The Inhibitory Effects of Roasted Black Bean (*Rhynchosia nulubilis*) Extracts on RANKL-Mediated RAW264.7 Cells Differentiation

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Abstract This study compared the inhibitory effects of roasted black bean (*Rhynchosia nulubilis*, *seomoktae*, RoS) extracts with raw *seomoktae* (RaS: control) extract on RANKL-mediated RAW264.7 cell differentiation after the determination of isoflavones content as well as antioxidant and anti-inflammatory activities. The RoS extracts (RoS90-20, RoS100-20, RoS110-20, and RoS120-20) had significantly ($p < 0.05$) higher isoflavones (genistein, daidzein, and glycitein) and better antioxidant activity than the RaS extract. Especially, RoS110-20, which was roasted at 110°C for 20 min, has the highest amounts of soy-derived compounds. The RoS110-20 extract significantly reduced LPS-induced nitric oxide (NO) and prostaglandin (PGE2) production compared to the RaS extract. The RoS110-20 extract showed a higher inhibitory effect on the expression of NFATc1 in RANKL-induced RAW 264.7 cells than the RaS extract without any evidence of cytotoxicity under western blotting and tartrate-resistant acid phosphatase staining (TRAP). The results of this study suggest that roasting increased the concentration of soy-derived compounds and improved the antioxidant and anti-inflammatory activities of RoS110-20 extract compared with RaS. In addition, the differentiation of RANKL-mediated RAW264.7 cells was effectively inhibited by the RoS110-20 extract due to its enhanced antioxidant and anti-inflammatory activities caused by roasting, confirming its potential for ameliorating bone loss.

Keywords: soy-derived compounds, roasted *Rhynchosia nulubilis*, RAW264.7 cells

Introduction

Osteoporosis is a major cause of fractures that can lead to complications and eventually death, especially in older people. Postmenopausal women are more susceptible to osteoporosis because of estrogen deficiency (1). Estrogen increases calcium absorption and optimizes bone mineral density (BMD), thus preventing the development of osteoporosis (1).

Oxidative stress is a condition that involves the excess formation of free radicals by physiological processes. Recent studies provide important evidence that oxidative stress is a primary pathogenic mechanism for skeletal degradation (2–4).

Osteoclasts are formed by the fusion of homeostatic cells of monocyte-macrophage lineage during the early stages of differentiation (5). Osteoclasts play a crucial homeostatic role in the modeling, remodeling, and destruction of bones in many pathological conditions. Receptor activator of NF- κ B ligand (RANKL) is important for osteoclastogenesis (6).

RANK-RANKL signaling is essential for osteoclast differentiation during bone resorption (7). Osteoprotegerin (OPG) competes with

RANKL and prevents the maturation of pre-osteoclasts, thus, inhibits bone resorption (7).

Traditional hormone replacement therapy (HRT) is shown to have beneficial effects on osteoporosis in postmenopausal women. Despite these beneficial effects, uncertainty remains regarding the risk/benefit ratio of HRT because of the increased risk of estrogen-dependent reproductive cancers (8).

Soy-derived compounds (genistein, daidzein, and glycitein) are structurally similar to estrogen and bind to estrogen receptors (ERs). These compounds can also prevent oxidation, regulate the host immune system, and affect cellular signaling, especially genistein. Consequently, they can prevent osteoporosis with the production of OPG in the bone tissue (9).

The potential importance of phytoestrogens, especially steroid-like molecules derived from soybeans, such as black bean, in humans is being actively investigated. Isoflavones derived from *yak-kong* (*seomoktae*) or black bean has been traditionally used as estrogen substitutes for preventing postmenopausal osteoporosis in Chinese medicine (10). Despite numerous reports, studies to increase the isoflavone aglycone content with respect to the physical activity of

black bean have not been performed.

Thermal processing of food prolongs its life span and improves its quality (11). Heat treatment or roasting of plants increases the concentration of phenolic compounds by chemical transformation (12). Among various methods used for thermal processing, roasting improves the sensory qualities of food, such as flavor and color. Moreover, roasting results in the break down, synthesis, and condensation of several functional components in the food (11).

