

Anti-adipogenic and Pro-osteoblastogenic Activities of *Spergularia marina* Extract

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ABSTRACT: For decades, *Spergularia marina*, a local food that is popular in South Korea, has been regarded as a nutritious source of amino acids, vitamins, and minerals. While several halophytes are reported to possess distinct bioactivities, *S. marina* has yet to be promoted as a natural source of bioactives. In this study, the effects of *S. marina* on the adipogenic differentiation of 3T3-L1 fibroblasts and the osteoblastic differentiation of MC3T3-E1 pre-osteoblasts and C2C12 myoblast cells were evaluated. The anti-adipogenic effect of *S. marina* was assessed by measuring lipid accumulation and adipogenic differentiation marker expression. *S. marina* treatment significantly reduced lipid accumulation and notably decreased the gene levels of peroxisome proliferator-activated receptor γ , CCAAT/enhancer-binding protein α , and sterol regulatory element binding protein 1c. In addition, *S. marina* enhanced osteoblast differentiation, as indicated by increased alkaline phosphatase activity and increased levels of osteoblastogenesis indicators, namely bone morphogenetic protein-2, osteocalcin, and type I collagen. In conclusion, *S. marina* could be a source of functional food ingredients that improve osteoporosis and obesity. Further studies, including activity-based fractionation, will elucidate the mechanism of action and active ingredients of *S. marina*, which would provide researchers with a better understanding of the nutraceutical and therapeutic applications of *S. marina*.

Keywords: osteoporosis, adipogenesis, osteoblastogenesis, halophyte, *Spergularia marina*

INTRODUCTION

Folk medicine has been used worldwide for decades. Many of the natural bioactives used in folk medicine come from plants (1-3). Throughout the past decade, halophytes have been studied as a source of bioactive materials. Halophytes are known for their resistance against the harsh environmental conditions of high salinity waters, mangrove swamps, and marshes (4). A large number of halophytes are thought to have bioactive effects because of their high content of polyphenols, which have therapeutic effects (5,6). One of the halophyte plant genera, *Spergularia*, is widely distributed throughout subtropical areas. Previous work had shown that *Spergularia* sp. is a source of therapeutic agents such as flavonoids and saponins (7,8). A prominent research trend to develop novel nutraceutical substances from natural plants has generated much interest in *Spergularia* sp., but a detailed mechanism of action for this halophyte has yet to be reported. For decades, *Spergularia*

marina, a local food that is popular in South Korea, has been regarded as a nutritious source of amino acids, vitamins, and minerals (9). However, aside from its nutritive value, published literature does not contain any reports on the potential health benefits of *S. marina*. In contrast, there have been reports indicating that other *Spergularia* species are important sources of several therapeutic agents. Considering the potential health benefits that *Spergularia* sp. holds, *S. marina* is a promising halophyte that should be investigated as a novel source of bioactive substances.

Osteoporosis and age-related osteopenia are reported to be associated with bone mass loss due elevated bone marrow adipogenesis (10). The bone mass imbalance that occurs during osteoporosis is triggered by increased adipogenic differentiation accompanied by decreased bone formation. These changes are thought to be similar to those that cause obesity-related increases in adipogenesis (11). The common idea behind osteoporosis treatments is to promote bone tissue formation by osteoblast differ-

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entiation while preventing bone volume loss through suppressed adipogenesis. Novel, natural sources of bioactive substances are at the center of the search for treatments that control osteoporosis more efficiently and with fewer side effects. Several studies have already revealed that marine-based natural compounds enhance osteoblastogenesis *in vitro*. Therefore, the potential of *S. marina* to suppress adipogenesis while enhancing osteoblastogenesis was tested in this study. The findings of this research will lead to proper utilization of *S. marina* as a treatment against osteoporosis.

MATERIALS AND METHODS

Plant materials and extract preparation

The *Spergularia marina* Griseb was purchased from Haenam in Jeollanam-do, Korea in February 2012. The sample was air-dried under shade, ground to a powder, and extracted three times in 20 volumes of ethanol. The extract was concentrated in a rotary vacuum evaporator (RV 10 Series, IKA Works, Inc., Wilmington, NC, USA) and dissolved in dimethyl sulfoxide (DMSO).

