

Effect of Steamed *Stauntonia hexaphylla* fruit on RAW 264.7 osteoclast and MC3T3-E1 osteoblast differentiation

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Abstract

Stauntonia hexaphylla (SH), a medicinal plant in the Lardizabalaceae family, holds traditional significance in East Asian countries for its therapeutic properties, particularly in treating rheumatism. Steam processing is a common method employed to augment the therapeutic properties of SH, prompting the need for a detailed investigation into the chemical and biological changes induced by this process. Comparative analyses revealed significantly higher 5-Hydroxymethylfurfural (5-HMF) content, total flavonoid and phenolic content, and antioxidant activity in steamed SH fruit (SHF) samples compared to unprocessed ones. Furthermore, the study explored the effects of steamed SHF extracts on bone health using an *in vitro* model simulating osteoclast in Raw 264.7 cells and osteoblasts in MC3T3E1 cells. Notably, steamed extracts demonstrated a dual effect on bone health by promoting alkaline phosphatase activity, calcium nodule, and collagen formation in osteoblasts, while inhibiting tartrate-resistant acid phosphatase (TRAP) activity in osteoclasts. Steamed SHF extracts were able to restore the aberrant gene expression of osteoclastogenesis indicators including *TRAP*, *NFTAc1*, *RANK*, *MMP9*, *c-Fos* and *TRAF6*. Additionally, the expression of the osteoblastogenesis biomarkers *ALP*, *Runx2*, *BGLAP*, *Col1a1*, and *OPG* were boosted by steaming to levels greater than those in the untreated control group, suggesting that steaming can be used to improve the properties of proteins. The component 5-HMF actively promotes ALP and inhibits TRAP activities. Hence, these results are expected to aid in confirming the efficacy of herbal medicines and identifying suitable materials for medicinal applications.

Introduction

Osteoporosis is characterized by a reduction in bone mass, diminished bone density, and degeneration of bone tissue microstructure due to the shortage of calcium and proteins in bone. It is characterized as a metabolic bone disease by a discrepancy in the restoration of bone in which the amount of bone resorption surpasses the amount of bone formation. Osteoporosis is a substantial health concern that has become more prevalent recently, primarily due to the increasing aging population [1]. Various factors contribute to bone abnormalities, including inadequate calcium and protein intake, hormonal imbalances associated with menopause, obesity, and reduced physical activity [2]. Unhealthy lifestyle habits such as the excessive use of steroids, frequent consumption of processed foods, and heavy smoking and drinking can also contribute to joint and bone diseases [3]. Although bone resorption inhibitors like bisphosphonates and denosumab were initially developed for osteoporosis treatment, individuals using them often encounter serious adverse effects, including breast cancer, uterine cancer, and osteonecrosis [4].

Recent investigations have unveiled the positive effects of natural compounds on bone formation and osteoclastogenesis [5, 6]. Notably, certain flavonoid constituents including quercetin, rutin, and naringenin have been found to prevent bone resorption and stimulate bone formation [7]. Additionally, isoflavones derived from soybeans have been found to enhance the osteogenic activity of osteoblasts [8]. Oxidative stress has also been linked inversely to decreased bone density, promoting bone resorption through the

activation of osteoclast differentiation [9]. These findings suggest that natural substances with antioxidant properties, such as flavonoids, may offer potential for osteoporosis prevention and treatment.

Stauntonia hexaphylla (SH) belongs to the Lardizabalaceae family and is a subtropical vine plant predominantly distributed in Korea, Japan, and China. Various parts of SH, including fruits, leaves, stems, and roots, have traditionally been used in oriental medicine for their analgesic, sedative, and diuretic attributes [10]. Recent research has reported SH's potential to enhance post-menopausal osteoporosis recovery [11], exhibit anti-inflammatory properties [10], mitigate alcoholic liver damage [12] and alleviate fatigue [13]. The fruit of SH is esteemed as a health food due to its rich content of vitamin C and vital minerals, such as calcium (Ca), potassium (K), and magnesium (Mg). It also contains various phytochemicals, such as flavonoids, polyphenols, and phenolic acids [14]. It has been reported that the promoting bone formation and inhibiting bone resorption activities of SH are caused by some flavonoids or phenolic acids existing in leaves or fruits [15]. SH has recently gained prominence in the health food markets of Korea, Japan, and China, where it is known by names like 'Mulkul,' 'Mube,' and 'Ye-mu- gua', respectively [10].

In the realm of food and natural product enhancement, a spectrum of techniques is employed, ranging from heating, drying, and microwave treatment to fermentation and enzyme treatment, aimed at bolstering their physiological attributes [16]. Among these methods, the steaming process is utilized to amplify the content and bio-accessibility of valuable phytochemicals, including phenolic compounds, while preserving the inherent constituents of the raw materials. Traditional Oriental medicine encompasses a practice referred to as 'Bupjae,' in which primary processed medicinal ingredients undergo reprocessing according to specific procedures to heighten their quality and effectiveness. Steaming processing is one of the methods traditionally associated with 'Bupjae' [17]. Phenolic compounds in natural products, fruits, vegetables, and foods can be found in both free and bound forms [18]. The application of steaming or heat treatment to these materials can result in an increase in bound phenolic compounds and pseudo-melanin substances, leading to browning due to the oxidation reaction of o-diphenols [19]. Indeed, it has been demonstrated that steaming chestnuts leads to a significant increase in both bound and free phenolic compounds [20].

Hence, our study was conducted with the expectation that dynamic modifications in phenolic compounds and other bio-active substances would transpire when SHF is subjected to steaming processes. It is anticipated that these altered constituents will exert a positive impact on physiological activities linked to osteoporosis.

Materials and Methods

SH fruit preparation and steaming

SH fruits used in this study were harvested in Jangheung-gun, Jeollanam-do, republic of Korea at the end of November 2022. The SH were washed with tap water, cut into pieces about 1.0 cm thick, and dried at

45~50°C for 24 hours. Each group, 0.5 kg of dried SH was added into a steamer (Cuckoo M-OC9600) and steamed them for 2 hours. After steaming, the fruits were dried at 55~60°C for 3 hours. The same conditions were used for two or three iterations of the steaming procedure.

The dried steamed fruits and the un-steamed control were each extracted with 10 volumes (w/v) of purified water at $90 \pm 3^\circ\text{C}$ for 6 hours, and the extraction was carried out twice. The two extracts were merged and filtered, and a concentrate was prepared using a rotary evaporator and used as an analysis sample.

Isolation of 5-HMF and identification.

The isolation of unknown peak that highly increased from steamed SHF was carried out by Waters preparative HPLC system equipped with Waters 1525 binary pump (manual injection valve) and 2489 UV/Visible detector. First, the extract of steamed SHF was added into separating funnel with ethyl acetate in the ratio 1:1(v/v) to carry out separation of unknown peak by solvent partition. And the ethyl acetate layer was evaporated using a rotary evaporator. After evaporation, HPLC grade methanol was added into evaporated sample to dissolve it, then was subjected to prep-HPLC to separate unknown peak. The Waters $\mu\text{Bondapak}^{\text{TM}}$ C18 Prep column (19 x 300mm, 10 μm) was used as stationary phase. The mobile phase was composed of 0.1% acetic acid aqueous solvent (A) and methanol (B). The flow rate was 12.28 ml/min and elution composition were initially 98% A and 2% B. The gradient elution composition was as follows: (0–20 min, 2% B; 20–30 min, 2–5% B, 30–40 min, 5% B; 40–50 min, 5–10% B; 50–51 min, 10–70% B; 51–60 min, 70% B). The injection volume was 1 ml and wavelength of UV/VIS detector was 260 nm. The 1st separation of unidentified peak was carried out between 3- and 10-min. The separated sample was evaporated and dissolved in methanol to be a target for performing 2nd separation. Again, the sample was separated at 1min-intervals from 6.5 to 10min. And the fractions from 8 to 10min was combined and evaporated. The weight of isolated samples was 18 mg and it was used as a sample for NMR analysis. The determination of the molecular structure of this compound was performed by Molecular Structure Determination Research Institute (MSDRI) of Kyung Hee University, Republic of Korea.

