

# *Anemarrhena asphodeloides* Bunge ameliorates osteoporosis by suppressing osteoclastogenesis

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**Abstract.** *Anemarrhena asphodeloides* Bunge has been traditionally used in Korean medicine for its antipyretic, diuretic, sedative, and antitussive effects. In the present study, the effects of an ethanol extract of *A. asphodeloides* Bunge (AAB) on osteoporosis and its underlying mechanisms on bone remodeling were investigated. Osteoporosis was induced in ICR strain mice by ovariectomy. The mice were divided into four groups: Sham, ovariectomized, 17 $\beta$ -estradiol and 100 mg/kg AAB. The treatment was continued for 4 weeks. Bone mineral density (BMD) and bone mineral content (BMC) were measured using dual-energy X-ray absorptiometry. In addition, Raw 264.7 cells were treated in the presence of 0.1, 1 and 10  $\mu$ g/ml AAB with 100 ng/ml receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) to induce osteoclast formation and stained with tartrate resistant acid phosphatase. In addition, levels of osteoclast-related factors were analyzed to investigate the signaling cascades in osteoclasts. The results demonstrated that AAB treatment reversed the decreases of both BMD and BMC in osteoporotic femurs. Additionally, the formation of osteoclasts was significantly suppressed by the AAB treatment in RANKL-stimulated Raw 264.7 cells. Compared with cells treated with RANKL alone, the AAB-treated osteoclasts had significantly decreased tumor necrosis factor- $\alpha$  and interleukin-6. The protein levels of c-fos were also decreased in the AAB-treated osteoclasts. Furthermore, the RANKL-induced nuclear translocation of nuclear factor- $\kappa$ B was attenuated in osteoclasts by the AAB treatment compared with cells treated with RANKL alone. Finally, AAB treatment downregulated the phosphorylation of mitogen-activated protein kinases. The

present results demonstrated that AAB exhibited ameliorative effects on osteoporosis by inhibiting osteoclastogenesis, and suggested that AAB may be a potential candidate for the treatment of osteoporosis.

## Introduction

Osteoporosis is a skeletal disease, which has the characteristics of decreased bone mass and deterioration of bone tissue (1). Increased bone fragility and fracture risk in patients with osteoporosis leads to disability, diminution of quality of life and premature death. In addition, the ever-rising population of the elderly is leading to a socioeconomic burden estimated to be \$25.3 billion each year (2).

There are many reasons for the loss of bone, including genetics, age, nutrition and lifestyle (3). In particular, the deficiency of sex hormones is closely associated with the increased activity of osteoclasts and loss of bone (4). The homeostasis of the bone matrix is maintained between the formation of the bone matrix by osteoblasts and bone resorption by osteoclasts (5). With age, the homeostatic balance of the bone shifts towards osteoclasts (6). Because estrogen has a role promoting apoptosis of osteoclasts and is involved in the differentiation of osteoblasts, its deficiency leads to greater osteoporotic changes in menopausal women (7). Therefore, the prevalence of osteoporosis is higher in women aged 50 years and older (8).

Currently, the treatments for osteoporosis include hormone replacement therapy (HRT), bisphosphonates, recombinant human parathyroid hormone (PTH) and supplements of Calcium and Vitamin D (9). Due to the critical roles of sex hormones in osteoporosis, HRT used to be the primary treatment for osteoporosis. However, no net benefits were observed between fracture risk reduction and burden to other parts of the body according to the results from the Women's Health Initiative, in which increased risk of stroke, coronary heart disease, pulmonary embolism and invasive breast cancer was the outcome (10). Calcium and Vitamin D supplements were the conventional first step to osteoporosis; however, the evidence was deemed insufficient by the US Preventive Task Force in 2013 to assess their benefits and/or potential harm (11). In addition, bisphosphonate is reported to inhibit bone resorption by osteoclasts. However, there is controversy over the use of bisphosphonates in the treatment of osteoporosis, especially due to incidences of unexpected serious

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adverse events, such as osteonecrosis of the jaw and atypical fracture of the femur (12). In summary, the effects of the current treatments for osteoporosis are beneficial in some and deleterious in others (13), requiring further studies. Traditional herbal medications exhibiting therapeutic effects against osteoporosis in both clinical and experimental conditions could be used as novel treatments.

The rhizome of *Anemarrhena asphodeloides* Bunge (Liliaceae) has been traditionally used in Asia for its antipyretic, diuretic, sedative, and antitussive effects (14). The steroidal saponins from *A. asphodeloides* Bunge exhibit anti-osteoporotic effects by increasing bone formation in ovariectomized (OVX) rats (15). Bu-Shen-Ning-Xin Decoction, a Traditional Chinese Medicine formula containing *A. asphodeloides* and seven other herbs has been demonstrated to have some positive effects in suppressing osteoclast differentiation (16). Nevertheless, the efficacy of *A. asphodeloides* on the amelioration of osteoporosis and its mechanisms of action remain unclear. In the present study, the effects of an *A. asphodeloides* Bunge extract on osteoporotic indexes were assessed and its mechanisms on osteoclast and osteoblast remodeling were determined.

## Materials and methods

**Sample preparation.** The rhizome of *A. asphodeloides* was purchased from Jung-do Herb (Seoul, Korea). Fifty grams of the herb were soaked in 500 ml of 70% ethanol for 24 h. The solvent was separated, evaporated and vacuum dried using a freeze-dryer to obtain the extracted powder. The extract of *A. asphodeloides* Bunge (termed here AAB) was 15.11 g, indicating that the yield was 30.22%. A voucher specimen (OP-AAB70) was deposited at our laboratory.

