USA). After another washing three times with PBS, cells were incubated with the following secondary antibodies: Alexa fluor488-labeled goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody (A11008, 1:800; Thermo Fisher Scientific, USA) and Alexa fluor488-labeled goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody (A11001, 1:800; Thermo Fisher Scientific, USA). Nuclei were stained using 40 μg/mL Hoechst 33342 (Sigma Aldrich, USA) for 10 min at room temperature and mounted with ProLong™ Gold antifade mountant (Thermo Fisher Scientific, USA). Fluorescence was visualized using an LSM 700 Zeiss confocal laser scanning microscope (400× magnification; Carl Zeiss, Germany).

2.6. Statistical analysis

Data were expressed as mean ± standard deviation (SD) and analyzed by one-way ANOVA with Tukey's *post hoc* test. $P<0.05$ was considered a statistical significance. All statistical tests were performed using GraphPad PRISM software version 8.0 (GraphPad Software, USA).

3. Results

3.1. Effect of dieckol on the viability of RAW 264.7 cells and RANKL-promoted TRAP activity

As shown in Figure 1, 6,6′-bieckol, 6,8′-bieckol, 8,8′-bieckol, and dieckol were non-toxic to RAW 264.7 cells at the concentrations up to 25 μM. Next, to demonstrate the effect of these compounds on RANKL-promoted osteoclast differentiation, TRAP, an osteoclast-related factor, was determined. As shown in Figure 2, RANKL significantly promoted osteoclast differentiation, as evidenced by TRAP-positive cells compared with the control group ($P<0.001$). However, dieckol markedly reduced TRAP-positive cells ($P<0.001$). In contrast, other compounds did not affect RANK-induced osteoclast differentiation and TRAP activity. Therefore, dieckol at different concentrations (6.25, 12.5, and 25 μM) was used for further experiment. The results showed that dieckol at 12.5 and 25 μM markedly inhibited RANKL-induced TRAP activity ($P<0.05$) (Figures 3A-B).

3.2. Effect of dieckol on RANKL-stimulated expression of osteoclastogenesis-related factors in RAW 264.7 cells

RANKL markedly induced CTR and TRAP expression compared with the control group ($P<0.001$) (Figure 3C). However, dieckol at all tested concentrations markedly suppressed CTR and TRAP expression levels ($P<0.001$). Moreover, RANKL promoted the expression of the transcriptional factors including c-Fos, c-Jun, and NFATc1 (Figure 4). In contrast, treatment with dieckol significantly suppressed the induction of these factors ($P<0.001$).

3.3. Effect of dieckol on RANK-activated NF-κB, ERK, and JNK signaling pathways in RAW 264.7 cells

We further evaluated the effect of dieckol on the activation of NF-κB, ERK, and JNK signaling pathways. Dieckol markedly

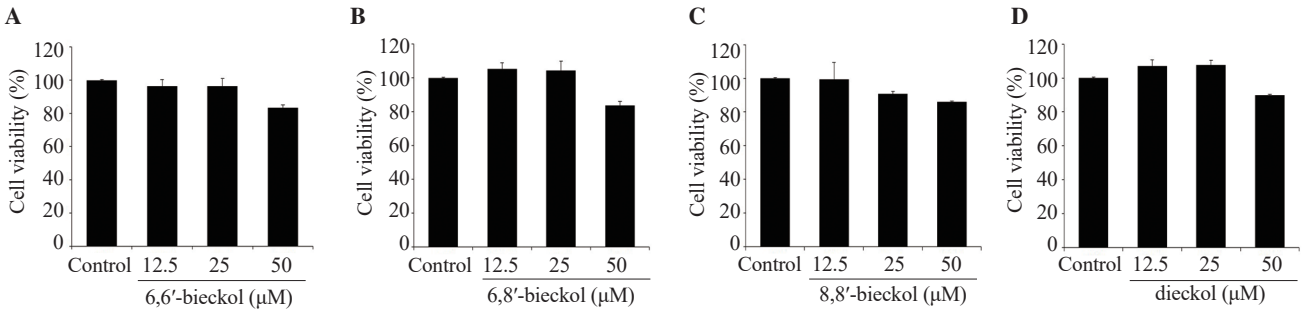

Figure 1. Effect of (A) 6,6′-bieckol, (B) 6,8′-bieckol, (C) 8,8′-bieckol, and (D) dieckol on the viability of RAW 264.7 cells. Cell viability was assessed by MTT assays. The data are expressed as mean ± SD of at least three independent experiments.

suppressed RANKL-induced IκB, p65, and p105 activation and p65 nuclear translocation (P<0.001) (Figures 5A-B). Moreover, RANKL activated both ERK and JNK signaling pathways, which were prominently blocked by dieckol (P<0.001) (Figure 6).