Measurement of 5- HMF and phenolic compounds content

The quantification of 5-Hydroxymethylfurfural (5-HMF), chlorogenic acids (CGAs), total phenolic compounds (TPC), and total flavonoid compounds (TFC) in SHF was conducted using the following methods.

For CGA, neo-CGA, and crypto-CGA contents, an HPLC (High-Performance Liquid Chromatography) system (Agilent Infinity 1260) was employed. The system comprised an Agilent 1260 Infinity Quaternary Pump (G1311B), Agilent 1260 Infinity Standard Auto Sampler (G1329B), Agilent 1260 Infinity Column Thermostat Compartment (G1316A), Agilent 1260 Infinity Variable Wavelength Detector (G1314F), and ZORBAX Eclipse Plus C18 (4.6mm $\times$ 10mm, 5 μm) served as the stationary phase. HPLC conditions included an injection volume of 5 μL , a temperature of 35°C, a flow rate of 1mL/min, and a wavelength of

260nm. The mobile phase consisted of solvent A (0.4% phosphoric acid) and solvent B (acetonitrile), with an initial elution composition of 95% A and 5% B. A gradient elution was performed as follows: (0–10 min, 5–9% B; 10–30 min, 9–9% B; 30–60 min, 9–30% B; 60–62 min, 30–50% B). Standards for CGA, neo-CGA, cryptog-CGA, and 5-HMF were sourced from Sigma-Aldrich (USA) and Wuhan ChemFaces Biochemical Co., Ltd (China).

For TPC analysis, 30 μL of the extraction solution was mixed with 150 μL of a 10% solution of 2N Folin-Ciocalteu reagent. After vigorous shaking, the mixture stood at room temperature for 5 minutes, and then 160 μL of a 7.5% (w/v) sodium carbonate solution was added. The mixture was rested for 30 mins in dark, and its absorbance was measured at 715 nm using a spectrophotometer. A calibration curve using gallic acid as the reference standard allowed the computation of TPC, expressed as $\mu\text{g GAE/mg}$ of the extract.

For TFC quantification, the extract (30 μL) was combined with distilled water (110 μL). Subsequently, 5% sodium nitrite (8 μL) was added, followed by the introduction of 10% aluminum chloride (8 μL) after 5 minutes, allowed to stand for 6 minutes. Then, 1M sodium hydroxide (50 μL) and distilled water (70 μL) were added, and the absorbance was evaluated at 510 nm. A calibration curve using rutin as the standard reference material enabled the calculation of TFC, expressed as $\mu\text{g RE/mg}$ of the extract.

Measurement of free radical scavenging ability

The assessment of free radical scavenging capability was performed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. Specifically, each sample was appropriately diluted, and 180 μL of a 0.2 mM DPPH solution was combined with 20 μL of the sample within a 96-well plate. Following a 30-minute incubation period in the absence of light, the absorbance was determined at 520 nm employing an ELISA reader (BioTec, USA). The radical scavenging capacity was quantified and expressed as $\mu\text{gGAE/g}$ dry weight.

Cell viability assay

MC3T3-E1 Pre-osteoblast and RAW264.7 macrophage cell-lines were plated in 96-well culture plates at 1×10^4 cells per well. After over-night incubation period, cells were subjected to treatment with several doses (between 31.25 $\mu\text{g/mL}$ and 500 $\mu\text{g/mL}$) of steamed SH extracts for a duration of 24 to 48 hours. Cell viability was assessed using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay solutions after 2 days incubation.

To conduct the MTT assay, 20 μL of MTT solution (5 mg/mL) was added per well, and the plates were left to incubate for 3 to 4 hours. Following this incubation period, the MTT solutions were aspirated, and 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan product. Subsequently, the absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The relative cell viability was expressed as a percentage in comparison to the untreated control cells.

Osteoblast cell culture and differentiation

A pre-osteoblast cell line, MC3T3-E1 (RCB1126, from C57BL/6 mouse calvaria) was obtained from the RIKEN Cell Bank (Tsukuba, Japan) and was maintained α -MEM medium (Gibco-BRL, Grand Island, NY). The media were supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 1% penicillin-streptomycin (Gibco) which is called complete medium (CM). Cells were then allowed to adhere at 37°C in a humidified 5% CO₂ incubator (Thermo Electron Corporation, USA). After reaching 80–90% confluence the cells were seeded in a 12 well plate at a density of 5×10^3 . The cell differentiation was induced by adding 10 mM glycerophosphate and 50 μ g/ml ascorbic acid in CM which is called differentiation medium (DM) for an additional 6 to 24 days.

Alkaline phosphatase (ALP) activity assay

The ALP activity of differentiated osteoblast was measured using an ALP assay kit from Sigma Chemical, St. Louis, MO, USA following the manufacturer's instruction. Briefly, MC3T3-E1 cells were differentiated in a 12 well plate using 10 mM glycerophosphate and 50 μ g/mL ascorbic acid in CM with or without SH. After 12 days, the cells monolayers were lysed with 0.1% Triton X-100/PBS followed by the PBS wash for 3 times. After that cell lysate were centrifuged at 12,000 rpm for 5 minutes at 4°C and supernatant was collected in 1.5 mL tubes for ALP activity measurement. The cell supernatant and pNPP substrate were added in a 96-well plate and incubated for 10 minutes at 37°. The reaction was stopped using a stopped solution after 10 minutes. Finally, the OD values at 405 nm were recorded using an ELISA reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Lastly, the ALP activity was normalized using BCA kit (Sigma Chemical, St. Louis, MO, USA).

Alizarin Red staining

MC3T3-E1 cells were differentiated in the presence or absence of SH extracts, as detailed in previous protocols. After 24 days, the cells were first meticulously rinsed twice with PBS, preserved with para-formaldehyde (4%) for 20 minutes, and subsequently dyed with 2% Alizarin Red S (ARS) dye solution (Sigma-Aldrich) adjusted to P^H 4.2 at room temperature for 10 minutes. The extracellular matrix mineralization nodules were photographed using a digital camera attached to an inverting microscope.

Subsequently, to analyze the ARS staining, a resolving solution containing 10% acetic acid and 20% methanol was used to dissolve the cells. After drying, the resulting liquid was transferred to a 96-well plate 15 minutes later. The absorbance was measured at a wavelength of 450 nm using an ELISA reader (Epoch TM Microplate Spectrophotometer: BioTek Instruments Inc., Winooski, VT, USA). Each reaction was carried out in triplicate.

Collagen content

MC3T3-E1 cells were cultivated using the same protocols used for the ALP assays to measure the amounts of cellular collagen. After an overnight incubation period and confluence, cells were treated with DM (with or without SH extracts) to start the differentiation process for a further 12 days. Every two days, the cultural medium was refreshed.

After the 12-day treatment period, the collagen content was quantified using a Sirius Red-based colorimetric assay. In brief, the cells were rinsed twice with PBS and then exposed to Bouin's solution for 1 hour. After removing Bouin's solution, the cells were rinsed by running tap water over the culture plates for ten to fifteen minutes. The plates were allowed to air-dry and then stained with a reagent containing Sirius Red dye for further 1 hour with gentle agitation. After this staining time, 0.01 N HCl was used to wash the cells to remove any remaining dye. After dissolving the dyed material in 0.1 N NaOH, the absorbance of the resultant solutions was measured with a microplate reader at 550 nm.