Adipocyte differentiation

Murine 3T3-L1 pre-adipocytes were seeded in 6-well plates at a density of 2×10^5 cells/well. Cells were grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂. At 1 day post-confluence (designated day 0), cell differentiation was induced with a mixture of 3-isobutyl-1-methylxanthine (0.5 mM), dexamethasone (0.25 M), and insulin (5 µg/mL) in DMEM containing 10% FBS. After 48 h (i.e., on day 2), the induction medium was removed and replaced with DMEM containing 10% FBS supplemented with insulin (5 µg/mL). This medium was changed every 2 days. *S. marina* extract was added to the culture medium from day 0 to day 6. The formation of adipocytes was monitored by the appearance of lipid droplets under a microscope.

Oil Red O staining

Cells were fixed with 10% fresh formaldehyde in phosphate buffered saline (PBS) for 1 h at room temperature and stained with 1 mL filtered Oil Red O solution (60% isopropanol and 40% water) for at least 1 h. After staining, the Oil Red O staining solution was removed and the plates were washed with distilled water or PBS and dried. Images of the lipid droplets present in 3T3-L1 adipocytes were collected with an Olympus microscope (Olympus Corporation, Tokyo, Japan).

Osteogenic differentiation

Murine osteoblastic MC3T3-E1 and myoblastic C2C12

cells were seeded in 6-well plates at a density of 1×10^5 cells/well. MC3T3-E1 and C2C12 cells were grown to confluence in α -Modified Minimal Essential Medium (α MEM) and DMEM, respectively, supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 100 units/L penicillin, and 100 mg/L streptomycin at 37°C in a humidified atmosphere of 5% CO₂. At confluence, cell differentiation was initiated with culture medium containing 50 µg/mL ascorbic acid and 10 mM β -glycerophosphate for 5 days with or without *S. marina* extract (12).

Cell proliferation assay

Cells were seeded in 96-well plates in triplicate at a density of 1×10^3 cells/well. After 24 h, the cells were washed with fresh medium and treated with control medium or medium supplemented with *S. marina* extract. During the differentiation periods, a different plate was used for each time point of interest. The medium was changed and the samples were re-treated every 2 days. At each time point, cells in the appropriate plate were washed, 100 µL of MTT solution (i.e., a final concentration of 1 mg/mL) was added, and the plate was incubated for 4 h. Finally, 100 µL of DMSO was added to solubilize the formazan crystals, which were quantified by measuring the absorbance of each well at 540 nm using a GENios[®] microplate reader (Tecan Austria GmbH, Grödig, Austria). The viability of cells was quantified as a percentage of the control. Dose response curves were developed with this assay.

Alkaline phosphatase (ALP) activity assay

Cellular ALP activity was measured in cells that had been treated with *S. marina* extract for 7 days and in untreated, control cells. The cell monolayer was gently washed twice with PBS and lysed with 0.1% Triton X-100, 25 mM carbonate buffer. The lysates were centrifuged at 4°C and 12,000 g for 15 min. ALP activity was measured in a reaction solution containing the supernatant fraction and an enzyme assay buffer (15 mM p -nitrophenyl phosphate, 1.5 mM MgCl₂, and 200 mM carbonate buffer). The absorbance of the reaction solution was measured at 405 nm. Absorbance values were plotted after normalizing the values to blank wells that only contained the reaction buffers and the appropriate samples.

RNA extraction and reverse transcription-polymerase chain reaction analysis

TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) was used to isolate total RNA from *S. marina*-treated and untreated 3T3-L1 adipocytes, MC3T3-E1 osteoblasts, and C2C12 osteoblasts. To synthesize cDNA, RNA (2 µg) was added to a mixture of oligo (dT) primers in RNase-