**Animal experiments.** Six-week-old female ICR strain mice were provided by RAONBIO Inc. (Yongin, Korea) and adapted for 1 week prior to the experiment. The mice were housed at a temperature of 20±2°C and a humidity of 50±5% under a 12-h light/dark cycle. The experimental protocols were approved by the Institutional Animal Ethics Committee of Kyung Hee University in Korea [approval no. KHUASP(SE)-15-093].

A total of 28 mice (n=7) were under the anesthetic Zoletil (Virbac Lab, Carros cedex, France). A total of 21 mice were surgically OVX, while seven normal mice in the sham group were subjected to sham surgery. To recover and induce post-menopausal osteoporosis, all mice were left for 7 weeks. After that, the mice were divided into 4 groups: Sham, sham-operated mice receiving daily oral PBS as the normal control; OVX, OVX mice receiving daily oral PBS as the negative control; E2, OVX mice receiving intraperitoneal injection of 17β-estradiol (E2) as the positive control; AAB, OVX mice receiving orally AAB. The Sham and OVX groups were administrated orally with 100 µl of vehicle PBS daily. The experimental AAB group was administrated orally with 100 mg/kg of AAB daily. This dose of AAB was selected based on previous references regarding the effect of the medicinal herb on osteoporosis (17-19). The E2 group was intraperitoneally injected with 10 µg/kg E2 daily. All treatments were continued daily for 4 weeks. Subsequently, all the mice were sacrificed.

**Measurement of bone mineral density (BMD) and bone mineral content (BMC).** The proximal femurs were excised and fixed in 10% neutralized formalin for 18 h. To analyze the BMD and BMC, the connective tissue was cleanly detached from the femurs. The collected bone tissues were scanned by dual-energy X-ray absorptiometry with the InAlyzer instrument (MEDIKORS, Seoul, Korea).

**Cell culture.** Murine macrophage-like Raw 264.7 cells (Korean Cell Line Bank, Seoul, Korea) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 100 units/ml penicillin until confluence. The cells were incubated at 37°C under an atmosphere of 5% CO<sub>2</sub> in a 100 mm culture dish. All cells were passaged no more than 10 times.

**Cell viability assay.** To assess the cell cytotoxicity of AAB, a cell viability assay was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Raw 264.7 cells were seeded in 96-well plates. Each well was treated with various concentrations of AAB (0.1, 1 and 10 µg/ml) suspended in DMEM culture medium for 24, 48 and 72 h. Then, 2 mg/ml of MTT solution was added to form formazan crystals. Following incubation, dimethyl sulfoxide was added and cell viability was measured at an absorbance of 570 nm using a microplate reading instrument (BioTek Instruments, Inc., Winooski, VT, USA). Cell viability was calculated as a % relative to untreated cells. The experiments were performed three independent times for reproducibility.

**Osteoclast differentiation in vitro.** To differentiate from macrophage-like cells to osteoclasts, Raw 264.7 cells were cultured with 100 ng/ml receptor activator of nuclear factor κB ligand (RANKL) in α-minimal essential medium supplemented with 10% heat inactivated FBS for 7 days. ABB (0.1, 1 and 10 µg/ml) was added into the differentiation media during those 7 days. Fresh differentiation media containing RANKL and/or AAB was replaced on day 3. The cells were fixed with 10% neutralized formalin and stained with acid phosphatase, leukocyte kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. The total tartrate-resistant acid phosphatase (TRAP) activity was measured at an absorbance of 405 nm using a microplate-reading instrument. The experiments were performed three independent times for reproducibility.

**ELISA.** Osteoclasts treated with or without AAB were cultured with RANKL for 7 days, as aforementioned. The supernatants were collected and cleared by centrifugation at 27,000 x g for 10 min. The concentrations of interleukin (IL)-6 and tumor necrosis factor (TNF)-α were determined by ELISA kits (BD Biosciences, San Jose, CA, USA; cat. nos. 555240 for IL-6 and 555268 for TNF-α, respectively) according to the manufacturer's instructions. Cytokine levels were estimated at an absorbance of 450 nm using a microplate-reading instrument.