The effects of roasting on isoflavones and the antioxidant activities of soybeans were recently reported (11-13). However, few studies focused on the effect of roasting on improving antioxidant and anti-inflammatory activities for preventing or ameliorating bone loss. Therefore, this study was conducted to determine the isoflavones content, antioxidant activities using DPPH and ABTS radical scavenging activities (IC_{50}) and anti-inflammatory activities based on the production of LPS-induced NO and PGE2. In addition, the suppressive effects on RANKL-mediated RAW264.7 cell differentiation were determined using western blotting and tartrate-resistant acid phosphatase staining (TRAP).

Materials and Methods

Materials *Seomoktae* (*Rhynchosia nulubilis*) was purchased from Chorocmaeul Co. (Seoul, Korea). All solvents used in the chromatographic analysis were HPLC grade and were obtained from Thermo Fisher Scientific (Pittsburgh, PA, USA). Isoflavone standards (daidzein, genistein, and glycitein) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade. Na_2CO_3 , Tannic acid, Vitamin C, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Phosphate buffered saline (PBS) and Folin Ciocalteu's phenol reagent were purchased from Sigma-Aldrich. RAW 264.7 cells (Mouse leukemic monocyte macrophage cell line, ATCC TIB71™) were purchased from American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Cellgro (Manassas, VA, USA). 3-(4,5-Dimethylthiazol-yl)-diphenyl tetrazoliumbromide (MTT) was purchased from DUCHEPA Biochemie (Haarlem, Netherlands). The receptor activator of NF- κ B ligand (RANKL) was purchased from R&D System Inc., (Minneapolis, MN, USA). Tartrate-resistant acid phosphatase (TRAP) staining kit was purchased from Sigma-Aldrich. Modified RIPA buffer was purchased from Biosesang Inc., (Sungnam, Korea). Protease inhibitor cocktail was purchased from Roche, IN, USA. Recombinant mouse anti-NFATc1 was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. Mouse anti-beta actin was purchased from Cell Signaling, Danvers, MA, USA. Experimental details will be published elsewhere. Chemiluminescent reagent was purchased from Millipore Corp. (Burlington, MA, USA).

Thermal processing of *seomoktae* Before roasting, 500 g of

seomoktae was rinsed with water for 1 min and then strained for 30 s to remove the water. Roasting conditions (temperature: 90, 100, 110, and 120°C and time: 20 min) were set considering previous studies (11-13). Roasting was performed using a roaster (Proaster THCR-01; Taehwan Automation Industry Co., Bucheon, Korea).

Determination of the soy-derived compounds (genistein, daidzein, and glycitein) The roasted *seomoktae* (RoS: RoS90-20, RoS100-20, RoS110-20, and RoS120-20) and the unroasted *seomoktae* (RaS: control) were ground separately using a grinder (Caimano, ANFIM, Milan, Italy). The powdered samples were extracted with 70% aqueous ethanol (1:10, w/v) by vortexing for 2 h at room temperature and by centrifuging for 10 min at 10,620×g. The final extract was filtered through a 0.22 μ m PIPE filter unit (Millipore Corp.). The concentrations of the soy-derived compounds of genistein, daidzein, and glycitein in RaS and RoS extracts were determined using a method described by Coward *et al.* (14), with some modifications. The concentrations of genistein, daidzein, and glycitein were determined using a SUPELCO™ LC-18 column (particle size: 5 μ m, L×id: 250×4.6 mm, SUPELCO™, Bellefonte, PA, USA) with a flow rate of 1.0 mL/min. The linear gradient mobile phase was 1% (v/v) aqueous acetic acid solution. This was replaced using a 510 HPLC pump with gradient control of acetonitrile for 20 min in the linear gradient (Waters, Milford, MA, USA). In all, 20 μ L of the sample was injected (Rheodyne injector, Rheodyne LLC, Rohnert Park, CA, USA) through the column, and the concentrations of genistein, daidzein, and glycitein were qualified by measuring their absorbance at 254 nm with a UV/Vis detector (UV-2077; Jasco, Tokyo, Japan). Fluorescein was used as an internal standard.

