Original Article

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Sargassum serratifolium attenuates RANKL-induced osteoclast differentiation and oxidative stress through inhibition of NF-κB and activation of the Nrf2/HO-1 signaling pathway

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Summary ***Sargassum serratifolium* C. Agardh is a marine brown alga that has long been used as an ingredient for food and medicine by many people living along Asian coastlines. Recently, various beneficial effects of extracts or compounds isolated from *S. serratifolium* have been reported, but their efficacies against bone destruction are unclear. Therefore, in this study, we investigated the inhibitory property of an ethanol extract of *S. serratifolium* (EESS) on osteoclast differentiation by focusing on the receptor activator of nuclear factor-κB ligand (RANKL)-stimulated osteoclastogenesis model using RAW 264.7 macrophages. Our results demonstrated that EESS reduced RANKL-induced osteoclast differentiation in RAW 264.7 cells, by inhibiting tartrate-resistant acid phosphatase (TRAP) activity and destroying the F-actin ring formation. EESS also attenuated RANKL-induced expressions of key osteoclast-specific genes, such as nuclear factor of activated T cells cytoplasmic 1 (NFATC1), TRAP, cathepsin K and matrix metalloproteinase-9. These effects were mediated by impaired nuclear translocation of nuclear factor (NF)-κB and suppression of IκB-α degradation. In addition, EESS effectively inhibited the production of reactive oxygen species (ROS) by RANKL, which was associated with enhanced expression of nuclear translocation of nuclear factor-erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1). Overall, our findings provide evidence that EESS suppresses RANKL-induced osteoclastogenesis and oxidative stress through suppression of NF-κB and activation of Nrf2/HO-1 signaling pathway, indicating that *S. serratifolium* has a potential application the prevention and treatment of osteoclastogenic bone disease.**

Keywords: *Sargassum serratifolium*, osteoclasts, RANKL, NF-κB, Nrf2/HO-1

1. Introduction

Bone remodeling or bone metabolism is a physiological process involving bone resorption and synthesis by osteoclasts and osteoblasts, respectively. Osteoclasts are specialized multinucleated cells that differentiate from hematopoietic stem cells to induce bone resorption (*1,2*). Under normal physiological conditions, they play an important role in the maintenance of calcium homeostasis and normal bone remodelling (*3,4*). However, excessive bone resorption compared to bone formation causes an imbalance in

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bone remodeling. This may occur due to menopause, aging, oxidative and inflammatory stress. The net result may be a progression to a variety of metabolic bone diseases, such as osteoporosis, autoimmune arthritis, hypercalcemia, and Paget's disease (*5-7*). Currently, hormones and anti-resorptive therapies are commonly used to treat them. However, long-term use of estrogen after menopause may increase the risk of various types of cancer in addition to blood clotting (*8-10*). In addition, specific inhibitors of osteoclastic activity such as bisphosphonates, which are widely used as reabsorption therapies, can cause serious side effects including hypocalcemia and osteonecrosis (*9-11*). Therefore, the discovery of substances capable of inhibiting osteoclast differentiation is a useful strategy for developing therapeutic agents for bone resorption-related diseases.

Receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) are known to be essential cytokines that play key roles in osteoclast differentiation and maturation (*12,13*). RANKL, a member of the tumor necrosis factor (TNF) superfamily, is a critical regulator for signaling to induce osteoclast differentiation and to facilitate the activation of precursor cells through interaction with its receptor RANK (*13,14*). On the other hand, M-CSF secreted from osteoclasts is involved in the survival and proliferation of osteoclast precursors and induction of RANK expression (*12,15*). Activation of RANK by RANKL stimulates transcriptional activation of osteoclast-specific factors, which are involved in osteoclast differentiation and osteoclastic activity through activation of various intracellular signal transduction pathways, thereby increasing bone resorption (*16-18*).

Oxidative stress caused by excessive production of reactive oxygen species (ROS) has been reported to inhibit osteoblast survival and function, thereby inhibiting bone formation (*19,20*). Excessive ROS can also cause bone destruction by exerting oxidative damage to all cellular biomolecules such as proteins, lipids and nucleic acids (*21,22*). In addition, the overproduction of ROS has been demonstrated to increase the expression of RANKL by osteoclasts, thereby activating osteoclasts and enhancing bone resorption capacity, indicating that ROS play an important role in osteoclastogenesis (*19,23,24*). Most cells have endogenous defense strategies to eliminate damage caused by excessive ROS production. Among them, the nuclear transcription factor erythroid-2-like factor 2 (Nrf2) is one of the critical antioxidant systems involved in the maintenance of the redox state (*25,26*). More importantly, many studies have shown that Nrf2 is a key regulator of bone homeostasis, because activation of Nrf2 signaling can promote the endogenous antioxidant response to ROS (*26-28*).