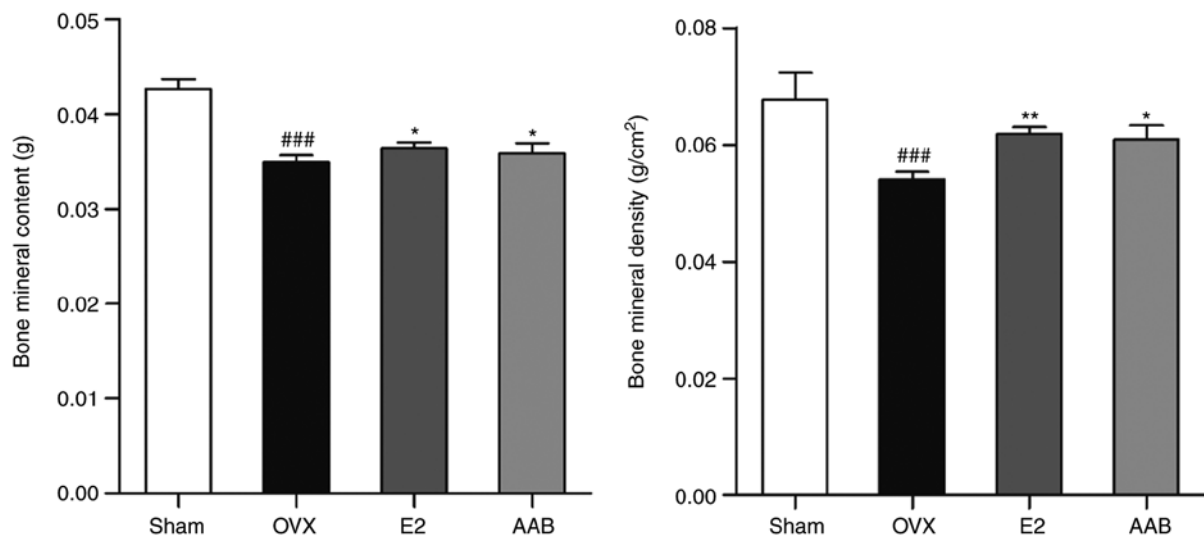


Figure 1. Effects of AAB on the BMC and BMD in OVX mice. BMC and BMD were measured in the proximal femurs of the experimental mice. Results are presented as mean  $\pm$  standard error of the mean. <sup>###</sup> $P < 0.001$  vs. Sham group; <sup>\*</sup> $P < 0.05$  and <sup>\*\*</sup> $P < 0.01$  vs. OVX group. AAB, *Anemarrhena asphodeloides* Bunge extract; BMC, bone mineral content; BMD, bone mineral density; OVX, ovariectomized; E2, 17 $\beta$ -estradiol.

**Western blot analysis.** Osteoclasts treated with or without AAB (0.1, 1 and 10  $\mu$ g/ml) were cultured with RANKL for 7 days, as aforementioned. Total protein lysates were extracted from the cultured cells with commercial lysis buffers. Radioimmunoprecipitation assay buffer (Tech & Innovation, Gangwon, Korea) was used for total protein extraction and a ReadyPrep protein extraction kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for cytoplasmic and nuclear protein extraction. All procedures for protein extraction were performed according to the manufacturer's instructions. The obtained cell lysates were used to determine the concentration of protein by Bradford assay. Protein samples (20  $\mu$ g) were subjected to SDS-PAGE on a 10% gel and transferred to polyvinylidene fluoride membranes. To block nonspecific sites, the membrane was incubated with 5% bovine serum albumin (Bio-Rad, Laboratories, Inc.) in a mixture of TBS and Tween 20 (TBS-T). Primary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA), against  $\beta$ -actin (cat. no. 3700), nuclear factor (NF)- $\kappa$ B (cat. no. 8242), NF- $\kappa$ B inhibitor  $\alpha$  (I $\kappa$ B- $\alpha$ ; cat. no. 4812), phosphorylated (p)-I $\kappa$ B- $\alpha$  (cat. no. 5209), Fos proto-oncogene (c-fos; cat. no. 4384) and LaminB (cat. no. 12255), diluted 1:1,000 in TBS-T, and extracellular signal-regulated kinase (ERK; cat. no. 4695), p-ERK (cat. no. 4370), c-Jun N-terminal kinase (JNK; cat. no. 9252), p-JNK (cat. no. 9251), p38 (cat. no. 9212) and p-p38 (cat. no. 9211), diluted 1:1,500 in TBS-T, were incubated with the membranes at 4°C overnight. Anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies diluted 1:4,000 in TBS-T (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. nos. sc-2357 and sc-516102, respectively) were used to bind to the primary antibody. After incubation for 2 h, enhanced chemiluminescence detection reagent (Amersham; GE Healthcare, Chicago, IL, USA) was added to visualize the protein bands.  $\beta$ -actin was used as an internal loading control for c-fos. LaminB was used as an internal reference protein for nuclear NF- $\kappa$ B. The total level of each total I $\kappa$ B- $\alpha$  and ERK was used as internal loading controls for phosphorylated forms. The band density

was quantified with ImageJ (National Institutes of Health, Bethesda, MD, USA) (20). The experiments were performed three times independently for reproducibility.

**Statistical analysis.** All data are expressed as the mean  $\pm$  standard error of the mean. Significance was determined by one-way analysis of variance, followed by Turkey's multiple comparison tests using GraphPad Prism 5 software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**AAB reverses the decrease of BMD and BMC in the osteoporotic femur.** In the osteoporotic femurs, there were significant decreases of the BMD and BMC levels compared with normal femurs ( $P < 0.001$ ; Fig. 1). In terms of the BMD, there was a 20.3% decrease in the animals of the OVX group compared with the sham group (Sham,  $0.068 \pm 0.005$  g/cm<sup>2</sup>; OVX,  $0.054 \pm 0.001$  g/cm<sup>2</sup>). The femurs of the AAB-treated mice exhibited a 12.7% recovery of the BMD compared with the OVX-induced osteoporotic femurs (E2,  $0.061 \pm 0.001$  g/cm<sup>2</sup>; AAB,  $0.061 \pm 0.002$  g/cm<sup>2</sup>). The BMC level in the OVX group was ~18.2% decreased compared with the sham group (Sham,  $0.0427 \pm 0.0009$  g; OVX,  $0.0349 \pm 0.0007$  g). AAB treatment significantly ameliorated the decrease of the BMC, to levels similar to the positive control group (E2,  $0.0365 \pm 0.0006$  g; AAB,  $0.0359 \pm 0.0010$  g).