Radical scavenging activity of DPPH and ABTS according to roasting condition After thermal processing, all the samples were ground and the resultant powder was extracted with water (1:20) for 3 h at 80°C by using a heating mantle (HM250C; Sercrim Lab Tech, Seoul, Korea). The final extract was filtered through a 0.22 μ m PIPE filter unit and was concentrated using a rotary vacuum evaporator (HS-2005S-N; Hahn Shin Scientific Co., Bucheon, Korea). Next, the samples were freeze-dried by storing in a deep freezer (WUF-500; Daihan Scientific Co., Wonju, Korea).

Radical scavenging activity of the samples was determined by using 1,1-diphenyl-2-picryl hydroxyl (DPPH) according to the method described by Naik *et al.* (15). Briefly, 10 μ L of 0.1 mM DPPH ethanol solution was mixed with 100 μ L of RaS and RoS extracts (RoS90-20, RoS100-20, RoS110-20, and RoS120-20), respectively and the mixture was maintained for 30 min at room temperature. The absorbance of the samples containing different concentrations of the extract was measured at 517 nm with ELISA reader (Tecan Infinite M200 Pro; GreenMate Bio, Seoul, Korea). The concentration of the extract that decreased the absorbance of DPPH by 50% (IC_{50}) was calculated.

Radical scavenging activity of the samples was determined by using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)

according to a method described by Jeong *et al.* (16). Briefly, 2.6 mM potassium phosphate was added to 7.4 mM ABTS solution, and the mixture was stored at -70°C for 24 h. Next phosphate-buffered saline (PBS) was diluted 15-fold and used as the ABTS reagent after absorbance at 732 nm (0.700 ± 0.03) was confirmed. Next, 50 μL aliquots of RaS and RoS extracts (RoS90-20, RoS100-20, RoS110-20, and RoS120-20) were added to 950 μL aliquots of the prepared ABTS reagent, respectively, and the solutions were left for 5 min at room temperature. The absorbance of the solutions was measured at 732 nm by using an ELISA reader (Tecan Infinite M200 Pro; GreenMate Bio). The concentration of the extract that decreased the absorbance of ABTS by 50% (IC_{50}) was calculated.

Cell culture Murine macrophage-derived RAW264.7 cells were purchased from American Type Culture Collection. The cells were grown overnight in Dulbecco's modified Eagle's medium (Cellgro) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO_2 , according to the manufacturer's instructions.

Sample preparation for cell culture Approximately 50 g of each sample was added to 1 L of distilled water and extracted for 3 h at 80°C by using a heating mantle attached to a reflux condenser. The extract was filtered through Whatman No. 2 filter paper and was concentrated under vacuum by using a rotary evaporator. The extracts were lyophilized by freeze drying at -70°C and were stored at the same temperature until further analysis. The extract was dissolved in PBS, then filtered and sterilized.

Cell viability RAW 264.7 cells were cultured at a density of 1×10^5 cells/well in a 96-well plate, then stabilized for 24 h under 5% CO_2 at 37°C . The cells were then treated with different concentrations (0, 10, 50, and 100 $\mu\text{g}/\text{mL}$) of RoS extracts (RoS110-20) or RaS extract. Following incubation for 24 h, the cells were mixed with MTT solution (120 $\mu\text{L}/\text{well}$) and cultured for an additional 2 h. Cell viability was determined based on measurement of the difference in absorbance at a wavelength of 570 nm (Vision Scientific, Daejeon, Korea). The reference wavelength was 600 nm.