Recently, there has been a growing interest in marine resources for prevention and treatment of various diseases. Among them, seaweeds are abundant as active substances with various pharmacological actions, which may have the potential to prevent bone loss through inhibition of osteoclastogenesis (*29-34)*. Sargassum is a genus of marine brown algae (Phaeophyceae) that is found in most seas around the world, and many coastal people, especially in Korea, Japan, and China, use it as a source for food and medicine (*35*). Recently, extracts or compounds isolated from *Sargassum siliquastrum*, *S. fusiforme* and *S. micracanthum* belonging to *Sargassum* spp. have been demonstrated to have potent inhibitory activities against osteoclast differentiation (*33,36-38*). For example, Komai *et al*. (*38*) reported that plastoquinones and chromene derivatives isolated from *S. micracanthum* contribute to inhibition of bone resorption by inhibiting the differentiation of osteoclast progenitors into osteoclast-like cells. In addition, sargachromanol G, a plastoquinone isolated from *S. siliquastrum*, was reported to have anti-osteoclastogenic effects while inhibiting the activity of NF-κB and mitogen-activated protein kinases by RANKL and interleukin-1β (*36,37*). However, up to now, the effectiveness of *S. serratifolium* in osteoclast formation has not been investigated. Therefore, this study was designed to examine the effects of an ethanol extract of *S. serratifolium* (EESS) on RANKL-induced osteoclast differentiation and oxidative responses, using RAW 264.7 cells as osteoclast precursor cells.

2. Materials and Methods

2.1. *Preparation of EESS*

The EESS used in this study was provided by the National Marine Biodiversity Institute of Korea (Seocheon, Republic of Korea). For the preparation of EESS, *S. serratifolium* was collected from offshore Jeju island, Republic of Korea in March 2016. Authentication of the brown algae was established by Dr. Dae-Sung Lee, National Marine Biodiversity Institute of Korea. Collected *S. serratifolium* was washed with tap water to remove slats, epiphytes, and sand attached to the surface of the samples, and then lyophilized. The dried sample of *S. serratifolium* (170 g) was pulverized, and extracted with 70% EtOH (1 : 10 w/v) for 1 h (five times) by sonication. The *S. serratifolium* extract (EESS) was obtained by evaporation under vacuum. Voucher specimen (AARC-2016-18) is deposited at the Anti-Aging Research Center, Dongeui University (Busan, Republic of Korea). The extract was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich Chemical Co., St. Louis, MO, USA), before use in the experiment.

2.2. *Cell culture*

The murine macrophage RAW 264.7 cell line was

obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (WelGENE Inc., Daegu, Republic of Korea), containing 10% fetal bovine serum (WelGENE Inc.) and 100 U/mL penicillin and streptomycin (WelGENE Inc.) at 37℃ in humidified air with 5% CO_2, and were subcultured every three days.

2.3. *Cell viability assay*

For the cell viability study, RAW 264.7 cells were cultured in 96-well plates at a density of 5×10^4 cells per well. After a 24-h-incubation, the cells were treated with various concentrations of EESS for 48 h. Afterward, the medium was removed, 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemical Co.) was added to each well and incubated at 37℃ for 3 h. The supernatant was then replaced with dimethyl sulfoxide (DMSO) to dissolve the blue formazan crystals. After 10 min, the optical density was measured at a wavelength of 540 nm, with an enzyme-linked immunosorbent assay (ELISA) microplate reader (Dynatech Laboratories, Chantilly, VA, USA).

2.4. *Flow cytometry assay*

For flow cytometry analysis, cells cultured under the same conditions as in the MTT assay were resuspended in phosphate-buffered saline (PBS) and fixed in 75% (v/v) ethanol at 4℃ for 1 h. After washing with PBS, the cells were incubated in cold propidium iodide solution (50 μg/mL, Sigma-Aldrich Chemical Co.) containing RNase A (0.1 mg/mL) in PBS (pH 7.4) in the dark for 30 min. The relative DNA contents of stained cells were analyzed by fluorescence intensities using a flow cytometer (BD Biosciences, San Jose, CA, USA), and cells belonging to the sub-G1 phase were calculated as apoptotic cells.