**AAB inhibits the formation of osteoclasts in RANKL-stimulated Raw 264.7 cells.** RANKL stimulation resulted in a significant induction of osteoclast formation activity in Raw 264.7 cells. TRAP-positive multinucleated cells were increased by 38.8% in the RANKL-stimulated cells compared with control cells (Fig. 2A). However, AAB treatment exhibited inhibitory effects on osteoclastogenesis. AAB treatment (0.1, 1 and 10  $\mu$ g/ml) decreased the formation of osteoclasts in

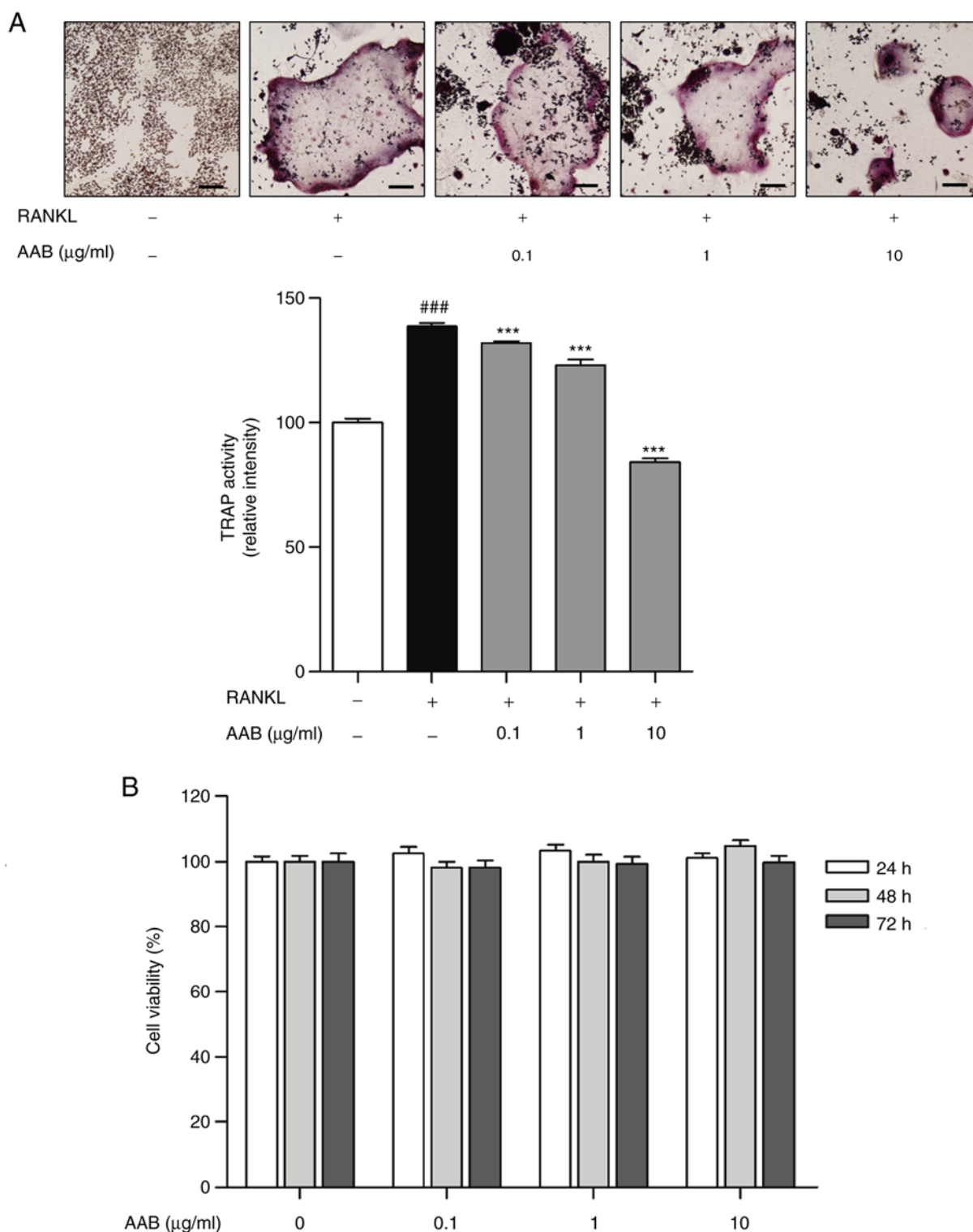


Figure 2. Effects of AAB on osteoclast formation in RANKL-induced Raw 264.7 cells. (A) Representative images and quantification of TRAP staining, indicating multi-nuclei osteoclast formation (scale bar, 200 µm; magnification, x100). (B) Cell viability was measured by MTT assay. Results are presented as mean ± standard error of the mean. ###P<0.001 vs. untreated cells; \*\*\*P<0.001 vs. RANKL-treated cells. AAB, *Anemarrhena asphodeloides* Bunge extract; RANKL, receptor activator of nuclear factor κB ligand; TRAP, tartrate-resistant acid phosphatase.

a dose-dependent manner, by 4.9, 11.2 and 39.4%, respectively, compared to the RANKL-treated cells (Fig. 2A). In addition, AAB showed no cytotoxicity at all the concentrations tested (0.1, 1 and 10 µg/ml) in the Raw 264.7 cells (Fig. 2B), indicating that AAB inhibited osteoclastogenesis without any toxic effects on the cells.