Measurement of NO production NO production was assayed based on measurement of the amount of nitrite in the supernatant of cultured RAW 264.7 cells using Griess reagent. Briefly, cells were cultured at a density of 1×10^5 cells/well in a 96-well plate, then stabilized for 24 h under 5% CO_2 at 37°C . Cells were washed with phosphate-buffered saline (PBS), replaced with fresh media, and incubated with 1 $\mu\text{g}/\text{mL}$ LPS in the presence or absence of RoS110-20 extract or RaS extract at different concentrations (0, 10, 50, and 100 $\mu\text{g}/\text{mL}$). After additional 24 h incubation, the media were collected and analyzed for nitrite accumulation as an indicator of NO production by the Griess reaction (17). The absorbance was measured at 570 nm (Vision Scientific) and nitrite concentration was calculated.

Measurement of PGE2 production Raw 264.7 cells were cultured in 24-well plates then incubated with or without 1 $\mu\text{g}/\text{mL}$ LPS in the absence or presence of RoS110-20 or RaS extract at different concentrations (0, 10, 50, and 100 $\mu\text{g}/\text{mL}$). After 24 h incubation, the PGE2 concentration in the culture medium was determined using an enzyme linked immunosorbent assay (ELISA) kit (R&D Systems Inc.) according to the manufacturer's instructions.

Western blotting RAW264.7 cells co-treated with RANKL (40 ng/mL) and optimally roasted *seomoktae* (RoS110-20) extracts were lysed in modified RIPA buffer (150 mM NaCl [pH 7.5], 50 mM Tris-HCl, 2 mM EDTA and 1% sodium-deoxycholate) supplemented with protease inhibitors made of 1 mM phenylmethane-sulfonylfluoride, and protease inhibitor cocktail tablet (Roche, IN, USA) on ice. Protein extracts were clarified by centrifuging at $13,500 \times g$ and 4°C for 15 min. The supernatant was transferred to fresh micro-centrifuge tubes, and protein concentrations were determined using the bicinchoninic acid (BCA) Protein Assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein (25 μg) were resolved by performing SDS-polyacrylamide gel electrophoresis. After electrophoresis, the separated proteins were transferred on polyvinylidene difluoride membranes (Millipore Corp.) by using Trans-Blot SD semi-dry transfer cells (Bio-Rad, Hercules, CA, USA). The membranes were then washed with Tris-buffered saline solution containing 0.2% Tween 20 (TBST) and were blocked for 2 h in TBST containing 5% skimmed milk. Next, the membranes were incubated overnight with mouse anti-NFATc1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse anti-beta actin (Cell Signaling) primary antibodies at 4°C . After the membranes were washed with washing buffer, they were incubated with adequate secondary antibodies for 2 h at room temperature. Signals were detected using a chemiluminescent reagent (Millipore Corp.) according to the manufacturer's instructions.

Tartrate-resistant acid phosphatase staining Tartrate-resistant acid phosphatase (TRAP) staining was performed to confirm the western blotting results. RAW264.7 cells were seeded in a 6-well plate (cell density, 1×10^5 cells/well) and were incubated simultaneously with RANKL (40 ng/mL) and the extracts (RoS110-20 and RaS) for 1 day. After incubation, the cells were fixed in citrate acetone and 3.7% formalin for 10 min. The fixed cells were washed three times with DPBS and were stained using TRAP staining kit (Sigma-Aldrich), according to the manufacturer's instructions, to determine TRAP activity. Initially, the cells were treated with fast garnet GBC base/sodium nitrite (1:1, v/v) for 3 min. Next, the cells were incubated with substrate solution (2.5 mM naphthol AS-BI phosphate, 100 mM acetate solution [pH 5.0], and 50 mM tartrate solution) for 1 h at 37°C in a humid and light-protected incubator. Finally, the cells were washed 3 times with distilled water and were counter-stained with hematoxylin for 2 min. TRAP-positive cells were analyzed and photographed using a microscope (TS-100 inverted microscope; Nikon, Tokyo, Japan).