2.5. *Tartrate-resistant acid phosphatase (TRAP) staining and activity assay*

RAW 264.7 cells were seeded in 48-well plates at a density of 1×10^5 cells per well, to investigate the effect of EESS on osteoclast differentiation. After 24-h-culture, 100 ng/mL RANKL (Abcam, Cambridge, MA, USA) with or without EESS (25 and 50 μg/mL) was added and further cultured for 5 days. The medium containing the relevant reagents was changed every three days within this period. After completion of the experiment, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich Chemical Co.) for 10 min and washed twice with PBS. The fixed cells were then permeabilized with 0.1% Triton X-100 for 1 min and stained for TRAP activity using a commercial kit (Sigma-Aldrich Chemical Co.), according to the manufacturer's instructions. TRAP-positive multinucleated cells containing three or more nuclei were classified as osteoclasts, counted and captured by a light microscope (Carl Zeiss, Oberkochen, Germany). At the same time, the culture medium was collected, and the activity of TRAP was measured using a TRAP assay kit (Sigma-Aldrich Chemical Co.) at 450 nm with an ELISA microplate reader. TRAP activity was calculated as a percentage of the untreated control, as previously described (*39*).

2.6. *F-actin ring formation assay*

The effect of EESS on the formation of F-actin rings was investigated in accordance with the methods of a previous study (*40*). Briefly, after treatment, the culture medium was replaced with a solution of 4% paraformaldehyde to fix the cells for 20 min at room temperature. The cells were then stained with fluorescein isothiocyanate (FITC)-phalloidin solution (Thermo Scientific, Waltham, MA, USA) for 45 min after 5 min treatment with a 0.1% Triton X-100 solution to permeabilize the cells. Nuclei were sequentially stained with 2.5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Chemical Co.) solution for 15 min and images were captured using a fluorescence microscope (Carl Zeiss).

2.7. *Protein isolation and Western blot analysis*

To extract whole-cell proteins, the cells were collected, washed twice with ice-cold PBS, and then lysed using the cell lysis buffer [25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% Nonidet-P40, 1 mM phenylmethylsulphonyl fluoride, and 5 mM dithiothreitol] for 1 h before cell debris was removed by centrifugation. The cytosolic and nuclear extracts were prepared using an NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology, Rockford, IL, USA) in accordance with the instructions of the manufacturer. The same amounts of protein (30-50 μg) were separated by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Schleicher and Schuell, Keene, NH, USA). The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and subsequently probed with primary antibodies overnight with gentle agitation at 4℃. After washing three times with Tris-buffered saline containing 0.1% Tween-20 for 5 min, the membranes were incubated with the corresponding horseradish-peroxidase-linked secondary antibodies (Amersham Biosciences, Westborough, MA, USA) for 2 h at room temperature. The membranes were visualized by enhanced chemiluminescence (ECL) solution (Amersham Biosciences) and exposed to X-ray films.

2.8. *Detection of the intracellular ROS levels*

The production of intracellular ROS was monitored using a cell-permeable fluorogenic probe, 5,6-carboxy-2',7'-dichlorofluorescein diacetate (DCF-DA). Briefly, the collected cells were stained with 10 μM DCF-DA (Sigma-Aldrich Chemical Co.) in the dark at 37℃ for 15 min. The cells were then rinsed twice with PBS, and 10,000 events were immediately analyzed using a flow cytometer (BD Biosciences, San Jose, CA, USA), with an excitation wavelength of 480 nm and an emission wavelength of 525 nm (*41*). To observe the degree of ROS production by fluorescence microscopy, the cells attached to the glass coverslips were stimulated with RANKL in the presence or absence of EESS. The cells were stained with 10 μM DCF-DA at 37℃ for 15 min, washed twice with PBS, and then fixed with 4% paraformaldehyde (pH 7.4) for 20 min. The fixed cells were washed twice with PBS and analyzed using a fluorescence microscope (Carl Zeiss).