*AAB inhibits the RANKL-induced TNF-α and IL-6 production.* RANKL-induced osteoclasts exhibited an obvious increase of pro-inflammatory cytokines related with osteoclastogenesis. The levels of TNF-α were increased by ~23.2-fold following RANKL stimulation in the Raw 264.7 cells (untreated cells, 3.16±0.52 pg/ml; RANKL-treated cells, 73.26±12.58 pg/ml;

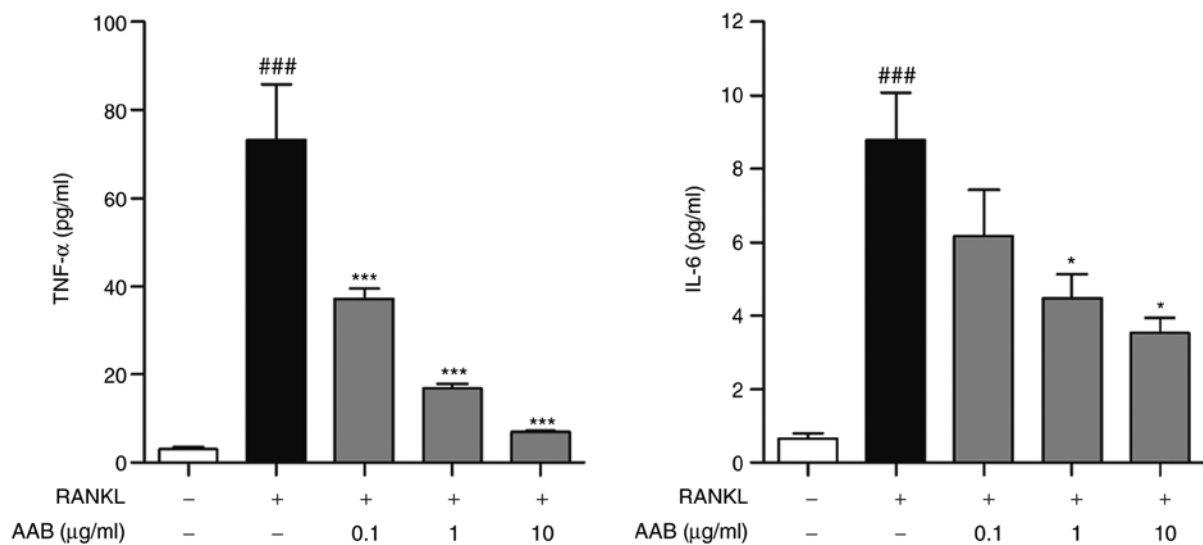


Figure 3. Effects of AAB on RANKL-induced TNF- $\alpha$  and IL-6 production. Raw 264.7 cells were treated with RANKL and/or AAB. Cytokine levels were measured in the supernatants by ELISA. Results are presented as mean  $\pm$  standard error of the mean. ###P<0.001 vs. untreated cells; \*P<0.05 and \*\*\*P<0.001 vs. RANKL-treated cells. AAB, *Anemarrhena asphodeloides* Bunge extract; RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-6, interleukin-6.

Fig. 3). In terms of the IL-6 levels, there was a ~13.3-fold increase in the RANKL-induced osteoclasts (untreated cells,  $0.66 \pm 0.14$  pg/ml; RANKL-treated cells,  $8.79 \pm 1.29$  pg/ml; Fig. 3). AAB treatment dose-dependently decreased both the TNF- $\alpha$  and IL-6 levels (Fig. 3). Compared with cells treated with RANKL alone, the 0.1, 1 and 10  $\mu$ g/ml AAB-treated osteoclasts exhibited a significant inhibition of TNF- $\alpha$  ( $37.31 \pm 2.26$ ,  $16.85 \pm 1.11$  and  $6.97 \pm 0.32$  pg/ml, respectively). In addition, RANKL-induced IL-6 production in Raw 264.7 cells was significantly reduced by AAB at the 0.1, 1 and 10  $\mu$ g/ml concentrations ( $6.19 \pm 1.25$ ,  $4.50 \pm 0.64$  and  $3.55 \pm 0.39$  pg/ml, respectively).

**AAB inhibits the RANKL-induced NF- $\kappa$ B nuclear translocation.** Based on a previous report (21), treatment with RANKL induces NF- $\kappa$ B translocation into the nucleus and I $\kappa$ B- $\alpha$  phosphorylation in the cytoplasm. In the present study, AAB treatment significantly inhibited the RANKL-induced NF- $\kappa$ B translocation into the nucleus (Fig. 4). In addition, p-I $\kappa$ B- $\alpha$  protein expression was significantly decreased following AAB treatment compared with the RANKL alone-treated cells (Fig. 4).

**AAB inhibits the RANKL-induced osteoclast-specific transcription factor expression.** As presented in Fig. 5, the protein expression levels of c-fos were upregulated in response to RANKL stimulation compared with untreated cells. AAB co-treatment in the presence of RANKL significantly reduced the expression of c-fos in the Raw 264.7 cells (Fig. 5).

**AAB inhibits the RANKL-induced mitogen-activated protein kinase (MAPK) pathway activation.** The phosphorylation status of the MAPKs, including ERK, JNK and p38, was assayed in the RANKL-induced osteoclasts by western blot analysis. RANKL stimulation resulted in significant increases in the phosphorylated levels of all three MAPKs, compared with untreated cells (Fig. 6). AAB treatment significantly

reversed the MAPK activation, by decreasing the protein expression levels of phosphorylated ERK, JNK and p38 compared with the RANKL alone-treated cells (Fig. 6).