Osteoclast cell culture and differentiation

The mouse macrophage-like RAW264.7 cells were obtained from Ginseng Bank (Kyung Hee University, Korea). The cells were cultured in a complete medium (CM) that consists of 89.5% Dulbecco's modified Eagle's medium, 10% fetal bovine serum (FBS), 100-U/mL penicillin- streptomycin and maintained in an incubator containing 5% CO₂ at 37°C. Cells were grown in 12 well plates (5 × 10³ cells/well) with or without 50 ng/mL of RANKL once they had reached 80–90% confluence. Cells were cultured for additional 3–7 days and multinucleated osteoblast cells were visible at day 7.

TRAP activity assay

The RAW264.7 cells were cultured and differentiated with 100 ng/mL RANKL in the presence or absence of SH extract for 5 to 7 days. After osteoblast cells differentiation, cell monolayers were washed with PBS and centrifuged at 12,000 rpm for 5 minutes followed by lysing with 0.5% Triton X-100 and. Supernatant was used for the quantification of activity using TRAP staining kit (Sigma Chemical, St. Louis, MO, USA) according to the manufacturer's protocol.

RNA isolation and real-time reverse transcription-PCR (qRT-PCR) analysis

Following the differentiation and treatment of MC3T3-E1 and Raw 264.7 cells in the presence or absence of SHF extracts, total RNA was extracted using TriZol LS reagents (Invitrogen, Carlsbad, CA, USA), adhering to the manufacturer's instructions. Moreover, employing the RevertAid First Strand cDNA Synthesis Kit from Thermo Fisher Scientific, USA, we generated 20 µL of cDNA from 2.5 ng of RNA. This was achieved by incubating the mixture at 42°C for 45 minutes, followed by a 70°C incubation for 5 minutes, as per the manufacturer's guidelines. The entire procedure was conducted within a PCR-clean environment.

Real-time reverse transcription-PCR (qRT-PCR) was carried out to gauge the gene expression using an Invitrogen SYBR Green qPCR Super Mix UDG kit and an R-Corbett Rotor-Gene Model 6000 (Mortlake, Australia). The relative expression of gene-specific products was assessed and normalized to the corresponding β -actin levels, applying the $2^{-\Delta\Delta C_t}$ method. These results were verified through three independent experiments. The list of primers can be found in Supplementary Table 1.

Statistical analysis

The data were presented as the mean $\pm$ standard error (SE) derived from a minimum of three distinct and independent experimental replicates. Analysis of the data was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, United States). To assess the overall differences between the treated and untreated (control) groups, statistical analyses including Student's t-test and two-way analysis of variance were employed. Statistical significance was determined at a significance level of $*p < 0.05$.

Results

The effect of steaming of SHF on quality

The color of SHF and its extracts was changed to dark-brown by the steaming and it was increased gradually with the number of steaming (Fig. 1). The texture of the fruits changed from a rough structure (ST-C, ST-1, ST-2) to a fine powder (ST-3) by the number of steaming process. The yield of the extract of SH fruits as a solid part was increased to 15% in ST-2, however, it was lowered below the control level in ST-3.

The effect of steaming of SHF on phenolic compounds content and free radical scavenging ability

Table 1 shows the content of CGAs including neo-CGA and crypto-CGA that is one of the major secondary metabolites of SHF. The content of neo-CGA, CGA and crypto-CGA of unsteamed fruits (ST-C) were 120, 100 and 108 ug/g, respectively, and that shows no changes until two times steaming. However, when the fruits were steamed three times under the same condition, the content of CGAs was greatly reduced. In all samples the ratio of three CGA isomer was not changed.

Table 1
The effect of steaming of SH fruit in content of chlorogenic acids

	Chlorogenic acid (µg/g)				
	Neo-CGA	CGA	Crypto-CGA	Sum	%
ST-C	120	100	108	328	100.0
ST-1	116	108	104	328	98.8
ST-2	104	129	102	331	99.4
ST-3	76	72	96	244	75.6

The TPC content of ST-C was 18.4 ± 0.02 µgGAE/mg extract. When the SH fruits were steamed once (ST-1) increased by 61% to 29.7 ± 0.03 µgGAE/mg extract, and when it was steamed twice (ST-2), it increased by 66%, but when it was steamed three times (ST-3), it increased by 33%.

In the case of TFC, it increased significantly from 1.46 ± 0.01 $\mu\text{RE}/\text{mg}$ extract of ST-C to 10.94 ± 0.02 $\mu\text{RE}/\text{mg}$ extract (747%) and 11.19 ± 0.02 $\mu\text{RE}/\text{mg}$ extract (766%) when steamed once and twice. However, when steamed three times, the increase in content was slightly reduced to 8.95 ± 0.01 $\mu\text{RE}/\text{mg}$ extract (613%).

The effect of the steaming process on the free radical scavenging ability of steamed SHF extract was evaluated and expressed as $\mu\text{gGAE}/\text{g}$. As shows in Fig. 2, the untreated SHF extract was 607 $\mu\text{gGAE}/\text{g}$ on a dry weight basis. When the SHF were steamed once, it increased by 59% to 965 $\mu\text{gGAE}/\text{g}$, and when it was steamed twice, it significantly increased by 84% to 1,118 $\mu\text{gGAE}/\text{g}$. However, when steamed three times, the increase rate decreased slightly to 917 $\mu\text{gGAE}/\text{g}$.

The effect of steaming of SHF on cell viability in MC3T3-E1 cells

This study set out to determine how these extracts affected on cellular proliferation, differentiation, and mineralization. Following a 24-hour exposure to extract of the steamed SHF, a dose-dependent proliferative effect was observed, with a noticeable increase in cell proliferation up to a concentration of 125 $\mu\text{g}/\text{mL}$. However, this proliferative effect was not evident at higher concentrations. Subsequently, following a 48-hour treatment period, it was observed that the proliferative effect of the steamed SH extracts was relatively diminished in comparison to the 24-hour treatment, particularly at higher concentration. Consequently, for subsequent experimental investigations, the concentration of up to 100 $\mu\text{g}/\text{mL}$ of SH extracts was chosen. This decision was based on the observation that compared to control group, it demonstrated a significant cellular proliferation effect. This concentration was deemed suitable for further experiments to study the effects of SH extracts on MC3T3-E1 cells (Fig. 3A and B).

The effect of steaming of SHF on ALP activity in MC3T3-E1 cells

In Fig. 3C, the results illustrate the impact of utilizing steamed fruit extract on ALP activity within MC3T3-E1 cells. ALP activity was assessed under non-cytotoxic conditions and expressed as a percentage relative to the untreated control group. The data revealed a dose-dependent increase in ALP activity when the cells were treated with steamed SH extracts. Specifically, ST-C led to a 6% increase in ALP activity at 25 $\mu\text{g}/\text{mL}$, which significantly rose to 26% at 100 $\mu\text{g}/\text{mL}$. Notably, ST-1 exhibited a remarkable ($p < 0.001$) 52% boost in ALP activity at 100 $\mu\text{g}/\text{mL}$ when compared to ST-C. Furthermore, ST-2 yielded a 14% increase in ALP activity at 25 $\mu\text{g}/\text{mL}$, escalating to 60% at 100 $\mu\text{g}/\text{mL}$. Additionally, ST-3 resulted in a 36% rise in ALP activity at 100 $\mu\text{g}/\text{mL}$.