free water. The reaction mixture was denatured at 70°C for 5 min and cooled immediately. RNA was reverse transcribed in a master mix (1× RT buffer, 1 mM dNTPs, 500 ng of oligo (dT) primer, 140 U of M-MLV reverse transcriptase, and 40 U of RNase inhibitor) at 42°C for 60 min and at 72°C for 5 min using an automatic T100™ Thermal Cycler (Bio-Rad, Oxfordshire, UK). The target cDNA was amplified using the following sense and antisense primers: forward 5'-TTT-TCA-AGG-GTG-CCA-GTT-TC-3' and reverse 5'-AAT-CCT-TGG-CCC-TCT-GAG-AT-3' for peroxisome proliferator-activated receptor γ (PPAR γ); forward 5'-TGT-TGG-CAT-CCT-GCT-ATC-TG-3' and reverse 5'-AGG-GAA-AGC-TTT-GGG-GTC-TA-3' for sterol regulatory element binding protein 1c (SREBP1c); forward 5'-TTA-CAA-CAG-GCC-AGG-TTT-CC-3' and reverse 5'-GGC-TGG-CGA-CAT-ACA-GTA-CA-3' for CCAAT/enhancer-binding protein α (C/EBP α); forward 5'-CCA-GCA-GGT-TTC-TCT-CTT-GG-3' and reverse 5'-CTG-GGA-GTC-TCA-TCC-TGA-GC-3' for ALP; forward 5'-GGA-CCC-GCT-GTC-TTC-TAG-TG-3' and reverse 5'-GCC-TGC-GGT-ACA-GAT-CTA-GC-3' for bone morphogenetic protein (BMP)-2; forward 5'-GCT-GTG-TTG-GAA-ACG-GAG-TT-3' and reverse 5'-CAT-GTG-GGT-TCT-GAC-TGG-TG-3' for Osteocalcin; forward 5'-GAG-CGG-AGA-GTA-CTG-GAT-CG-3' and reverse 5'-TAC-TCG-AAC-GGG-AAT-CCA-TC-3' for type I collagen (collagen-I); and forward 5'-CCA-CAG-CTG-AGA-GGG-AAA-TC-3' and reverse 5'-AAG-GAA-GGC-TGG-AAA-AGA-GC-3' for β -actin. The amplification cycles were carried out at 95°C for 45 s, 60°C for 1 min, and 72°C for 45 s. After 30 cycles, the PCR products were separated by electrophoresis on a 1.5% agarose gel for 30 min at 100 V. Gels were then stained with 1 mg/mL ethidium bromide and visualized by UV light using a Davinch-Chemi CAS-400SM (Davinch-K, Seoul, Korea).

Statistical analysis

The data were presented as mean \pm SD. Differences between the means of the individual groups were analyzed using the analysis of variance (ANOVA) procedure of Statistical Analysis System, SAS v9.1 software (SAS Institute, Cary, NC, USA). Duncan's multiple range tests were used for post-hoc analyses. The differences among means were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of *S. marina* extract on adipogenic differentiation of 3T3-L1 pre-adipocytes

The bone mass imbalance that occurs during osteoporosis is triggered by an increase in adipose tissue. These changes are mainly triggered by the same causes of obe-

sity-related adipogenesis (13). To evaluate ability of *S. marina* extract to prevent adipogenic differentiation, *S. marina* extract was introduced to the adipogenic cocktail used to stimulate the differentiation of 3T3-L1 cells. Oil Red O staining of 3T3-L1 cells at the end of differentiation (i.e. on day 6) revealed that *S. marina* extract treatment inhibited adipogenesis in a dose-dependent manner (Fig. 1A). Cell images taken after Oil Red O staining showed that *S. marina* extract-treated cells accumulated fewer lipid droplets, indicating failed differentiation. Lipid droplets, visible as red drops, were observed in control cells, indicating that the 3T3-L1 pre-adipocytes were able to differentiate into mature adipocytes.

Adipocyte differentiation is regulated by transcription factors such as PPAR γ , C/EBP α , and SREBP1c. In order to evaluate the possible mechanism responsible for the effect of *S. marina* on adipocyte differentiation, the effects of *S. marina* extract on the expression of adipogenic-specific genes were analyzed by RT-PCR (Fig. 1B). Differentiation of pre-adipocytes induced the up-regulation of PPAR γ , C/EBP α , and SREBP1c genes, which led to cytoplasmic triglyceride synthesis, and ultimately, fat accumulation. *S. marina* extract decreased the expression of these factors, namely PPAR γ , SREBP1c, and C/EBP α . For the most part, gene suppression was greater in cells that were treated with higher concentrations of *S. marina*. However, SREBP1c expression was not affected by *S. marina* in a completely dose-dependent manner. As indicated earlier, *Spergularia* sp. contains bioactive flavonoids, which have been reported to relieve hyperglycemic conditions (14). Previous reports have indicated that some flavonoids only affect SREBP1c expression at certain concentrations. Hence, it can be sug-