Fig. 1. Concentration of soy-derived compounds and free radical scavenging activity of roasted *seomoktae* against DPPH (IC₅₀) and ABTS (IC₅₀). RaS: not roasted *seomoktae*, 90°C: roasted at 90°C for 20 min, 100°C: roasted at 100°C for 20 min, 110°C: roasted at 110°C for 20 min, 120°C: roasted at 120°C for 20 min. Means with different letters (a-c) indicate significant ($p < 0.05$) differences among roasting conditions for each compound and antioxidant activity based on 1-way ANOVA followed by Duncan's multiple comparison test.

Statistical analysis All data ($n=3$) are expressed as mean $\pm$ standard deviation (SD). Differences among the groups were assessed using 1-way ANOVA followed by Duncan's multiple comparison tests. Correlation coefficients were analyzed by Pearson's correlation test. All analyses were conducted using Statistical Package for Social Sciences (SPSS) software for Windows (version 21.0; SPSS Inc., Chicago, IL, USA).

Result and Discussion

Concentrations of soy-derived compounds (genistein, daidzein, and glycitein) in RoS Soybeans contain isoflavones, a class of flavonoids that are well known for their beneficial properties. These flavonoids prevent many chronic diseases, including osteoporosis, and menopausal symptoms (18). The biological activity of isoflavones, which are structurally similar to 17-beta-estradiol and other synthetic estrogens (19), is presumed to be due to their ability to act as a weak estrogen and suppress the differentiation of osteoclasts by binding to estrogen receptors (ERs) because of their antioxidant activity (20).

The bioavailability of isoflavones is affected by their chemical forms in foods and their stability during processing (21). Interestingly, various soy isoflavones showed conversion after thermal treatment (21). Soy isoflavones include genistein, daidzein, and glycitein (to a lesser extent). The total isoflavone content in soy ranges from 10–300 mg/100 g of dry weight (22).

In this study, we first investigated the antioxidant activity and isoflavones content of *seomoktae* roasted at 90, 100, 110, and 120°C for 20 min. As shown in Fig. 1, thermal treatment at 110°C for 20 min (RoS110-20) significantly increased the concentrations of genistein, daidzein, and glycitein. However, further heating to 120°C decreased the concentrations of genistein, daidzein, and glycitein compared with the RoS110-20 sample. The concentrations of genistein, daidzein, and glycitein (300 mg/100 g, 380 mg/100 g, and 60.52 mg/100 g of dry weight, respectively) were significantly higher in the RoS110 sample than in the RaS sample and in samples roasted under different thermal conditions (RoS90-20, RoS100-20, and RoS120-20). This result indicated that thermal treatment at 110°C for 20 min (RoS110-20) effectively enhanced the concentration of soy-derived compounds (genistein, daidzein, and glycitein) in RoS samples.

Table 1. Correlation coefficients of soy-derived compounds of roasted *seomoktae* against free radical scavenging activities

Variables	Correlation coefficient value	
	DPPH (IC ₅₀)	ABTS (IC ₅₀)
Daidzein	-0.526* ¹⁾	-0.447*
Genistein	-0.473*	-0.507*
Glycitein	-0.534*	-0.541*

¹⁾* $p < 0.05$ significant difference among daidzein, genistein, DPPH (IC₅₀), and ABTS radical scavenging activity by Pearson's correlation test.

DPPH and ABTS radical scavenging activity (IC₅₀) of RoS DPPH radical scavenging activities (IC₅₀) of RoS (RoS90-20, RoS100-20, RoS110-20, and RoS120-20) and RaS samples are shown in Fig. 1. DPPH is a dark, crystalline powder. DPPH radicals interact with other radicals to produce a violet compound. Antioxidant compounds reduce the chemical interactions of DPPH. This decrease is determined by measuring the decrease in DPPH absorbance with a spectrophotometer. The DPPH assay is widely used to determine the antioxidant properties or radical scavenging activities of various compounds (23). The DPPH radical scavenging activity (IC₅₀) of the RoS110 sample (22 µg/mL) was superior to that of RaS (68 µg/mL), RoS90-20 (52 µg/mL), RoS100-20 (38 µg/mL), and RoS120-20 (21.34 µg/mL) samples. This was attributed to the increased concentrations of genistein, daidzein, and glycitein in the RoS110-20 sample.