## Discussion

Bone loss and the destruction of bone structure are the crucial hallmarks of osteoporosis. Osteoporotic patients have a lower BMD and BMC compared with healthy individuals. The WHO defines osteoporosis as a BMD <2.5 standard deviations from the average value (22). BMD is not the only the diagnostic criterion, but it also serves as valuable information to predict and prevent fractures in both genders (23). In addition, a low BMD has been reported to be associated with higher risk for almost every type of fracture (24). Several studies have demonstrated that OVX surgery results in a decreased BMD and BMC in a rodent model, and therefore the OVX rodent is a widely used model for postmenopausal loss of bone (25). In the present study, AAB was demonstrated to exhibit positive effects on preventing bone loss in a mouse model of osteoporosis, by ameliorating the BMD and BMC of the femurs.

The development and maintenance of bone are regulated by the continuous remodeling of the bone coordinated by the actions between osteoclasts and osteoblasts (26). Aging increases the stromal/osteoblast cell induced osteoclast genesis and expands its pool of precursors (27). Therefore, it promotes bone absorption and decreases bone formation. To explain the mechanisms underlying the anti-osteoporotic effects of AAB, the effects of AAB on bone metabolic cells including osteoclasts and osteoblasts were investigated.

Osteoclasts are capable of not only absorbing bone, but also making intimate connections with the bone-generators, such as osteoblasts, and cells from the immune system (28). Osteoclasts are specialized, multinucleated cells originating from the monocyte and macrophage lineage. They adhere to the bone matrix and degrade it through secretion of acids and enzymes (29). During osteoclastogenesis, there are two