2.9. *Statistical analysis*

All the experiments reported in this study were replicated in three independent experiments. The results are presented as the mean ± SD. Statistical significance was assessed by one-way analysis of variance. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. *Effects of EESS on RAW 264.7 cell viability*

RAW 264.7 cell viabilities were measured following treatment with various concentrations of ESS for 48 h, to assess the results under current experimental conditions. The cytotoxic effect of EESS, as shown in Figure 1A, was not induced at concentrations up to 100 μg/mL of the agent, but the cell viabilities were gradually reduced in the treatment groups with larger concentrations (≥150 μg/mL) of EESS compared to the control cells. Flow cytometry analysis showed no significant difference in the frequencies of cells of the sub-G1 phase, which means the presence of apoptosis in the groups treated with ≤100 μg/mL of EESS compared to the control group (Figure 1B). Therefore, the highest concentration of EESS was chosen as 50 μg/mL for subsequent studies.

3.2. *EESS inhibits osteoclast differentiation in RANKL-stimulated RAW 264.7 cells*

RAW 264.7 cells were treated with 25 and 50 μg/mL EESS, respectively, in the presence of RANKL and stained with TRAP, to determine the inhibitory effect of EESS on osteoclast differentiation. In the RANKL-alone treatment group, typical osteoclast morphological changes, in which many cells were aggregated and bundled, and an increased activation of TRAP were observed, suggesting that RAW 264.7 cells differentiated completely into osteoclasts (Figure 2A). However, EESS significantly reduced multinucleated osteoclast-like cell formation and TRAP activity in RANKL-stimulated RAW 264.7 cells in a concentration-dependent manner. In addition, RANKL-induced osteoclast differentiation was completely suppressed in the osteoprotegerin (OPG)-treated cells, as a positive control (Figure 2).

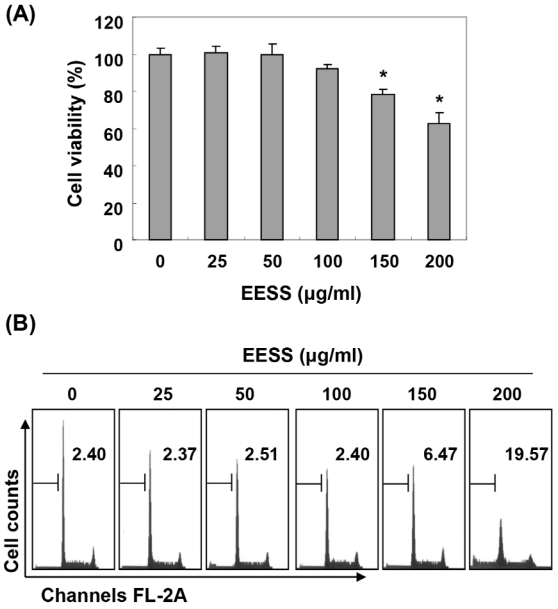

Figure 1. Effect of EESS on the cell viability in RAW 264.7 cells. Cells were treated with various concentrations of EESS for 48 h. **(A)** Cell viability was assessed by the MTT assay. The results are the means ± SD obtained from three independent experiments (*p < 0.05 compared with the control group). **(B)** To quantify the degree of apoptosis induced by EESS, the cells were evaluated by a flow cytometer to determine sub-G1 DNA content, which represents the cells undergoing apoptotic DNA degradation. The results are expressed as the mean of two different experiments.

3.3. *EESS disrupts the formation of F-actin ring structure in RANKL-stimulated RAW 264.7 cells*

To confirm the inhibitory effect of EESS on RANKL-induced osteoclastogenesis, the influence of EESS on formation of F-actin ring structures, known to beessential for bone resorption to occur (*42,43*), was investigated in this study. As shown in Figure 3, RANKL-treated RAW 264.7 cells showed the actin ring cytoskeletal structure; however, the size of the ring structure was markedly reduced in cells exposed to EESS compared with cells treated with RANKL alone, suggesting that EESS effectively inhibited osteoclast differentiation.