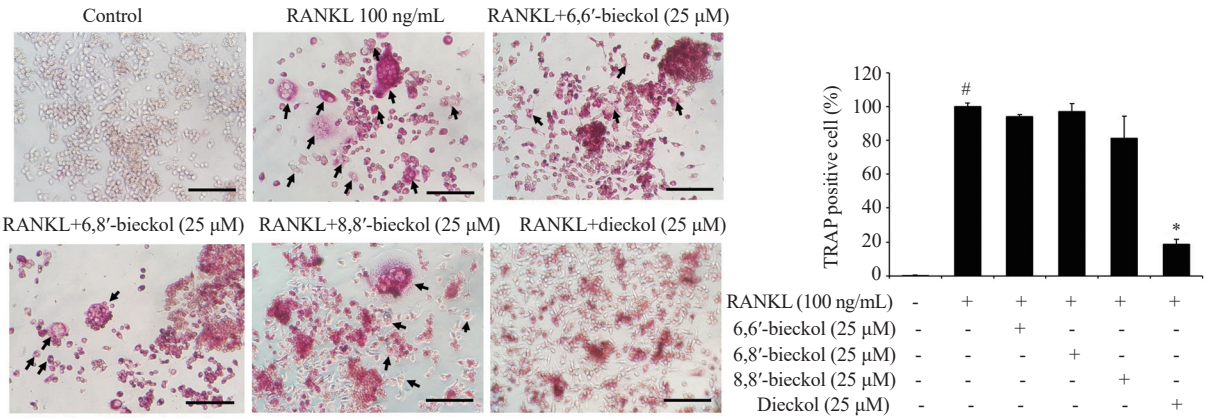

Figure 2. Effect of four phlorotannin compounds on tartrate-resistant acid phosphatase (TRAP) activity in RANKL-induced RAW 264.7 cells. TRAP activity was evaluated by TRAP staining (magnification: 100 ×; scale bar: 100 μm). Black arrows: TRAP-positive cells. All results are expressed as mean ±SD of at least three independent experiments. $^{\#}P < 0.001$ compared with the control group; $^{*}P < 0.001$ compared with the RANKL-stimulated group.