Effect of steaming of SHF on mineralization and calcium deposition

The mineralization of the extracellular matrix is a critical process in bone formation, closely linked to the development and differentiation of osteoblasts [21]. In order to assess the impact of extracts from steamed SHF on osteoblast development, we conducted ARS staining to measure calcium accumulation within the extracellular matrix.

Our research outcomes revealed that the application of steamed SHF extract led to a significant increase in calcified extracellular matrix after 24 days when compared to the control group. Following ARS staining, the nodules of mineralized calcium deposition displayed a striking orange-red color. Specifically, the mineralization process exhibited substantial improvement, ranging from $106.1 \pm 1.7\%$ to $120.6 \pm 1.36\%$ with ST-C treatment at concentrations ranging from 25 to 100 $\mu\text{g/ml}$. Similarly, treatment with ST-1 resulted in enhanced mineralization, with values ranging from $108.5 \pm 2.2\%$ to $131.3 \pm 3.0\%$ at concentrations of 25 to 100 $\mu\text{g/ml}$. Likewise, ST-2 treatment at concentrations of 25 to 100 $\mu\text{g/ml}$ increased the mineralization area from $111.4 \pm 1.8\%$ to $142.5 \pm 1.3\%$, and ST-3 demonstrated an increase in mineralization ranging from $109.1 \pm 0.89\%$ to $126.9 \pm 1.2\%$ (Fig. 4A and B).

Effect of steaming of SHF on collagen content

To assess collagen secretion, Sirius Red staining was conducted following a 14-day osteogenic induction period. Remarkably, the findings indicate a positive influence of steam-treated SH on collagen formation, as illustrated in Fig. 4C. The staining intensity of MC3T3-E1 cells exhibits a discernible dose-dependent trend. Concurrently, the quantitative outcomes presented in Fig. 4D demonstrate notable dose-dependent variations among ST-C and the ST-1, ST-2, and ST-3 groups, respectively.

On day 14, a discernible increase of approximately 32% (in ST-1 groups), 40% (in ST-2 groups), and 25% (in ST-3 group) was quantified across the concentration range of 0 to 100 $\mu\text{g/ml}$. These results highlight the positive impact of steam treatment on collagen synthesis and suggest a dose-dependent enhancement in the observed effects.

The effect of steaming of SHF on TRAP in RAW264.7 cells

We conducted further investigations to assess the impact of varying concentrations of steamed SH (ranging from 31.25 to 500 $\mu\text{g/mL}$) on osteoclast cell survival, osteoclastogenesis, and resorption activity. Following a 24-hour treatment in RAW264.7 cells, none of the tested dosages showed any detrimental effects. However, after 48 hours, cell viability began to significantly decrease, particularly beyond the 125 $\mu\text{g/mL}$ concentration (Fig. 5A and B). Consequently, we selected 100 $\mu\text{g/mL}$ as the highest concentration for subsequent experiments.

The results pertaining to the influence on TRAP activity, as measured in RAW264.7 cells, are illustrated in Fig. 6C. In the presence of RANKL, RAW264.7 cells generated multinucleated osteoclast-like (OCL) cells that exhibited TRAP positivity (Fig. 6A and B). Notably, the number of multinucleated cells showing TRAP positivity declined when exposed to steamed SHF extracts at concentrations of 25 and 100 $\mu\text{g/mL}$. It is worth mentioning that the treatment with ST-1, ST-2, and ST-3 exhibited distinct morphological changes

compared to control OCL cells, with reduced size and fewer nuclei. Specifically, ST-1 and ST-2 treatment significantly decreased the number of OCLs compared to RANKL-treated cells. Similarly, steamed SHF extracts significantly reduced TRAP activity in a dose-dependent manner, with TRAP activity expressed as a relative value, using the untreated group as a reference set at 100%. As depicted in the figure, even non-steamed fruits displayed a notable inhibitory effect on TRAP activity, amounting to approximately ($p < 0.05$). However, the inhibition of TRAP activity was more pronounced in steamed fruits, with a notable increase in inhibitory activity as the number of steaming cycles increased. The inhibitory effect was notably higher when the fruit was steamed twice ($p < 0.001$). However, the inhibitory activity of fruits steamed three times on TRAP showed a slight reduction.

The effect of steaming of SHF on the expression of genes related to differentiation and proliferation of osteoblasts and osteoclasts

To substantiate the promotion of osteogenic differentiation by steamed SH, we conducted an examination of osteogenesis-related markers through RT-PCR under 100 $\mu\text{g/ml}$ concentrations of ST-C, ST-1, ST-2, and ST-3 treatments after a 12-day osteogenic induction. The mRNA expression of Runt-related transcription factor 2 (Runx2) exhibited upregulation by 1.50 ± 0.18 (ST-C), 2.30 ± 0.31 (ST-1), 2.62 ± 0.20 (ST-2), and 1.98 ± 0.09 (ST-3) folds. Additionally, ALP, osteoprotegerin (OPG), type I collagen (COL-1), and Bone Gamma-Carboxyglutamate Protein (BGLAP) mRNA expressions were assessed by RT-PCR (refer to Fig. 7). Notably, ST-C, ST-1, ST-2, and ST-3 treatment groups exhibited significant upregulation in OPG, ALP, BGLAP, and COL-1 mRNA expressions, with ST-1 and ST-2 demonstrating particularly noteworthy increases. Overall, the osteogenesis-related gene expressions in steamed ST-1 and ST-2 groups were approximately 30–60% higher than those in the ST-C treatment groups. Furthermore, the expression of receptor activator of nuclear factor kappa beta (RANKL) exhibited a moderate decrease with ST-1, ST-2, and ST-3 treatments (Fig. 7A).

To delve into the molecular mechanisms of steamed SH in osteoclast differentiation, we examined the expression of TRAF6, revealing that ST-1, ST-2, and ST-3 inhibited TRAF6 expression compared to ST-C. Subsequently, we investigated the mRNA expression of NFATc1 and c-Fos, key regulators of osteoclast differentiation. ST-1 and ST-2 significantly inhibited the mRNA expression of NFATc1 ($P < 0.001$) and c-Fos ($P < 0.001$) compared to both ST-C and ST-3. Further confirmation of the inhibitory effect on osteoclast differentiation involved RT-PCR detection of RANK, TRAP, CtsK, and MMP9 expression (refer to Fig. 7B). ST-1, ST-2, and ST-3 demonstrated significant decreases in the expression levels of osteoclast differentiation-related genes compared to ST-C (Fig. 7B), indicating that the inhibitory impact of steamed SH on RANKL-mediated NFATc1 expression is followed by the downregulation of osteoclastogenic marker genes.

Osteotropic effect of 5-HMF, a compound increased in SHF by steaming process

Notably, our study represents a novel contribution as we have successfully identified the presence of 5-HMF in the steamed fruit of SH. The verification of 5-HMF was achieved through the utilization of preparative High-Performance Liquid Chromatography (prep-HPLC) in conjunction with Nuclear Magnetic Resonance (NMR) spectroscopy. This analytical approach ensures the accuracy and reliability of our findings, further substantiating the observed increase in 5-HMF levels following steaming. In our investigation, the content of 5-HMF in untreated SH fruits was 217µg/g as existence naturally, but it was significantly increased about two-fold by once steaming process and showed 4-folds by twice treatment. However, in three-time steamed SH fruit, it was reduced to almost unsteamed level. (Fig. 8A).

Figure 8B and C shows the effect of 5-HMF on ALP and genes related to the differentiation and proliferation of osteoblast cells. Interestingly 5-HMF increased remarkably ALP activity with dose dependent manner, namely the enzyme activity was increased 21% by 10µg/ml 5-HMF and 43% by 20µg/ml, respectively. 5-HMF also promoted the expression of OPG, Rux2 and RANKL genes that play a key role in the differentiation and or proliferation of osteoblast cells.