3.4. *EESS alleviates RANKL-induced NF-κB nuclear translocation and IκBα degradation in RAW 264.7 cells*

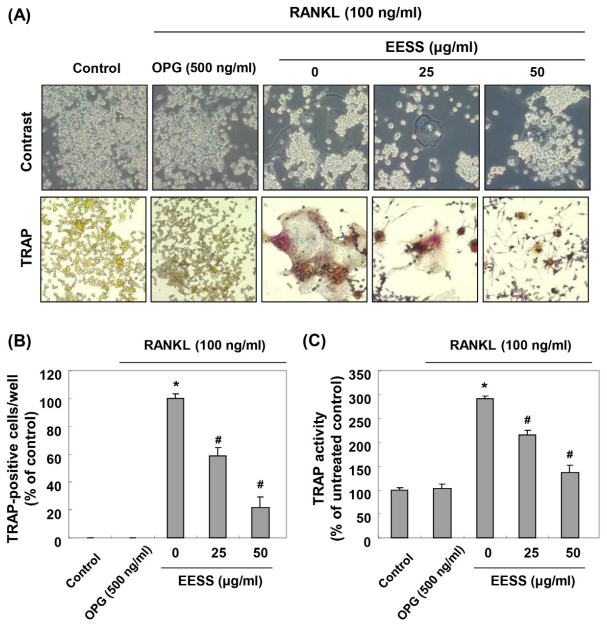

Figure 2. Inhibition of RANKL-induced osteoclast differentiation by EESS in RAW 264.7 cells. Cells were stimulated with 100 ng/mL RANKL in the presence or absence of EESS (25 and 50 μg/mL) for 5 days. Osteoprotegerin (OPG) was used as a positive control. **(A)** The fixed cells were stained for TRAP and identified using a microscope. **(B)** TRAP-positive multinucleated cells were counted to determine osteoclast numbers. **(C)** Supernatants were collected, and the TRAP activity was measured by an ELISA reader. **(B** and **C)** Each point represents the mean ± SD of three independent experiments (*$p < 0.05$ *vs.* the untreated control; #$p < 0.05$ *vs.* the RANKL-treated cells).

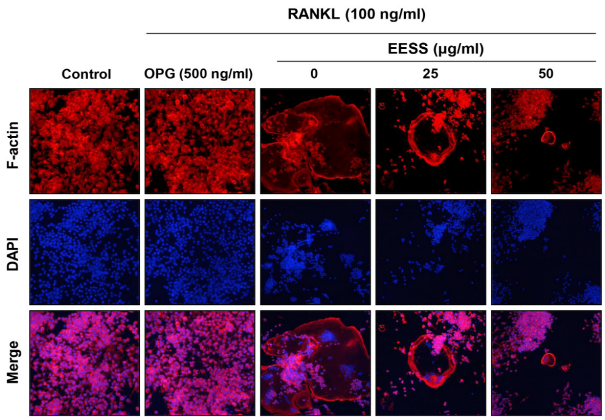

Figure 3. Suppression of F-actin ring formation by EESS in RANKL-stimulated RAW 264.7 cells. The cells were incubated with 100 ng/mL RANKL in the presence or absence of the indicated concentrations of EESS for 5 days. The cells were fixed and stained for F-actin ring with a FITC-phalloidin solution. Subsequently, the cells stained with DAPI solution and then imaged with a fluorescence microscope. OPG was used cells as a positive control.

We next determined whether EESS could attenuate RANKL-induced activation of NF-κB, which is critical for RANKL-mediated osteoclastogenesis (*13,14*). Immunoblotting data, using cytoplasmic and nuclear extracts, showed that the expression of the NF-κB p65 subunit in the nucleus was greatly increased after a