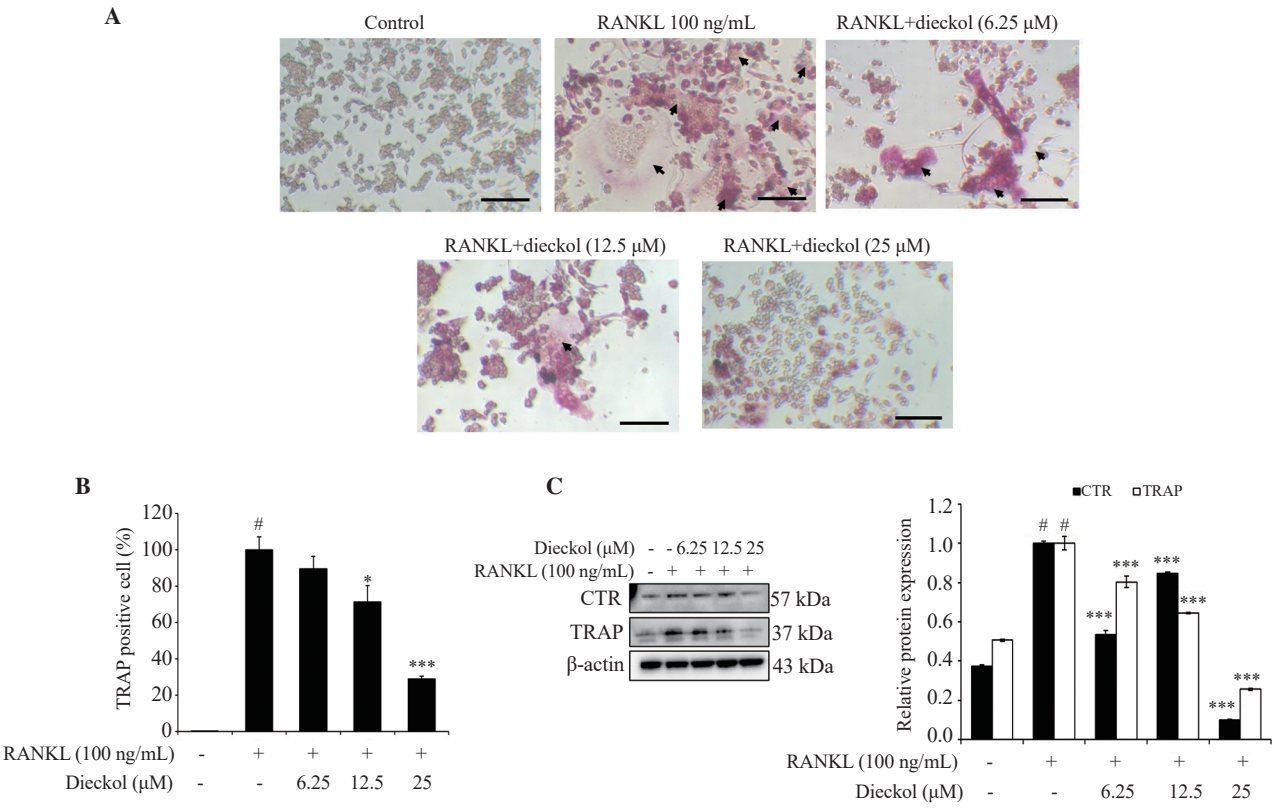

Figure 3. Effect of different concentrations of dieckol on TRAP activity and osteoclast-related factors in RANKL-induced RAW 264.7 cells. (A) TRAP activity was evaluated by TRAP staining (magnification: 100 ×; scale bar: 100 μm). Black arrows: TRAP-positive cells. (B) Quantitative results of TRAP activity. (C) The expression of calcitonin receptor (CTR) and TRAP protein was analyzed by Western blotting analysis. All results are expressed as mean ± SD of at least three independent experiments. $^{\#}P < 0.001$ compared with the control group; $^{*}P < 0.05$, $^{***}P < 0.001$ compared with the RANKL-stimulated group.