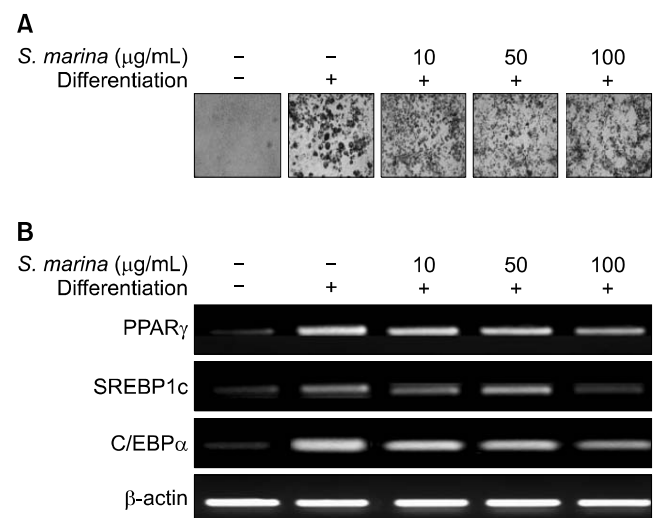


Fig. 1. Anti-adipogenic activity of *S. marina* extract in differentiated 3T3-L1 cells. Lipid droplet accumulation was determined by Oil Red O staining (A). The expression levels of PPAR γ , SREBP1c, and C/EBP α were determined by RT-PCR analysis (B).

gested that higher concentrations of extract could have decreased effects on the expression of SREBP1c because they contain different types of flavonoids. The significant decreases observed in all key factors of adipogenesis, including decreased lipid accumulation, suggests that *S. marina* extract effectively blocks adipocyte differentiation through the PPAR γ pathway.

Recent studies have defined osteoporosis as obesity of bone. If bone marrow cells undergo imbalanced differentiation towards adipogenesis rather than osteoblastogenesis, bone with less bone volume but high adipose tissue volume is formed (15). Moreover, bone marrow imbalance is also triggered with several diabetes drugs which increase glucose uptake by cells through the PPAR pathway, leading to increased adipogenic differentiation (16). Research indicates that the treatment of osteoporosis can occur via two mechanisms: blocked adipogenesis and enhanced osteoblast differentiation.

Effect of *S. marina* extract on osteogenic differentiation of MC3T3-E1 and C2C12 cells

After confirming its anti-adipogenic activity, the ability of *S. marina* extract to enhance the osteoblast differentiation of mouse MC3T3-E1 and C2C12 cells was tested. MC3T3-E1 cells are mouse pre-osteoblasts that provide a very efficient way to test bone mineralization. C2C12 cells are mouse myoblasts that are capable of differentiating into osteoblasts. While osteoblast differentiation occurs via the same pathways in these cell types, exploring the response of two different cell lines to *S. marina* extract enhances our understanding of its pro-osteoblastogenic potential.

Osteoblast differentiation has been extensively studied and well documented by several researchers. Pre-osteoblasts are induced to differentiate into osteoblasts through a pathway that involves the elevation of specific genes and proteins, such as osteocalcin and BMP-2 (17). Osteocalcin is an indicator of the final differentiation state of osteoblastogenesis. BMPs are known to enhance expression of ALP, collagen-I, and other non-collagenous bone proteins that serve as indicators of successful maturation into osteoblasts (18).

Previous work indicates that MC3T3-E1 mouse pre-osteoblasts are good models of *in vitro* osteoblast differentiation. Therefore, *S. marina* extract was introduced into the differentiation medium of MC3T3-E1 pre-osteoblasts. Differentiation of MC3T3-E1 pre-osteoblasts into mature osteoblasts was confirmed by measuring cell proliferation, ALP activity, osteoblastogenesis gene expression, and ALP protein expression. The presence of *S. marina* extract promoted osteoblastic cell growth and increased the ALP activity of osteoblastic cells (Fig. 2A and B). After 2, 3, and 5 days of incubation, the cell proliferation rates of MC3T3-E1 cells that had been treated