5-HMF strongly inhibited TRAP activity in RAW264.7 cells and also suppressed the expression of its related genes such as RANK, TRAP, and TRAF6. (Fig. 8D and E)

Discussion

In contemporary studies, there is a growing interest in exploring natural products that can activate osteoblasts or hinder osteoclastogenesis. In this particular investigation, we examine the potential of steamed SH fruits as an alternative approach to combat osteoporosis, shedding light on its mechanism of action with a specific emphasis on *in vitro* osteogenesis.

SH fruits were steamed under the conditions most commonly applied in the Korean food industry for the processing or 'Bupjae' of food and natural products such as rice, chestnuts, ginseng and *Pleuropterus multiflorus*, and investigated changes in physicochemical properties. The steaming treatment is usually steamed for 1 to 2 hours and repeated once to several times, up to nine times. The authors observed SH fruits by steaming them for 2 hours and repeating the process up to 3 times.

As seen in Fig. 1, it was found that when SH fruits were steamed, a dark browning occurred in the fruits and its extract, and this phenomenon became more intense as the number of steaming process continued. This color change seems to be due to the oxidation reaction of diphenols and/or Maillard reaction of compounds present in the SH fruits. This is because SH fruits contain proteins and amino acids along with various types of sugars [14], and these substances can cause the reaction during the steaming.

The yield of SH fruits extract increased by about 15% due to steaming two times. This is thought to be the result of the fruit's texture structure becoming somewhat loose and fine-powdered during steaming process. However, when steamed it three times, although the browning phenomenon increased, the extract yield was lower than that of un-steamed fruits. This result is believed to be due to an increase in

adhesive micronized particles in the fruit tissue due to an increase in the number of times of steaming, which increases the surface area of the particles and increases solubility.

In order to investigate the effect of steaming treatment on phenolic compounds and antioxidant activity of SH fruits, the contents of CGA, a representative phenolic acid, total phenolic compounds, and total flavonoid compounds were compared. Additionally, free radical scavenging ability was compared to evaluate antioxidant activity.

CGA is a naturally occurring phenolic compound found in all higher plants. CGA is a representative phytochemical present in the fruits as it is a compound with various physiological activities such as strong antioxidant activity as well as promoting bone formation and inhibiting bone resorption [22, 23]. CGA up-regulates collagen I mRNA and protein expressions and increase the collagen secretion in HDFs without affecting the cell viability, and reduce the accumulation of UVA-induced reactive oxygen species.

Potential uses of CGA are suggested in pharmaceuticals, foodstuffs, feed additives, and cosmetics due to its recently discovered biomedical activity. This finding caused new interest in CGA properties, its isomers, and its natural occurrence. In this study, the authors found that three types of isomers, including CGA, neo-CGA and crypto-CGA exist in similar amounts in SH fruits. the content of CGA in SH fruits was almost unchanged to two times steaming, but decreased by 25% only when treated three times compared to unsteamed control. In addition, the content and the ratio of 3 isomers of CGA were all maintained in the same pattern until the treatment of two times. This result indicates that CGAs, the major phenolic acids in SH fruit, is relatively stable under the steaming process at 100°C steam for 2hrs.

An interesting finding in this study is the remarkable increase in TPC and TFC content of SH fruits to 1.7 and 7.7 folds, respectively, by steaming two times.

Polyphenols are a representative phytochemical with antioxidant activity. Flavonoids also exert many positive effects on health and protect against various diseases. Therefore, since ancient times, much effort has been made to maintain or further increase the content of these and nutritional value of fruits and vegetables. In general, consumers tend to prefer non-thermal processing methods to maintain nutritional value and active ingredients. Although non-thermal processing methods have the advantage of maintaining ingredients with low stability, however, is can affect polyphenols negatively since the residual enzyme activity from the extracts [24]. In addition, in the case of the flavonoid luteolin-7-O- β -glucoside, it was observed that splenocyte proliferation, natural killer (NK) cell activities, and antioxidant capacity were improved when heat treated [25]. It has been reported that the content of free and bound form flavonoids in chestnuts was increased when those were steamed [26]. When shiitake mushrooms were heated under pressure, free flavonoids increased and bound forms decrease. In addition, secondary products with a higher activity may have been generated due to thermal reactions or isomerization in vegetables and or fruits rich in water [27].

Human bones undergo continuous bone formation and resorption and remodeling to be replaced with new bone [28]. During this process, bone resorption is higher than bone formation due to abnormalities in

hormone secretion such as estrogen or in metabolism of the related biomolecules, and when the balance is broken, bone density decreases, bone tissue degenerates, and osteoporosis develops. Means to increase bone density include diet such as intake of calcium and vitamin D, and exercise therapy, however, the most effective means is using drugs that increase bone formation and/or inhibit absorption. Therefore, to prevent and cure osteoporosis, strategies are needed to promote the differentiation of osteoblast cells or to suppress the differentiation of osteoclast cells. So, for, much effort has been made to develop drugs that regulate these biological functions. Interestingly, these two biological functions have coupling factors like RANK-RANKL and two essential enzymes, ALP and TRAP. These are used as markers of osteoclasts and osteoblasts, respectively.

In this study, SHF extract (100 µg/mL) increased the activity of ALP by approximately 26% in osteogenic cells (MC3T3-E1) without cytotoxicity. And that of steamed fruit significantly increased more the activity of this enzyme at the same concentration. Namely, ALP activity increased by 52% when steamed once, and by 60% when steamed twice. In order to know that the gene expression of this enzyme was examined to determine whether the increase in activity of this enzyme was due to the simple action of an activator due to a certain component in the extract or due to cellular expression. As a result, it was confirmed that the enhancement of ALP activity was due to promotion of gene expression.

The mineralization of the extracellular matrix is a critical process in bone formation, closely linked to the development and differentiation of osteoblasts [21]. In order to assess the impact of extracts from steamed SHF on osteoblast development, we conducted ARS staining to measure calcium accumulation within the extracellular matrix. The results of calcium nodule formation measured by ARS staining showed more clearly that steaming of SHF can increase bone density as shown in Fig. 3C. As another marker for mineralization, anabolic effect of the steamed fruits on differentiation and mineralization of osteoblast was measured by evaluation of the production collagen formation in MC3T3-E1 cells. As shown in Fig. 3C, the result of collagen production is almost the same pattern as ALP activity and formation of Calcium nodules. This consistently shows the clear effect of steamed SHF on promoting bone formation.

TRAP is a glycoprotein produced by activated dendritic cells, mature osteoclasts, and macrophages, and is recognized not only as a histochemical marker for osteoclasts, but also as a widely occurring molecule with functions in both the skeletal and immune systems [29].

Mouse macrophage is well known *in vitro* model for osteoclastogenesis and have been shown maintain the capability to differentiate into mature osteoclast-like cells through the stimulate a receptor activator of RANKL and M-CSF [30] when RAW264.7 cells treated RANKL, these cells produces high levels of osteoclast specific marker such as TRAP [31]. So further, the inhibitory effects of the steamed SH fruits on the regulation of RANKL-induced osteoclastogenesis in RAW264.7 cells were investigated.

SHF extract significantly inhibited the activity of TRAP, and the inhibitory activity of the enzyme was further increased in steamed fruits. We also confirmed that the inhibition of this enzyme's activity corresponded to the gene expression level by measuring the mRNA level. These results suggest that

steamed SHF have the activity of inhibiting the differentiation of osteoclast cells. From the above, we confirmed that steamed SHF extracts play a positive role in two enzymes that play a pivotal role in bone formation and resorption. Therefore, in order to find out how SHF extract acts on two functionally different enzyme systems, its effect on the expression of the major genes related osteoblastogenesis and osteoclastogenesis was investigated.