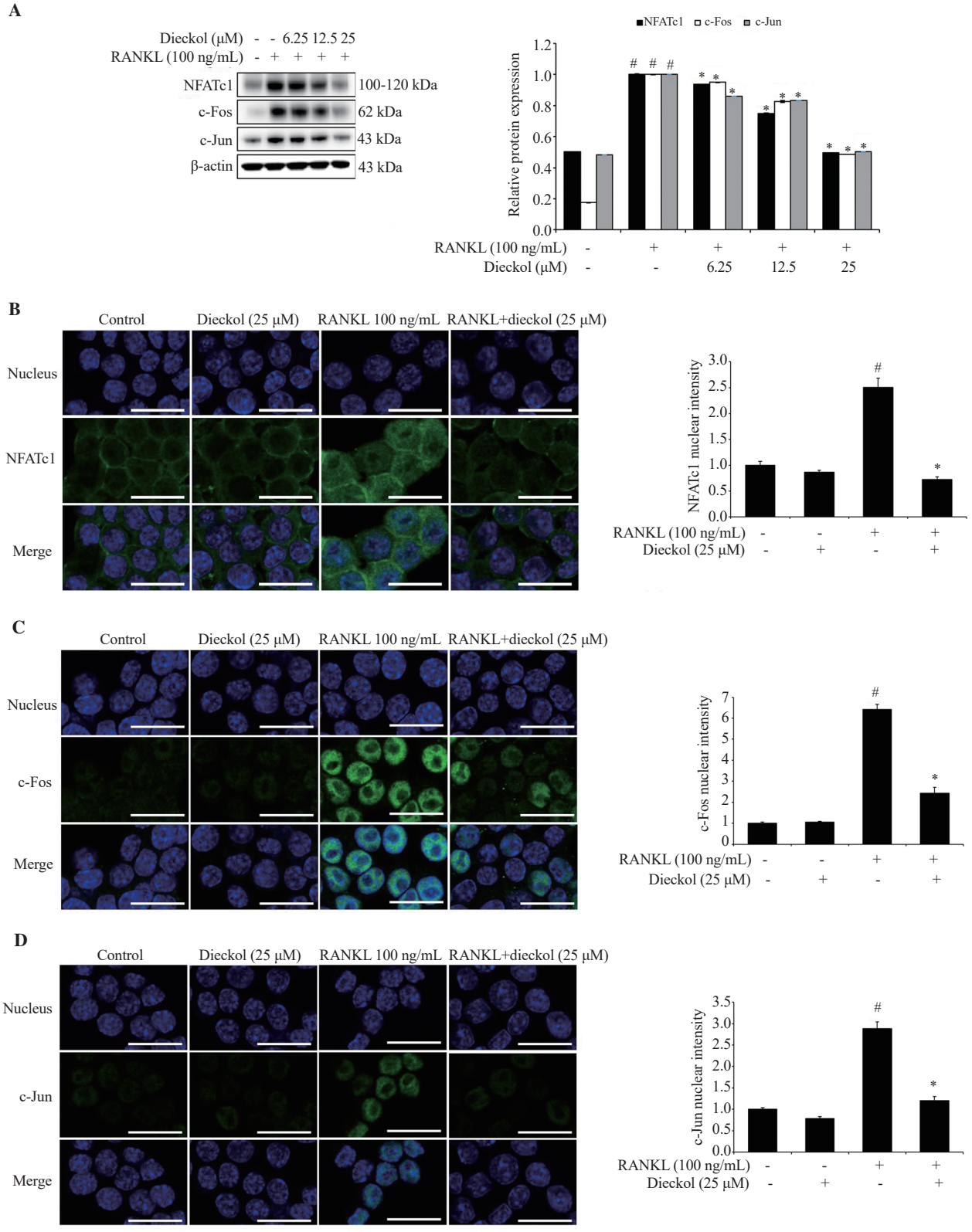

Figure 4. Effect of dieckol on the expression of osteoclast-related transcriptional factors in RANKL-induced RAW 264.7 cells. (A) Protein expression was analyzed using Western blotting analysis. Nuclear translocation of (B) NFATc1, (C) c-Fos, and (D) c-Jun in RANKL-induced RAW 264.7 cells. These images were observed with an anti-NFATc1, anti-c-Fos, anti-c-Jun, Alexa Fluor 488 goat anti-mouse antibody, and Alexa Fluor 488 goat anti-rabbit antibody using an LSM700 Zeiss confocal laser scanning microscope (magnification: 400 ×; scale bar: 20 μm). Nuclei were stained with Hoechst 33342. All results are expressed as mean ± standard deviation (SD) of at least three independent experiments. $^{\#}P < 0.001$ compared with the control group; $^{*}P < 0.001$ compared with the RANKL-stimulated group.