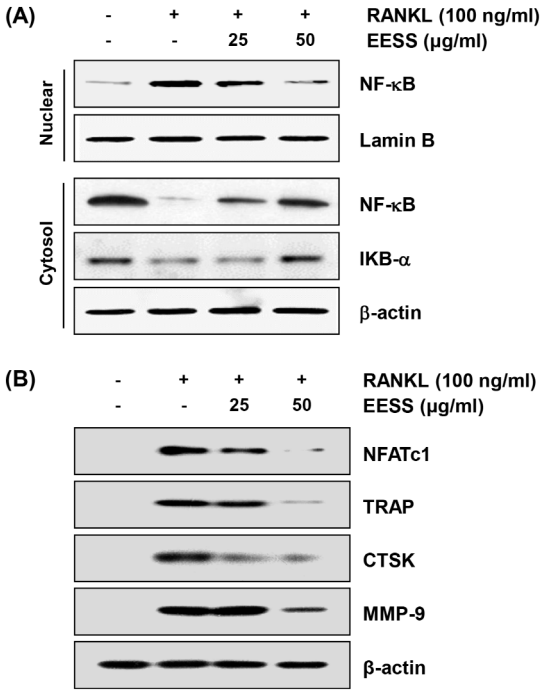

Figure 4. Effects of EESS on RANKL-induced activation of NF-κB and expression of osteoclast-specific genes in RAW 264.7 cells. (A) After 100 ng/mL RANKL treatment with or without EESS for 5 days, the nuclear and cytosolic proteins were isolated, and the expression of NF-κB and IκB-α was determined by Western blot analysis using an ECL detection system. Lamin B and β-actin were used as internal controls for the nuclear and cytosolic fractions, respectively. **(B)** The cellular proteins were isolated from cells cultured under the same conditions, and the expression of osteoclast-specific proteins was assessed by Western blot analysis. β-actin was used as an internal control. The results shown are representative of three independent experiments.

RANKL challenge, whereas there was a reduction in its expression in the cytoplasm. In addition, the expression of IκBα was reduced in the cytoplasm, indicating that NF-κB was activated by RANKL treatment (Figure 4A). However, the shift of NF-κB p65 to the nucleus induced by RANKL and the degradation of IκBα were abolished in a concentration-dependent manner in the presence of EESS, suggesting that EESS could inhibit the activation of NF-κB by RANKL.

3.5. *EESS suppresses the RANKL-induced expression of osteoclastogenesis-associated genes in RAW 264.7 cells*

We further investigated the effect of EESS on the expression of osteoclastic markers for the study of mechanisms involved in the inhibition of osteoclastogenesis by EESS. When RAW 264.7 cells were treated with RANKL, immunoblotting revealed that the major osteoclastogenesis factors, including nuclear factor of activated T cells cytoplasmic 1 (NFATC1), TRAP, cathepsin K (CTSK) and matrix metalloproteinase-9 (MMP-9), were dramatically upregulated in the cells (Figure 4B). However, the increased levels of these genes by RANKL were

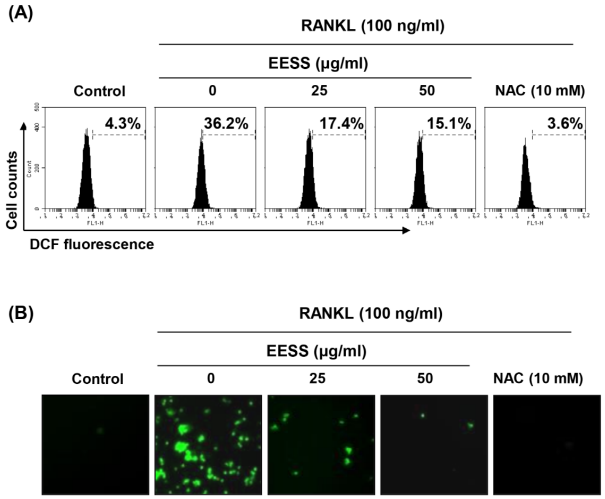

Figure 5. Effects of EECC on the RANKL-induced ROS generation in RAW 264.7 cells. The cells were treated with 100 ng/mL RANKL in the presence or absence of EESS (25 and 50 μg/mL). **(A)** The cells were incubated with DCF-DA, and DCF fluorescence was measured by flow cytometry. The values represent the means of two independent experiments. **(B)** After staining with DCF-DA, images were obtained using a fluorescence microscope. These images are representative of at least three independent experiments.

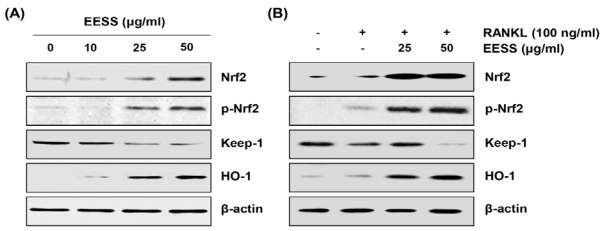

Figure 6. Effects of EECC on the expression on Nrf2 and its regulatory proteins in RAW 264.7 cells. The cells were treated with the indicated concentrations of EESS for 5 days **(A)** or treated with 100 ng/mL RANKL in the presence or absence of EESS (25 and 50 μg/mL) for 5 days **(B)**. The cellular proteins were probed with the indicated antibodies and were visualized using an ECL detection system. Equal protein loading was confirmed by analysis of β-actin. The results shown are representative of three independent experiments.

reduced by addition of EESS in a concentration-dependent manner.