Multiple anabolic signaling pathway, such as Wnt, BMP and Runx2 are positive regulators osteoblast differentiation. Among these Runx2 is a vital transcriptional regulator of osteoblast differentiation and bone formation [32]. Runx2 pathway has been shown to not only activate mRNA expression ALP but also stimulate the expression of OCN, a late-stage marker of osteoblast differentiation. Runx2 critically regulates many of the gene that control the development of osteoblast differentiation such as ALP, Coll-I and OCN [33]. Therefore, we evaluated the effect of SHF extract on the expression of Runx2, ALP, OCN, RANKL, and Coll-I by measuring the mRNA levels in MC3T3-E1 cells. Our results clearly show that steamed SHF extract may stimulates osteoblast differentiation and mineralization by signaling through the Runx2 pathway.

To determine the inhibitory mechanism of SHF extract against osteoclast-specific markers, the mRNA expression of transcription factors such as RANK, TRAP, NFATc1, c-fos, TRAF6, Ctsk and MMP9 were analyzed by RT-PCR analysis. Osteoblasts and bone marrow stromal cells produce RANKL, a key factor in bone regulation. RANKL binds to RANK on monocytes/macrophages, initiating receptor oligomerization. This process recruits signaling adapter molecules like TNF receptor-associated factor- κ B ligand 6 (TRAF6) [34]. The subsequent association of TRAF6 with TAK1 activates downstream signaling pathways, including NF- κ B and activator protein 1 (AP-1) with c-Fos/c-Jun dimer, leading to the expression of NFATc1, the master regulator of osteoclasts [35]. Modulating these pathways could potentially prevent excessive osteoclast formation and mitigate pathological bone loss as shown in Fig. 4b, the finding of our results suggest that SHF extract has a strong anti-osteoclastogenic activity *in vitro*. This is because all measured gene expressions induced by RANKL were strongly suppressed. The particularly strong inhibitory activity of SHF extract on c-fos and TRAF6 expression suggests that it regulates the early stages of osteoclast differentiation. The above results clearly show that the steamed SHF extract has the effect of promoting osteoblast differentiation and inhibiting osteoclast differentiation. So, what ingredient in SHF extract exerts such positive effects?

First, we can consider the possibility that these are already well-known active substances. As mentioned earlier, honey contains a wide variety of phytochemicals. Among SH extracts, compounds reported to be effective against osteoporosis include rutin [36], luteolin-7-o-glucoside [37], quercetin [38], prunin [39], CGA [40] and gallic acid [41]. In addition, methanol extract of SH leaves is also reported to promote ALP activity and inhibit TRAP activity [42].

However, in this study, the authors confirmed that total flavonoid compounds and phenolic compounds increased when the SHF were steamed, but the identification of individual components and their activities

were not verified. Therefore, it is unknown which flavonoid component contributed to the osteotropic activity and to what extent.

Secondly, we therefore paid special attention to the components that increase during steaming of SHF. It was observed that 3~4 peaks of compounds increased on the HPLC chromatogram. Among them, the one ingredient that increased most noticeably was separated and purified, and its chemical structure was confirmed using NMR. The compound was 5-HMF, one of the Maillard reaction products. The authors investigated the effect of this ingredient on ALP and TRAP activity and gene expression related to osteoblast and osteoclast differentiation. Surprisingly, this component remarkably promoted ALP activity and strongly inhibited TRAP activity. Additionally, the results of gene expression experiments showed that this substance positively and effectively participates in their expression.

Therefore, the authors concluded that 5-HMF, which was significantly increased during the steaming process of SHF, may be a cause of the increase in osteogenic activity.

5-HMF is widely available in different food products which are thermally processed and it serves as a well-established indicator of nonenzymic browning, frequently employed to assess deteriorative changes resulting from heating and/or prolonged storage of food products [41]. It exhibits biphasic effects, positive and negative activities in biological systems. Namely, 5-HMF has anti-cancer, antioxidant, anti-proliferative, anti-lesion, anti-inflammatory, anti-allergic, and anti-apoptotic activities [43]. On the other hand, SMF derived from 5-HMF exhibited genotoxicity [44]. However, no toxic effects have been observed at daily doses ranging from 80 to 100 mg/kg body weight [45]. To date, there are no conclusive human studies confirming the toxicity of 5-HMF.

In the food industry, 5-HMF content is commonly regarded as a quality indicator, signifying overheating during processing or inadequate storage conditions. The content of 5-HMF in food products varies widely based on ingredients, formulation, and processing methods. It is found in various foods and fruits such as malt, coffee, vinegar, honey, and fruit juice. In the case of fresh foods, 5-HMF levels are typically negligible, approaching zero, but it can be formed in fruits containing carbohydrates during storage. 5-HMF content is proportional to the lycopene content in processed tomato products. 5-HMF content in fruit ranges from 35–79 mmol/kg and it doubles during maturation from the green stage to the deep red stage [46].

In our studies, following one and two rounds of steaming, there was a substantial increase in HMF levels in SHF, however, a diminishing trend was observed after steamed 3 times. Therefore, the simple steaming of SHF can increase the physiological activity against osteoporosis, and since the fruit is high in sugar, it is also beneficial for long-term storage.

In summary, steaming SHF increases osteogenic activity, and 5-HMF produced during steaming appears to contribute significantly. It seems that some components, including 5-HMF, present in SHF extract promote bone formation and inhibit bone resorption by regulating the expression of genes involved in the RANKL-RANK-OPG signal.

Conclusion

This study is the first to demonstrate *in vitro* the positive effects of SHF extract on osteoblastogenesis and osteoclastogenesis during osteoporosis. Steaming of SHF increases yield of the extracts without affecting the content of CGA which is one of the main components. It also significantly increases the contents of phenolics including flavonoids, desirable Maillard reaction products as well as antioxidant activity. More interestingly, this process significantly improved the activity of promoting bone formation and inhibiting bone resorption in cellular level.

This study provided valuable information on dynamic changes of phenolic profiles and the ability of bone cells differentiation in SHF under a steaming process, which could offer one possible guidance for improving biochemical activity of SHF.

Declarations

Author contributions

The concept originated from Reshmi Akter, Muhammad Awais and Dong Wook Lee. Reshmi Akter, Jong Chan Anh, and Dong Wook Lee were responsible for the original writing, drafting, and figure preparation, while Jong Hak Kim, and Md Nijal Morshed contributed to formal analysis. Fund acquisition was spearheaded by Dong Wook Lee, with support from Deok Chun Yang, Kong Byoung Man.

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Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

Data Availability

The experimental data are included in the manuscript and is available from the corresponding author upon request.

Ethics declarations

Not Applicable.