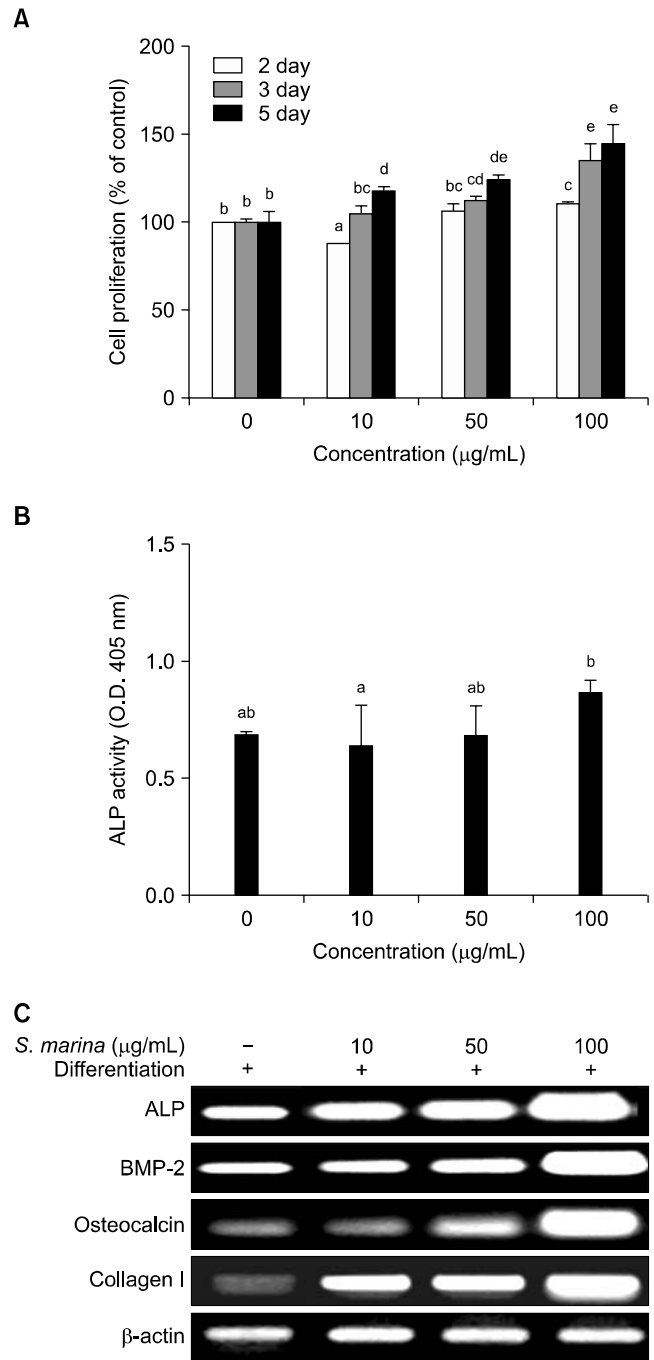


Fig. 2. Effect of *S. marina* extract on osteogenic differentiation of MC3T3-E1 pre-osteoblasts. Cell proliferation (A), ALP activity (B), and mRNA expression levels of key osteoblastogenesis markers (C) were determined in MC3T3-E1 osteoblasts. Means with different letters (a-e) are significantly different ($P < 0.05$) by Duncan's multiple range test (O.D.: optical density).

with a 10 µg/mL *S. marina* concentration were 87.2%, 104.1%, and 117.5% of the control cell proliferation rates, respectively. Similarly, 2, 3, and 5 days of incubation with a 50 µg/mL *S. marina* treatment enhanced the proliferation of MC3T3-E1 cells to 106.1%, 112.3%, and 123.7% of control cell proliferation, respectively. After 2, 3, and 5 days of incubation with the highest *S. marina* concentration (100 µg/mL), MC3T3-E1 pro-

liferation was enhanced to 110.5%, 134.8%, and 144.4% of the rate of the control cells, respectively. These results indicated that *S. marina* extract stimulated MC3T3-E1 cell proliferation in a time- and dose-dependent manner. Longer incubation periods resulted in significantly increased cell proliferation.