3.6. *EESS attenuates RANKL-induced ROS production in RAW 264.7 cells*

As oxidative stress also plays a critical role in the osteoclast differentiation and bone resorption (*19,20*), we then investigated whether EESS could suppress RANKL-induced generation of ROS. Our flow cytometry results indicated that the level of ROS markedly increased in RANKL-stimulated cells; however, the increase in ROS content was reduced in a concentration-dependent manner by addition of EESS. Moreover, as expected, the co-treatment of N-acetyl cysteine (NAC), a ROS scavenger, completely blocked ROS production by RANKL (Figure 5A). The effect of preventing ROS formation was confirmed in our studies using a fluorescence microscope. Consistent with the results from flow cytometry, the increase in DCF-DA fluorescence intensity observed in the cells treated with RANKL was greatly weakened by pretreatment of EESS, as shown in Figure 5B.

3.7. *EESS enhances the expression of Nrf2 and HO-1 in RAW 264.7 cells*

Furthermore, the effect of EESS on the expression of Nrf2 and its regulatory gene heme oxygenase-1 (HO-1) was investigated because it is well known that activation of the Nrf2/HO-1 signaling pathway plays an important role in antioxidant activity for bone homeostasis (*26,44*). The immunoblotting results showed that the expression of Nrf2 and HO-1 protein gradually increased in a concentration-dependent manner on EESS treatment, but conversely, Kelch-like epichlorohydrin-associated protein 1 (Keap1) expression decreased with EESS treatment (Figure 6A). Phosphorylation at serine 40, in particular, is important for activation and stabilization of Nrf2, also increased with EESS treatment, demonstrating that EESS activated Nrf2/HO-1 signaling in RAW 264.7 cells. In addition, the expression and phosphorylation of Nrf2 in RANKL-treated cells were not increased or induced to a lower level than untreated control cells, its phosphorylation, as well as expression, were markedly elevated in cells co-treated with EESS and RANKL (Figure 6B). Furthermore, HO-1 expression was also much increased in treated cells compared to cells treated with EESS, and expression of Keap1 was further reduced, suggesting that the antioxidant effect of EESS may be related to Nrf2/HO-1 axis activation.

4. Discussion

In the current study, murine macrophage RAW 264.7 cell line was used to evaluate the effect of ethanol extract of *S. serratifolium* (EESS) on RANKL-induced osteoclast differentiation. We demonstrated that EESS could effectively attenuate RANKL-induced osteoclast differentiation by inhibiting TARP activity and F-actin ring formation accompanied by suppression of RANKL-induced expression of osteoclast-associated marker genes through inhibition of nuclear translocation of NF-κB. In addition, EESS abolished RANKL-induced oxidative stress by inhibiting ROS production related to the activation of Nrf2/HO-1 signaling pathway.

The destruction of metabolic balance of the bone is due to increase of bone resorption by osteoclasts rather than new bone formation by osteoblasts. RANKL is a key pro-osteoclastogenic cytokine that plays an essential role in the induction of osteoclast differentiation from hematopoietic cells of monocyte-macrophage lineage (*12,13*). Terminal differentiation into osteoclasts by

RANKL involves the following steps: 1) activation of TRAP, a representative osteoclast marker involved in bone resorption, and 2) formation of multinuclear osteoclasts by combining TRAP-positive cells with actin ring formation. To perform bone resorption by activated TRAP of mature osteoclasts, formation and maintenance of actin rings on the bone surface must be continued (*45,46*). Results of this study revealed that EESS could inhibit TRAP activation and F-actin ring formation by RANKL, suggesting that EESS might be able to inhibit bone resorption through blocking early stages of differentiation from precursor cells to osteoclasts.

As noted in many studies, NF-κB is a key transcription factor that plays a role in RANKL-induced osteoclast differentiation (*12,14*). Normally, NF-κB is retained in the cytoplasm in an inactive form coupled with IκB-α, an inhibitory subunit. When IκB-α is phosphorylated and degraded by a ubiquitin-dependent pathway upon binding of RANKL and RANK, NF-κB translocates from the cytosol into the nucleus and triggers transcriptional activation of several osteoclastogenesis-related genes (*13,14*). Results of the current study indicated that NF-κB translocation to the nucleus and cytoplasmic degradation of IκB-α were increased by RANKL stimulation. However, EESS reduced these changes. Therefore, blockade of nuclear translocation of NF-κB, an essential step in NF-κB activation, can be proposed as one of the mechanisms involved in EESS-mediated anti-osteoclastogenic effect.