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Figures

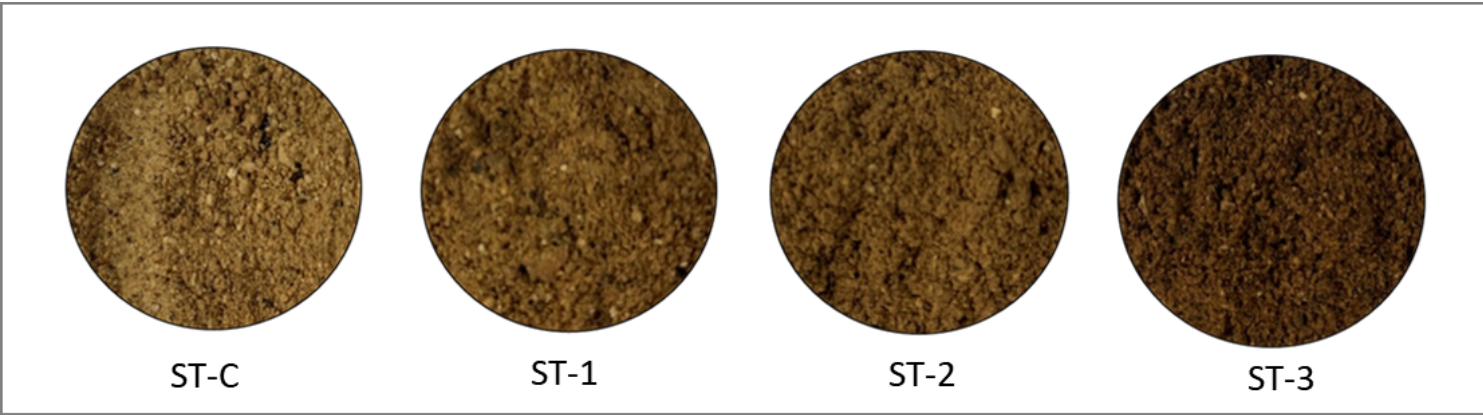


Figure 1

Change in color of SH fruits by steaming

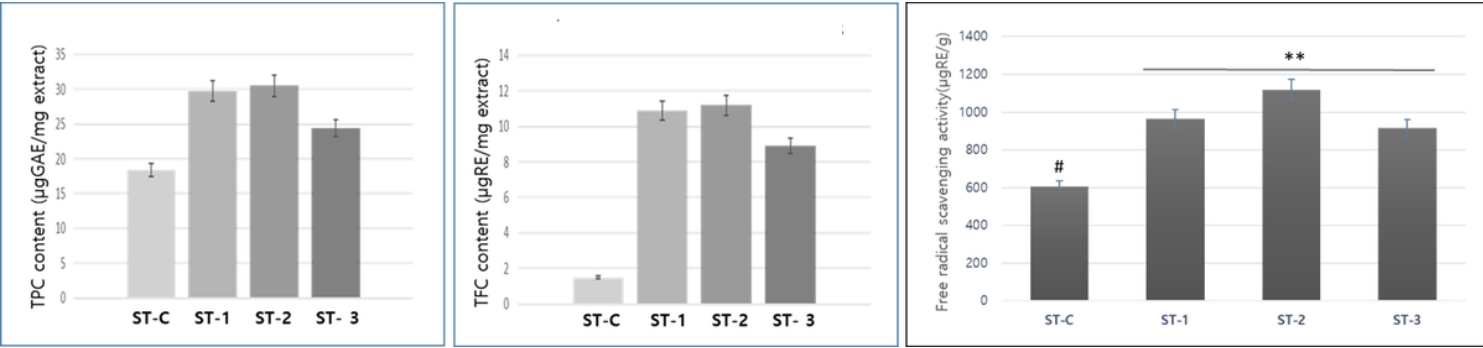


Figure 2

Changes in the content of total phenolic and flavonoid compounds, and free radical scavenging ability of SHF by steaming treatment.

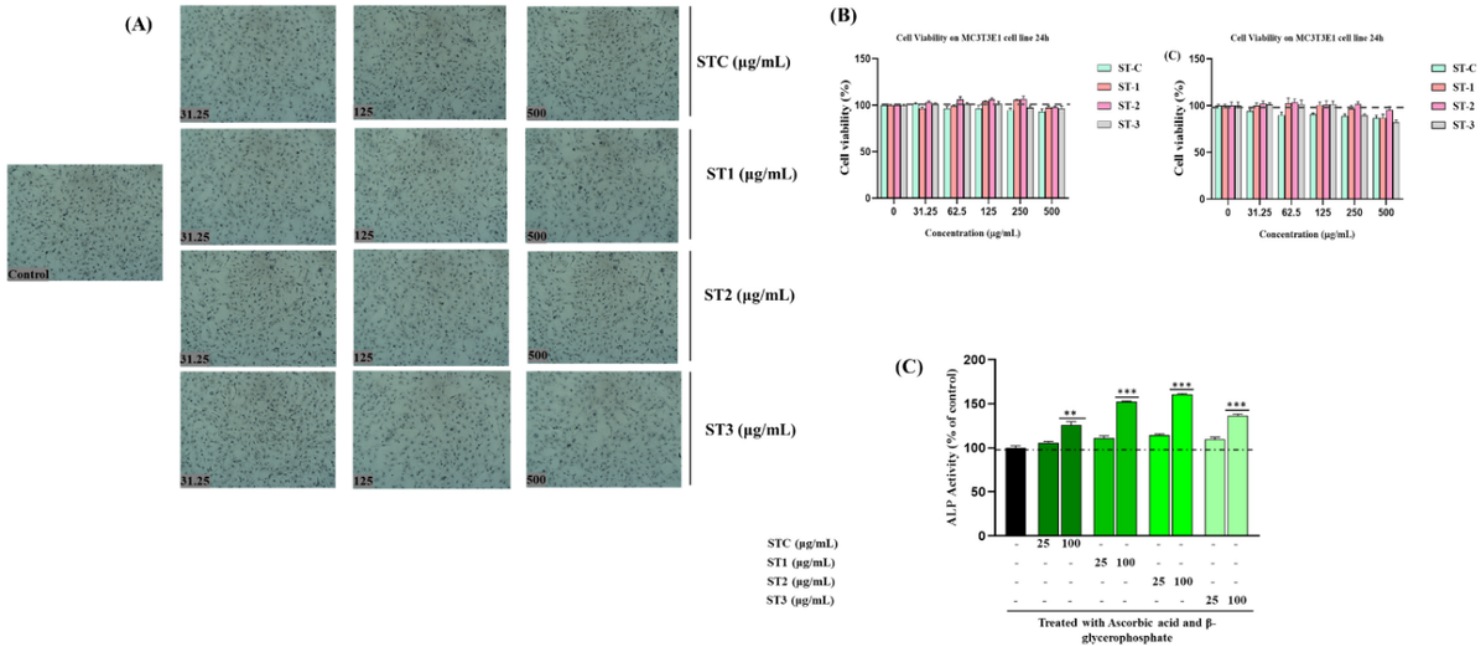


Figure 3

MC3T3-E1 pre-osteoblast cells (1×10^4 cells/well) were treated with SHF (0–500 $\mu\text{g ml}^{-1}$) for 2 days. (A) Cell viability was determined by an MTT assay on 24h and 48h. (B) Cellular images were captured using phase-contrast microscopy ($\times 20$). Scale bar = 100 μm . (C) Effects of SHF on ALP activity in MC3T3–E1 cells. Cells were cultured with different concentrations (25 to 100 $\mu\text{g/mL}$) of SHF extract for 7 days. The demonstrated data consists of the mean $\pm$ standard deviation (SD) from three different experiments. Statistical analysis revealed significant differences, denoted as $**p < 0.01$, $***p < 0.001$ when compared with the indicated ascorbic acid and β -glycerophosphate treated group.