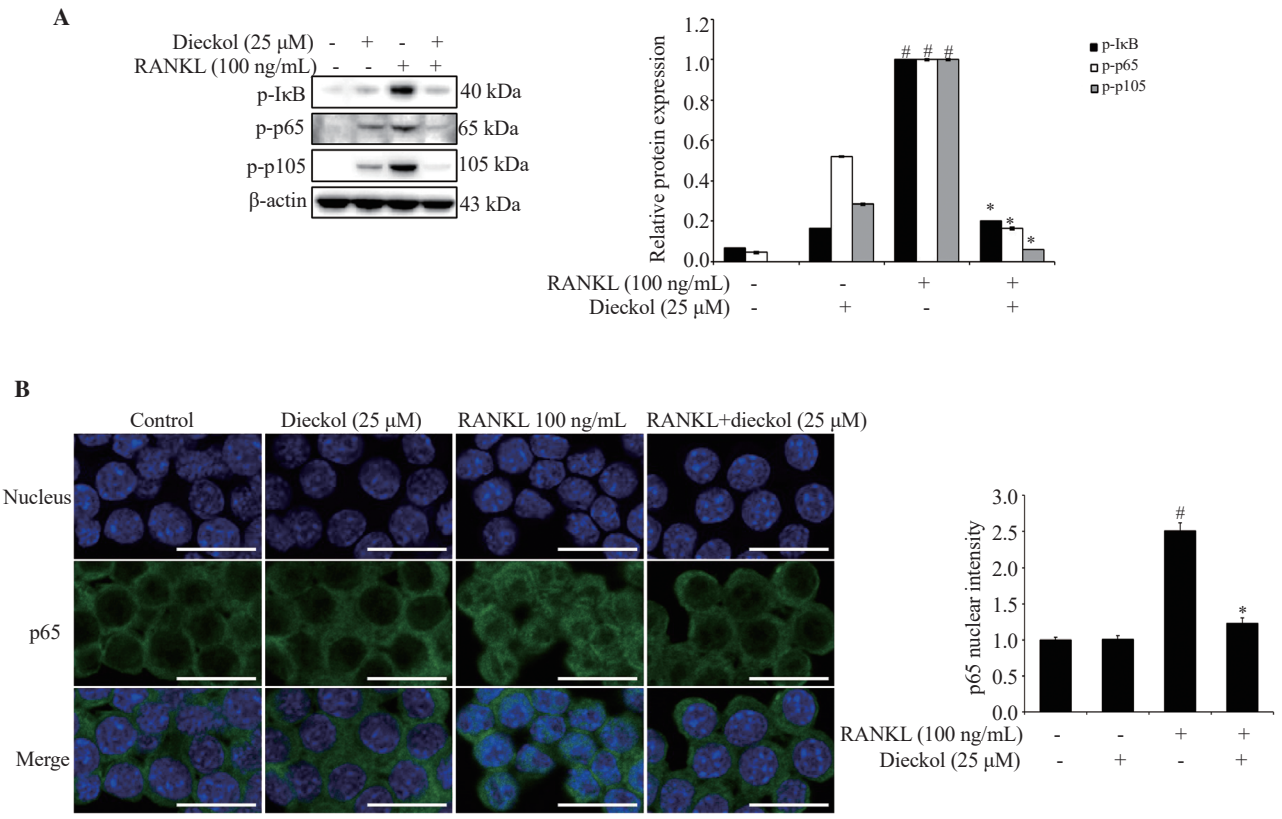

Figure 5. Effect of dieckol on activation of the NF-κB signaling pathways in RANKL-induced RAW 264.7 cells. Cells were pre-treated with 25 μM dieckol for 2 h and then treated with RANKL for 5 min. (A) Protein expression was analyzed using Western blotting analysis. (B) Nuclear translocation of p65 in RANKL-induced RAW 264.7 cells. These images were observed with an anti-p65 and Alexa Fluor 488 goat anti-rabbit antibody using an LSM700 Zeiss confocal laser scanning microscope (magnification: 400 ×; scale bar: 20 μm). Nuclei were stained with Hoechst 33342. All results are expressed as mean ± standard deviation (SD) of at least three independent experiments. $^{\#}P < 0.001$ compared with the control group; $^{*}P < 0.001$ compared with the RANKL-stimulated group.

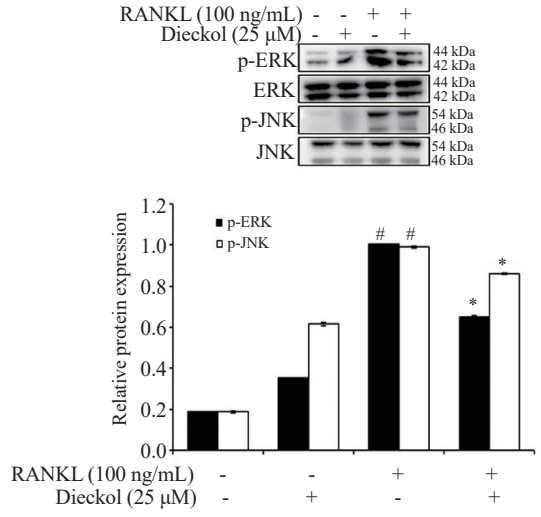

Figure 6. Effect of dieckol on the activation of ERK and JNK signaling pathways in RANKL-induced RAW 264.7 cells. Protein expression was analyzed using Western blotting analysis. All results are expressed as mean ± standard deviation (SD) of at least three independent experiments. $^{\#}P < 0.001$ compared with the control group; $^{*}P < 0.001$ compared with the RANKL-stimulated group.