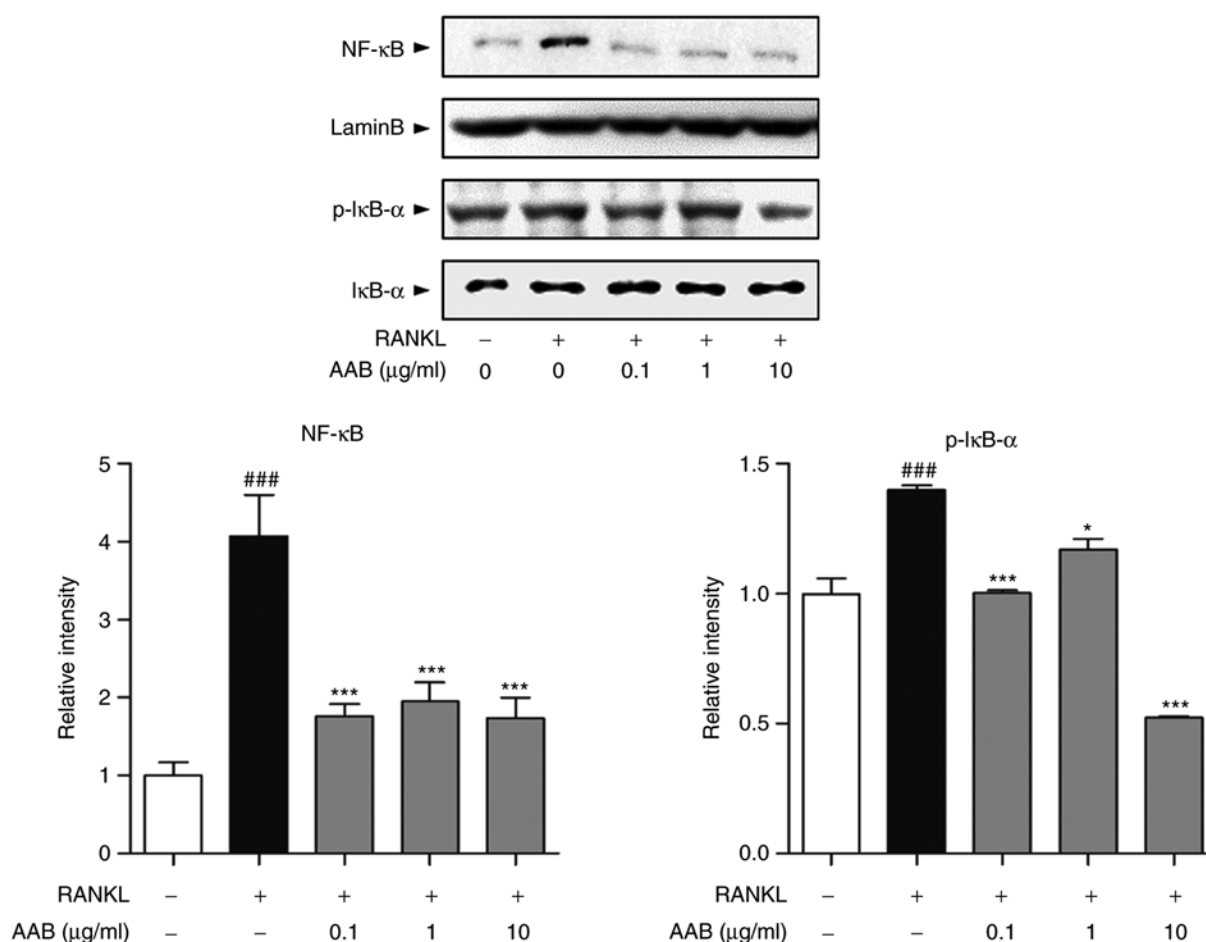


Figure 4. Effects of AAB on RANKL-induced NF- $\kappa$ B nuclear translocation and I $\kappa$ B- $\alpha$  phosphorylation. Raw 264.7 cells were treated with RANKL and/or AAB, and the expression levels of the indicated proteins were measured by western blotting. Intensities were calculated relative to LaminB for NF- $\kappa$ B, and relative to total I $\kappa$ B- $\alpha$  for p-I $\kappa$ B- $\alpha$ . Results are presented as mean  $\pm$  standard error of the mean. <sup>###</sup> $P < 0.001$  vs. untreated cells; <sup>\*</sup> $P < 0.01$  and <sup>\*\*\*</sup> $P < 0.001$  vs. RANKL-treated cells. AAB, *Anemarrhena asphodeloides* Bunge extract; RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; NF- $\kappa$ B, nuclear factor- $\kappa$ B; I $\kappa$ B- $\alpha$ , NF- $\kappa$ B inhibitor  $\alpha$ ; p-, phosphorylated.

factors that are required, RANKL and the colony-stimulating factor-1 (30). The most central and critical regulator of osteoclasts is the RANKL/RANK pathway with its decoy receptor OPG. The binding of RANKL to RANK leads to osteoclast activation and differentiation through multiple signaling pathways (31). Mature osteoclasts can be observed by staining for their histochemical marker TRAP (32). In the present study, cells treated with AAB had significantly suppressed TRAP activity in a dose-dependent manner, suggesting that AAB inhibited the RANKL-induced osteoclast formation from its precursors without any cytotoxicity.

During the differentiation of osteoclasts, the inflammatory cytokine TNF- $\alpha$  mediates the stimulation of the RANK by its ligand through an autocrine mechanism (33). TNF- $\alpha$  also stimulates the production of IL-6 in osteoblasts, which has synergistic effects in the bone resorption activity with TNF- $\alpha$ , regardless of OPG (34). The current results demonstrated that the production of TNF- $\alpha$  and IL-6 were reduced following AAB treatment in a dose-dependent manner, indicating that AAB may have inhibitory effects on inflammatory cytokines during osteoclast differentiation.

NF- $\kappa$ B is a transcription factor with pleiotropic features involved in the formation, action and survival of

osteoclasts (35). The regulation of NF- $\kappa$ B is critically related to the I $\kappa$ B- $\alpha$  kinase complex, with phosphorylation of I $\kappa$ B- $\alpha$  being a central factor in the activity of NF- $\kappa$ B (36). The degradation of I $\kappa$ B- $\alpha$  releases NF- $\kappa$ B, activating its translocation into the nucleus (37). In the present study, AAB was demonstrated to inhibit NF- $\kappa$ B activation and I $\kappa$ B- $\alpha$  phosphorylation in RANKL-induced osteoclasts. The RANK signaling pathway also involves the MAPK cascades, ERK, JNK and p38 (38). The MAPK cascades together promote osteoclast activation. The MAPKs are also associated with osteoclast formation through activation of the transcription factor AP-1 complex, which contains Fos and Jun (39). c-fos serves as the critical switch component in control of osteoclast differentiation from its progenitor (40). In the present study, AAB was demonstrated to have inhibitory effects on c-fos and the MAPKs, indicating that osteoclastogenesis is the target of the ameliorative effects of AAB in osteoporosis by regulating related factors, such as cytokines, NF- $\kappa$ B, MAPKs and c-fos.

In summary, AAB had ameliorative effects on osteoporosis *in vivo* and *in vitro*. *In vivo*, AAB treatment improved BMD and BMC levels, while *in vitro*, AAB treatment inhibited several osteoclastogenic markers, including TNF- $\alpha$ , IL-6,