Transcription factor NFATc1 as a master regulator in osteoclastogenesis. It also plays an important role in regulating the expression of osteoclast-specific genes upon RANKL signaling. Its expression is enhanced by binding to transcription factors including NF-κB on the promoter at early stage of osteoclast differentiation (*16,17*). Thus, genes regulated by NFATc1 including TRAP, CTSK, and MMP-9 are highly expressed at RANKL-induced terminal differentiation stage, thereby promoting bone resorption (*25,47*). Our results showed that RANKL-induced expression of NFATc1 was significantly suppressed by EESS in a concentration-dependent manner. Expression levels of most osteoclast-related marker genes were also reduced by EESS. Although further studies should be conducted to determine whether NF-κB plays a direct role in the regulation of NFATc1 expression, these results suggest that EESS-mediated blockade of NFATc1 expression associated with inhibition of NF-κB activation might play a central role in inhibiting osteoclast differentiation and bone resorption activity.

Up to date, targets of ROS that arise during osteoclast differentiation by RANKL signaling remain unclear. However, they certainly play important signaling roles in the process of osteoclastogenesis (*21,22*). Many previous studies have shown that ROS accumulation can increase the expression of RANKL in osteoclasts and enhance their survival and proliferation (*19,23,24*). In addition, ROS can act as upstream molecules for activation of NF-κB and NFATc1 to promote transcription of osteoclast-specific genes at the onset of osteoclastogenesis. On the other hand, excessive production of ROS will inhibit the differentiation of osteoblasts and induce death of osteocytes as well as osteocytes, leading to bone formation imbalance (*19,20*). Therefore, inhibiting or eliminating ROS production can prevent pathological causes of various diseases associated with functional activation of osteoclasts by oxidative stress. As can be seen from results of the present study, EESS could significantly weaken RANKL-induced accumulation of ROS. The strong ROS scavenging ability of EESS might be another mechanism involved in its inhibitory effect on osteoclast differentiation.

Accumulated evidence suggests that Nrf2 signaling pathway plays a critical role in protecting cells from oxidative damage by promoting the expression of antioxidant enzymes in most cells. It is also an attractive target in bone metabolism homeostasis (*26,44*). Under physiological conditions, Nrf2 binds to Keap1 and becomes sequestered in the cytoplasm. However, under a situation in response to oxidative stress, Nrf2 is disassociated from Keap1 and then translocates to the nucleus. In this process, phosphorylation of Nrf2 is accompanied. It is an essential step for transcriptional activation of its target genes. HO-1 is one of cytoprotective enzymes that act as a rate-limiting enzyme. It is regulated by Nrf2 and catalyzes the degradation of heme to biliverdin, carbon oxide, and iron (*44,48*). Previous studies have shown that increased expression of HO-1 by transcriptional activation of Nrf2 plays a central role in the removal of ROS generation by RANKL (*26,44*). In the present study, expression and phosphorylation of Nrf2 were significantly increased in cultured cells co-treated with RANKL and EESS compared to those in cells treated with EESS alone while the expression of Keap1 was reversely decreased. Moreover, the expression of HO-1 was significantly upregulated in RANKL and EESS co-treatment cells, indicating that EESS could activate the Nrf2/HO-1 antioxidant pathway. Although further experiments are needed to determine the inhibition of ROS production and activation of Nrf2/HO-1 axis, results of this study show that the Nrf2/HO-1 signaling pathway might contribute to the protective ability of EESS against RANKL-mediated oxidative stress.

In summary, our data demonstrate that EESS can suppress RANKL-induced osteoclast differentiation through inactivation of NF-κB and inhibition of NFATC-1 expression. EESS also attenuated RANKL-induced oxidative stress associated with activation of Nrf2/HO-1 signaling pathway. Results of the current study suggest that EESS might have therapeutic potential for treating bone loss-related disorders. However, additional experiments such as reassessment of the anti-osteoclastogenic potential of EEES using

animal models and validation of major bioactive components of EESS are required.

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