4. Discussion

Polyphenolic compounds, such as bromophenol, phenolic terpenoids, and phlorotannins, are abundant in seaweeds and exhibit several biological activities[26,29]. Previous studies have reported the osteoclastogenesis-inhibitory activities of polyphenolic compounds. Ha *et al*. demonstrated that fucoxanthin isolated from *E. cava* inhibits osteoclastogenesis by blocking MAPK and Nrf2 signaling pathways in RAW 264.7 cells[32]. Further, Bang *et al*. proved that fucosterol isolated from *Undaria pinnatifida* suppresses RANKL-activated osteoclast differentiation in bone marrow-derived macrophages (BMMs)[33]. Phlorotannins are polyphenolic compounds found in brown seaweeds[34]. Their inhibitory effects on osteoporosis have been reported in literature. Ihn *et al*. demonstrated that diphlorethohydroxycarmalol from *Ishige okamurae* inhibits osteoclastogenesis by hindering NF-κB activation *in vitro*[35]. In a previous study, Oh *et al*. revealed that phlorofucofuroeckol A from *E. cava* enhances osteoblastogenesis *in vitro*[36]. Moreover, an extract from the brown alga *E. cava* containing dieckol inhibits RANKL-promoted osteoclast differentiation by downregulating MAPK and NF-κB activation and activating heme oxygenase-1 *in vitro*[37]. Therefore, this study demonstrated the effect of dieckol on RANKL-stimulated osteoclast differentiation using RAW 264.7 cells.

Many studies have been conducted on the inhibition of osteoclastogenesis using RANKL-treated RAW 264.7 cells[38,39]. Osteoclastogenesis-related factors were generated *via* RANKL treatment in RAW 264.7 cells. In the present study, the effect of phlorotannin compounds (6,6′-bieckol, 6,8′-bieckol, 8,8′-bieckol, and dieckol) was evaluated at a non-cytotoxic concentration. The results showed that among four compounds, only dieckol significantly inhibited RANKL-promoted TRAP activity. Furthermore, dieckol reduced the expression of TRAP and CTR protein at 6.25-25 μM.

Osteoclasts require RANKL for their proliferation, survival, differentiation, and activation[40]. The binding of RANKL to RANK causes the recruitment of TRAF6, thereby activating downstream signaling cascades including MAPK and NF-κB[38,40]. Among the MAPK family members, ERK and JNK promote the activation of c-Fos and c-Jun, respectively[41,42]. c-Jun produces the activator protein-1 complex with c-Fos, an important transcription factor for osteoclastogenesis[42]. Moreover, TRAF6 mediates inhibitor of κB (IκB) kinase (IKK) activation and subsequently IκB phosphorylation[43]. Phosphorylated IκB is degraded *via* the ubiquitin/proteasome pathway, and NF-κB is translocated from the cytoplasm to the nucleus[44]. NFATc1, induced by activator protein-1 and NF-κB, plays an essential role in osteoclastogenesis-related gene expressions, such as *TRAP* and *CTR*; hence, it controls osteoclast differentiation[13,14]. Our study showed that dieckol suppressed RANKL-induced TRAP and CTR generation in cells. Additionally, dieckol significantly inhibited RANKL-generated expression of the nuclear transcriptional factors c-Fos, c-Jun, and NFATc1. Dieckol also significantly inhibited the phosphorylation of ERK, and JNK and NF-κB signaling.

Other phlorotannins, such as diphlorethohydroxycarmalol (25, 50, and 75 μg/mL), also inhibit the TRAP-positive cells and osteoclast differentiation in bone marrow-derived macrophages[35]. In addition, they significantly inhibit osteoclastogenesis-related genes including TRAP (*Acp5*) and NFAFc1 (*Nfatc1*)[35]. The effective inhibitory activity of phlorotannins is controlled by NF-κB and MAPK signaling pathways. The effect of dieckol observed in this study was similar to that in a previous study, indicating that dieckol may inhibit osteoclastogenesis in bone marrow-derived macrophages. However, further studies are required to prove this effect.

The main limitations of this study are that we are not yet able to perform additional experiments using human osteoclast precursor cells and an ovariectomized mouse model. These experiments are essential to demonstrate the effect of osteoporosis. Therefore, further studies are also needed to prove the effect of dieckol on human osteoclast precursor cells and in an ovariectomized mouse model.

In conclusion, our study proved that dieckol suppresses RANKL-induced osteoclastogenesis-related factors including TRAP and CTR, by inhibiting c-Fos, c-Jun, and NFATc1 and by downregulating the ERK, JNK, and NF-κB signaling pathways. Therefore, dieckol may be useful for inhibition of osteoclastogenesis.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Funding

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT; No. NRF-2020R1A2C1008527).

Authors' contributions

JA and KNK supervised and designed the study. SHC, THK, HJ, JSK, SRK, MSJ, SP, MC, and JHW performed experimental analysis. THK provided the resources. SHC, THK, JA, and KNK wrote the manuscript.

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