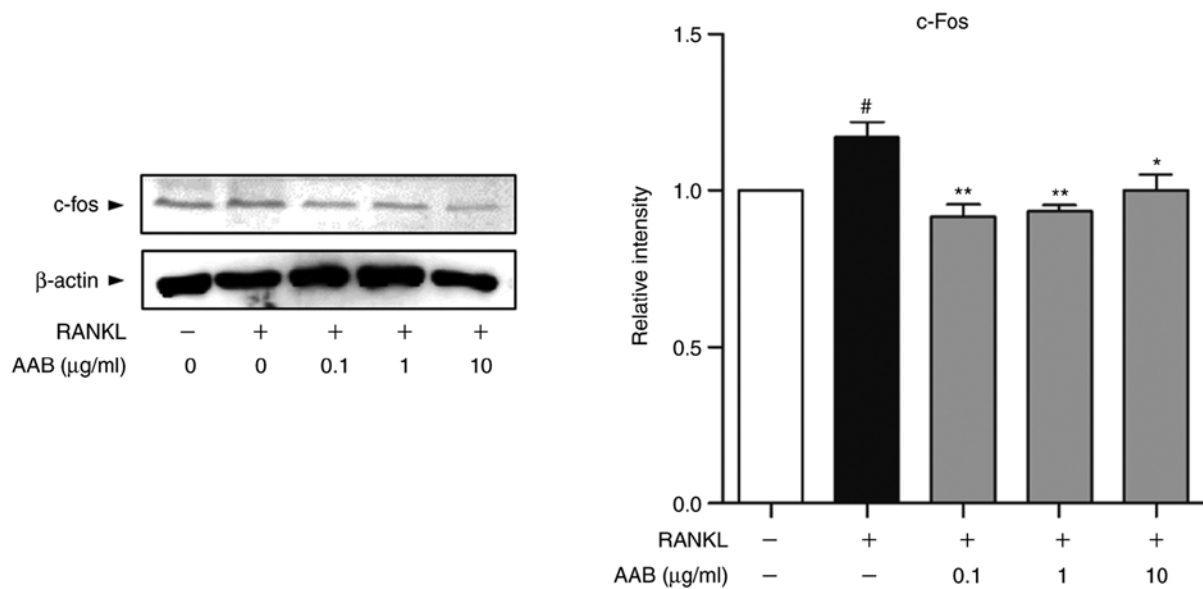


Figure 5. Effects of AAB on RANKL-induced c-fos protein expression in Raw 264.7 cells. Results are presented as mean  $\pm$  standard error of the mean. <sup>#</sup>P<0.001 vs. untreated cells; <sup>\*</sup>P<0.05 and <sup>\*\*</sup>P<0.01 vs. RANKL-treated cells. AAB, *Anemarrhena asphodeloides* Bunge extract; RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; c-fos, Fos proto-oncogene.

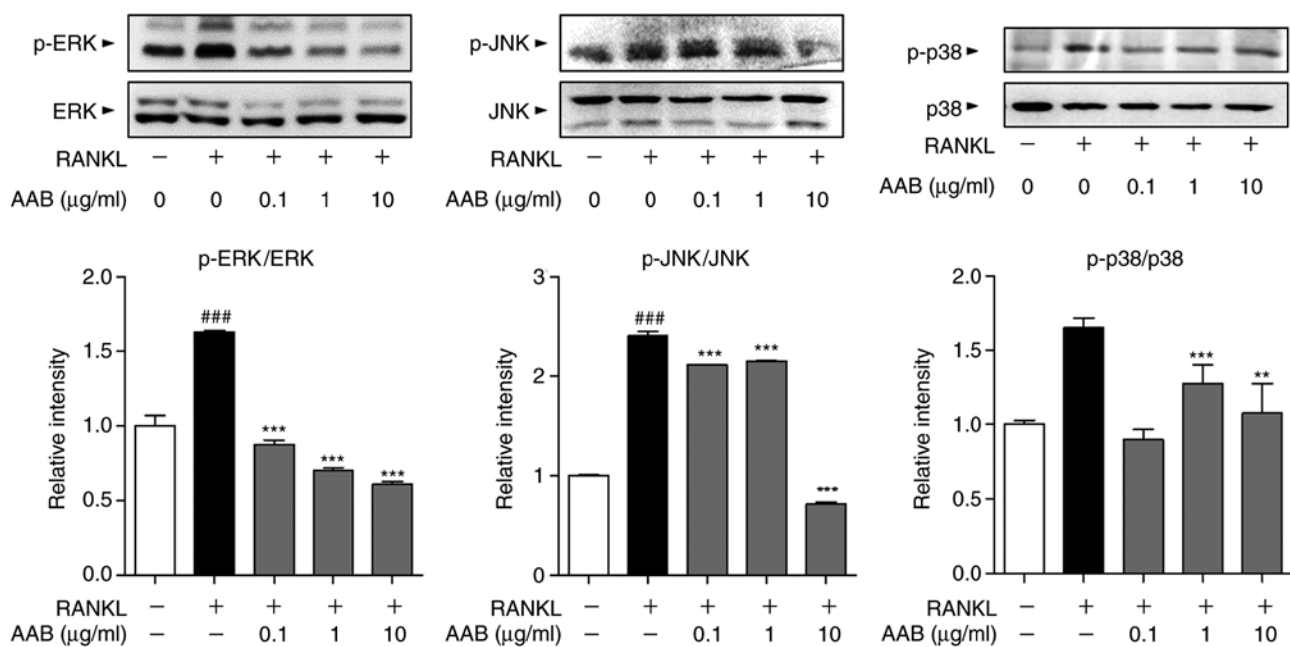


Figure 6. Effects of AAB on RANKL-induced mitogen-activated protein kinase pathway activation. Raw 264.7 cells were treated with RANKL and/or AAB, and the expression levels of the indicated proteins were measured by western blotting. Intensities of the phosphorylated proteins were calculated relative to the total protein. Results are presented as mean  $\pm$  standard error of the mean. <sup>##</sup>P<0.01 and <sup>###</sup>P<0.001 vs. untreated cells; <sup>\*\*</sup>P<0.01 and <sup>\*\*\*</sup>P<0.001 vs. RANKL-treated cells. AAB, *Anemarrhena asphodeloides* Bunge extract; RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p-, phosphorylated.

NF- $\kappa$ B, MAPKs and c-fos. However, the potential single ingredient or combination of phytochemicals of AAB involved in its inhibitory effect on osteoclastogenesis remains unknown. Further investigations at mechanistic and preclinical levels may provide useful additional insight for the development and optimization of advanced treatments for osteoporosis. The present findings suggest that AAB may be a novel candidate for the treatment of osteoporosis by inhibiting the formation of osteoclasts.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

JSL, MHK and WMY contributed to the study design. JSL, MHK and HL performed experiments and analyzed data. JSL, MHK and WMY drafted the manuscript. WMY supervised the study. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Experimental protocols involving animals were approved by the Institutional Animal Ethics Committee of Kyung Hee University in Korea [approval no. KHUASP(SE)-15-093].

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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