The 10 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ *S. marina* doses altered ALP activity, which is reported as blank-normalized optical density (O.D.), from 0.685 (measured in wells containing untreated control cells) to 0.637 and 0.682, respectively. However, at the 100 $\mu\text{g/mL}$ *S. marina* concentration, ALP activity was increased by 26.2%, from 0.685 (measured in wells containing untreated control cells) to 0.865. Although the ALP activity of MC3T3-E1 cells was not significantly affected by the lower *S. marina* concentrations tested, the increase in ALP activity observed at the 100 $\mu\text{g/mL}$ *S. marina* dose was significant. Previous research indicates that the timing of the ALP activity response is affected by the cell line source (19). Accordingly, a relatively low increase in ALP activity was expected in the MC3T3-E1 cell line because this cell line does not typically present significantly elevated ALP activities after shorter incubation times. In the present study, it is likely that the 100 $\mu\text{g/mL}$ *S. marina* concentration stimulated the differentiation of MC3T3-E1 cells more than any other dose, which resulted in the highest increase in ALP activity. These results clearly suggested that *S. marina* extract had a notable effect on osteoblast differentiation.

To evaluate the mechanism responsible for *S. marina*-induced osteoblast differentiation, the mRNA expression of key osteoblastogenesis markers was measured in the presence and absence of *S. marina* extract. Even the lowest *S. marina* concentration (10 $\mu\text{g/mL}$) was associated with strongly enhanced mRNA expression levels of BMP-2, ALP, osteocalcin, and collagen-I (Fig. 2C). These results indicate that *S. marina* extract enhances osteoblast differentiation through its effects on osteoblastogenesis factors.

C2C12 myoblasts are able to differentiate into myotubes under myogenic conditions or into osteoblasts under osteogenic culture conditions (12,20). Hence, the ability of the *S. marina* extract to induce osteoblast differentiation was also examined with C2C12 cells. The effect of the *S. marina* extract on C2C12 cell proliferation was also measured (Fig. 3A). In order to evaluate the time-dependent effects of *S. marina* extract treatment on C2C12 cells, the C2C12 cells were incubated in medium containing *S. marina* extract for 2, 3, or 5 days. C2C12 cell proliferation was not affected by a 2-day incubation in medium containing 10 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, or 100 $\mu\text{g/mL}$ *S. marina* concentrations. However, after the third day of incubation a notable elevation in C2C12 cell proliferation was observed. The cell proliferation rates of C2C12 cells that were incubated in medium containing