The ABTS radical scavenging activities (IC₅₀) of RoS (RoS90-20, RoS100-20, RoS110-20, and RoS120-20) and RaS samples are shown in Fig. 1. The ABTS method is suitable for monitoring antioxidants such as genistein, daidzein, and glycitein (24). The ABTS radical scavenging activity (IC₅₀) of RoS110-20 sample (38.10 µg/mL) was superior to that of the RaS (78.71 µg/mL), RoS90-20 (60.10 µg/mL), RoS100-20 (58.00 µg/mL), and RoS120-20 (42.12 µg/mL) samples. Similar to the DPPH radical scavenging activity (IC₅₀), this result indicated increases in the concentrations of genistein, daidzein, and glycitein might play an important role in the enhanced ABTS radical scavenging activity (IC₅₀).

Correlation coefficients of soy-derived compounds and free radical scavenging activities

The activity of isoflavones (genistein, daidzein, and glycitein), which are structurally similar to 17- β -estradiol and synthetic estrogens (18), is presumed to be attributed to their ability to act as weak estrogens and bind to ERs as well as their antioxidant activity (19). The correlation between soy-derived compounds (genistein, daidzein, and glycitein) and antioxidant activities (IC₅₀ of DPPH and IC₅₀ of ABTS) was analyzed. The correlation coefficients for each comparison is shown in Table 1. Genistein, daidzein, and glycitein were negatively correlated with DPPH radical scavenging activity (IC₅₀) ($r = -0.526, -0.473$, and -0.534 , respectively) and ABTS scavenging activity (IC₅₀) ($r = -0.447, -0.507$, and -0.541 , respectively). Some studies have shown that the potent effects of black bean extracts may be closely related to their high antioxidant activity and isoflavone content compared with extracts of other legumes (25,26). In the present study, the RoS110-20 sample contained significantly higher amounts of genistein, daidzein, and glycitein and had higher antioxidant activity than the RaS extract. These results suggested that RoS110-20 extract, roasted at 110°C for 20 min, increased the health promoting properties by increasing the amounts of soy-derived compounds (genistein, daidzein, and glycitein) and the associated antioxidant activities.

Effects of roasted *seomoktae* on cell viability The effects of RoS extracts on the viability of RAW264.7 cells were investigated to evaluate the cytotoxic effects caused by roasting. To determine the cell survival rate after exposure to RoS extracts or RaS extract, the extracts were treated at different concentrations (0, 10, 50, and 100 µg/mL) for 24 h. For all the conditions, there was a survival rate of 94% or higher, confirming the absence of a cytotoxic effect on RAW 264.7 cells (Fig. 2).

Inhibitory effects of roasted *seomoktae* on NO and PGE2 production

After stimulation with LPS, large amounts of pro-inflammatory mediators, NO and prostaglandin E2 (PGE2) are generated (27). Nitric

Fig. 2. Effects of roasting samples on cell viability of RAW264.7 cells. RAW 264.7 cells were treated with roasting samples extract at different concentrations (0, 10, 50, and 100 µg/mL) for 24 h. Cell viability was tested using the MTT assay. RaS, not roasted *seomoktae*; RoS90-20, roasted at 90°C for 20 min; RoS100-20, roasted at 100°C for 20 min; RoS110-20, roasted at 110°C for 20 min; RoS120-20, roasted at 120°C for 20 min

Fig. 3. Inhibitory effects of roasting samples on LPS-stimulated NO production in RAW 264.7 cells. RAW 264.7 cells were treated with different concentrations of roasting samples (0, 10, 50, and 100 µg/mL) extract, followed by LPS (1 µg/mL) for 24 h. The culture supernatant was analyzed for NO production based on the Griess reaction, RaS, not roasted seomoktae; RoS110-20, roasted at 110°C for 20 min; Values are the mean±SEM of three independent Experiments. Significant differences between treated groups were determined using the Student's t-test, ### represents $p<0.001$ compared to the control, *** represents $p<0.001$ compared to the normal, +++ represents $p<0.001$ compared to the RoS110-20.

oxide (NO) is the major inflammatory mediator and its production is known to be related to inflammation (28). Therefore, the inhibition of NO production may have potential therapeutic value when related with inflammation. There have been several studies to investigate the inhibitory activity of flavonoids, a large family of plant constituents. Genistein, an isoflavone, shows mild inhibitory activity on NO production in LPS-activated RAW264.7 cells (29).