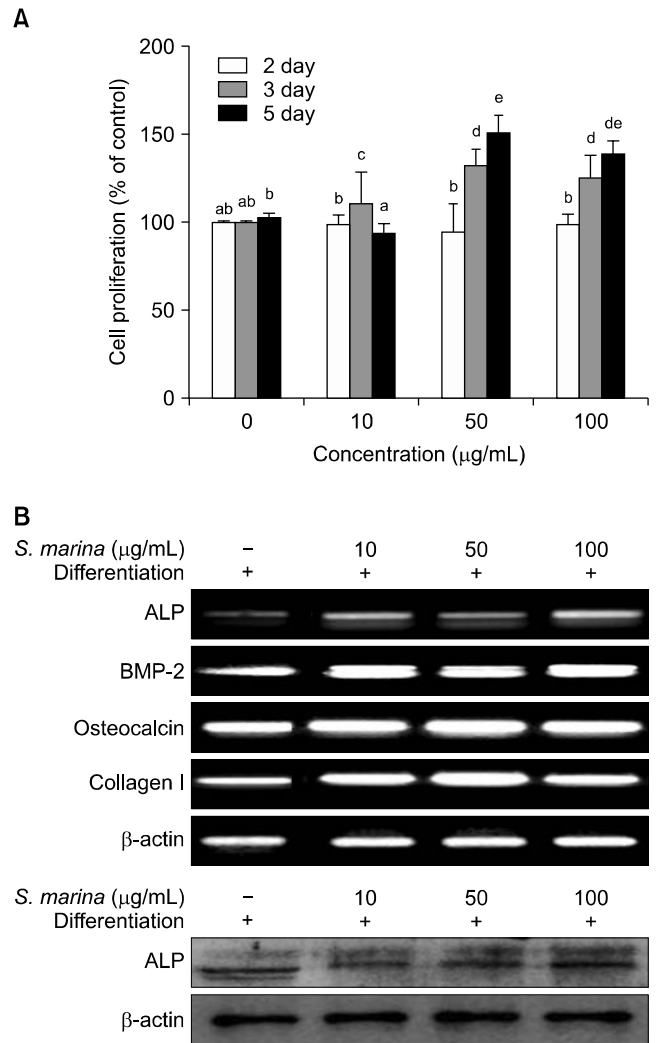


Fig. 3. Effect of *S. marina* extract on osteogenic differentiation of C2C12 cells. Cell proliferation (A) and mRNA and protein expression levels of key osteoblastogenesis markers (B) were measured in osteoblast differentiated C2C12 cells. Means with different letters (a-e) are significantly different ($P < 0.05$) by Duncan's multiple range test.

10 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, and 100 $\mu\text{g/mL}$ *S. marina* concentrations for 3 days were increased to 110.4%, 132.0%, and 124.7% of the proliferation rate of untreated control cells, respectively. Similarly, the cell proliferation rates of C2C12 cells that were incubated in medium containing 10 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, and 100 $\mu\text{g/mL}$ *S. marina* concentrations for 5 days were 93.4%, 150.7%, and 138.5% of the proliferation rate of the control cells, respectively. These results indicated that the *S. marina*-induced increase in C2C12 cell proliferation was dependent on incubation time.

The effects of *S. marina* extract on C2C12 cell expression of key osteoblastogenic mRNA markers and ALP protein were assayed by RT-PCR and western blotting, respectively (Fig. 3B). The presence of the extract increased the expression of osteoblastogenesis-specific genes, namely ALP, BMP-2, osteocalcin, and collagen-I.

A similar, dose-dependent effect on mRNA expression was observed for all treatment concentrations (i.e., 10 µg/mL, 50 µg/mL, and 100 µg/mL). In addition, the *S. marina* extract increased ALP protein levels in a dose-dependent fashion. Taken together, these findings indicate that *S. marina* exerts significant osteoblastogenic effects.

Overall, the results of the present study indicate that *S. marina* extract inhibits adipogenesis while enhancing bone formation through elevated osteoblast differentiation. RT-PCR analysis confirmed that the activity of *S. marina* extract was exerted by interaction with key differentiation factors that were known to play crucial roles in the initiation of adipogenesis and osteoblastogenesis, namely PPAR γ and BMP-2, respectively. Therefore, *S. marina* extract can be promoted as a promising natural bioactive compound that possesses the ability to act against osteoporosis and obesity by simultaneously blocking adipogenesis and enhancing osteoblastogenesis.

S. marina was reported to enhance osteoblast differentiation along its potential ability to induce adipogenesis. Future studies to evaluate the efficiency and mechanism of action of *S. marina* on the stimulation of stem cell differentiation away from adipocytes and towards osteoblasts are urged. In addition, because *S. marina* is able to inhibit adipogenesis while enhancing osteoblastogenesis, its active components need to be elucidated. Nevertheless, *S. marina* is a potential source of natural bioactive compounds that can be used to control age-related obesity and bone disorders. Published reports credit the bioactivity of *Spergularia* sp. mainly to flavonoid content (8). Marine plant-based flavonoids have also been reported to possess anti-adipogenic and pro-osteoblastogenic activities. The *S. marina* bioactivities that were observed in this study were considered to be due to the presence of bioactive flavonoids. In addition, saponins, were also presumably included in *S. marina* extract, could be responsible for the reported bioactivities (7).

In conclusion, we prepared extracts of *S. marina* in order to evaluate its effect on adipogenesis and osteoblastogenesis. The purpose of this research was to discover novel natural bioactive substances. The results of the present study showed that *S. marina* extract inhibits adipogenesis in pre-adipocytes while enhancing the differentiation of pre-osteoblasts to osteoblasts. These results clearly indicate that *S. marina* contains substances that protect against osteoporosis by controlling the balance of bone mass through the regulation of adipogenesis/osteoblastogenesis balance. Further studies to isolate the active components of *S. marina* and elucidate their mechanisms of action are recommended, as they will pave the way towards the use of natural products as treatments for osteoporosis.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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