Figure 3 and 4 show that RoS 110-20 extracts (0, 10, 50, and 100 µg/mL) effectively inhibited the production of NO and PGE2 in LPS-activated RAW 264.7 cells compared with control extract, and did so in a dose-dependent manner. The inhibitory effect of RoS extracts on NO and PGE2 production may contribute to their anti-inflammatory activity. This result was attributed to increased amounts of isoflavones caused by roasting, confirming the potential of RoS extracts for ameliorating inflammation.

Amelioration effects of roasted seomoktae extracts on the expression of NFATc1 during RANKL-induced RAW 264.7 cells differentiation Osteoclasts are unique multinucleated cells responsible for bone resorption and are derived from hematopoietic stem cells (4). RANKL is a member of the tumor necrosis factor (TNF) family and binds to its receptor, RANK, expressed in osteoclast precursor cells (30). Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) is a downstream transcription factor in the RANKL/RANK signaling pathway and plays a key role in osteoclastogenesis by turning on a series of osteoclast-specific genes, including the gene encoding TRAP (31). Here, western blotting was used to determine the level of NFATc1 in RANKL-induced RAW 264.7 cells. TRAP staining was performed to confirm the results (Fig. 5). RoS110-20 extract

Fig. 4. Inhibitory effects of roasting samples on LPS-stimulated PGE2 production in RAW 264.7 cells. RAW 264.7 cells were treated with different concentrations of RaS or RoS110-20 (0, 10, 50, and 100 µg/mL) extract, followed by LPS (1 µg/mL) for 24 h. The culture supernatant was analyzed for PGE2 production using an enzyme linked immunosorbent assay (ELISA) kit. Control, not treated LPS; RaS, not roasted seomoktae; RoS110-20, roasted at 110°C for 20 min; Values are the mean±SEM of three independent Experiments. Significant differences between treated groups were determined using the Student's t-test, ### represents $p<0.001$ compared to the control, *** represents $p<0.001$ compared to the LPS, +++ represents $p<0.001$ compared to the RoS110-20.

reduced the formation of TRAP-positive multinucleated cells in a dose dependent manner compared with RaS extract (Fig. 5). This raises the possibility that RoS110-20 extract may inhibit RANKL-induced NFATc1 expression during RANKL-mediated RAW264.7 cells differentiation. It was concluded that RoS110-20 extract contains increased amounts of soy-derived compounds (genistein, daidzein, and glycitein), which might decrease bone resorption by enhancing antioxidant and anti-inflammatory activities during RANKL-mediated RAW264.7 cells differentiation.

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Disclosure An author declare no conflict of interest.

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Fig. 5. Effects of RoS110-20 compared with RaS on the expression of NFATc1 and TRAP in RANKL stimulated RAW 264.7 cells. RAW 264.7 cells were treated with different concentrations of RoS110-20 (0, 10, 50, and 100 µg/mL) extract compared with RaS (0, 10, 50, and 100 µg/mL) extract, followed by RANKL (40 ng). (A) western blotting with primary antibody against NFATc1 and β -actin, (B) TRAP, Normal: not treated RANKL, RaS: not roasted seomoktae, RoS110-20: roasted at 110°C for 20 min, Values are the mean $\pm$ SEM of three independent Experiments. Significant differences between treated groups were determined using the Student's t-test, ^{###} represents $p < 0.001$ compared to the normal, ^{***} represents $p < 0.001$ compared to the control, ⁺⁺⁺ represents $p < 0.001$ compared to the RoS110-20.

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