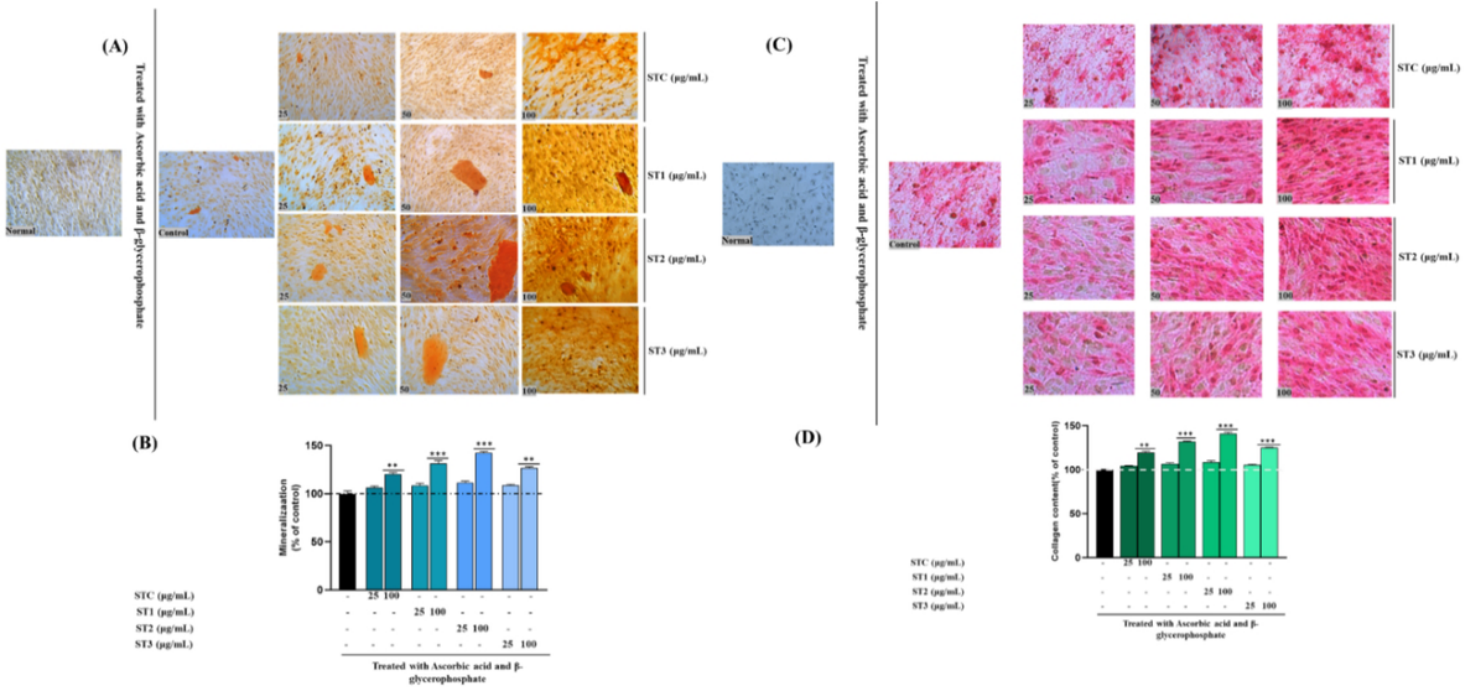


Figure 4

Effects of SHF extracts on mineralization in MC3T3–E1 cells. Cells were treated with or without differentiation media with extracts at concentrations ranging from 25 to 100 $\mu\text{g/mL}$ for 12 days. (A) Alizarin red staining (ARS) was performed and visualized by microscopy ($\times 100$ magnification). (B) Mineralization (%) was evaluated by measuring absorbance at 562 nm. (C) Picro–sirius red staining was performed and visualized by microscopy ($\times 100$ magnification). (D) Collagen content was evaluated by measuring absorbance at 550 nm. The demonstrated data consists of the mean $\pm$ standard deviation (SD) from three different experiments. Statistical analysis revealed significant differences, denoted as $**p < 0.01$, $***p < 0.001$ when compared with the indicated ascorbic acid and β -glycerophosphate treated group.

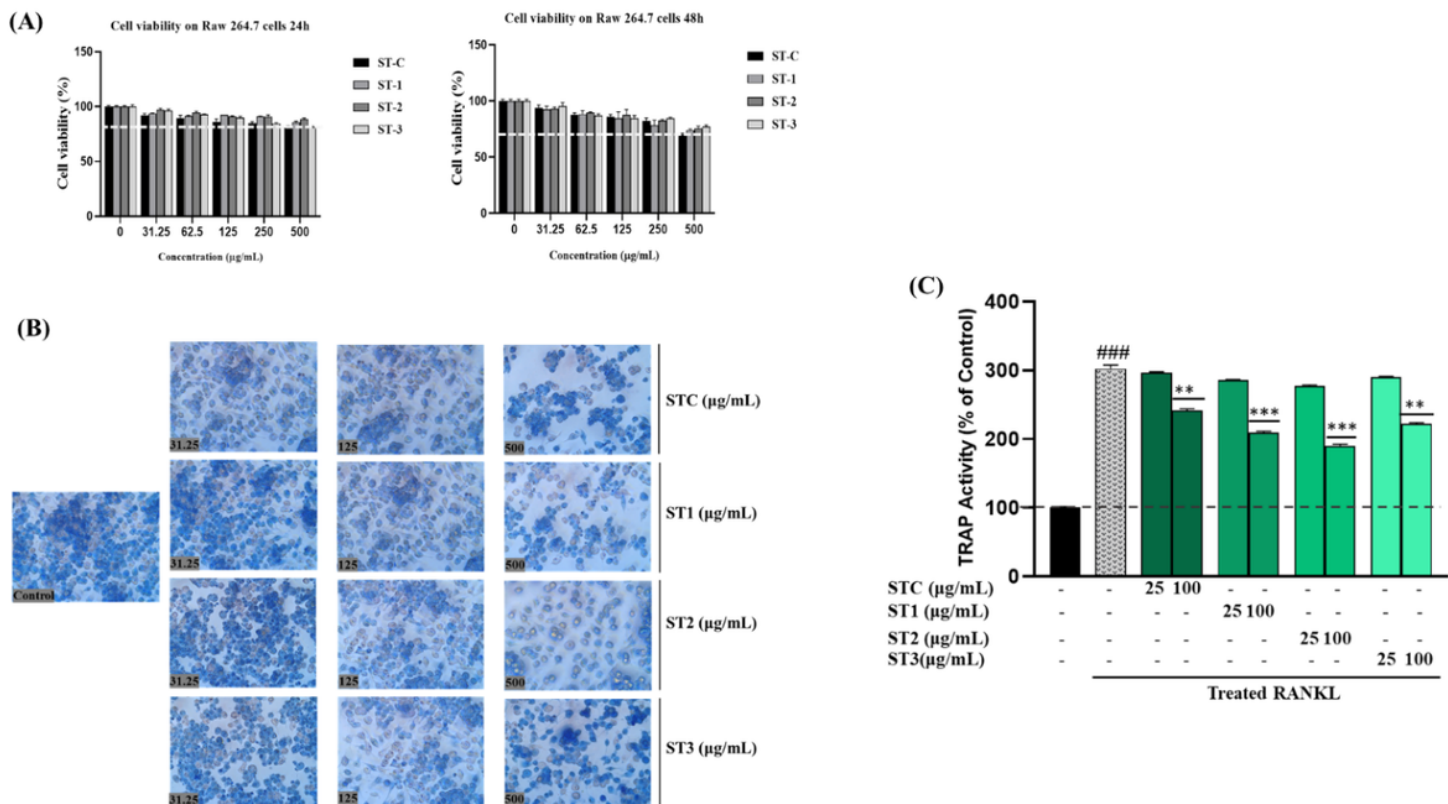


Figure 5

Raw 264.7 macrophage cells (1×10^4 cells/ml) were treated with SHF (0–500 µg/ml) for 2 days. (A) Cell viability was determined by an MTT assay on 24h and 48h. (B) Cellular images were captured using phase-contrast microscopy ($\times 20$). Scale bar = 100 µm. (C) Differentiation of RAW 264.7 cells into osteoclasts was induced by stimulation with RANKL in the presence of SHF (25 to 100 µg/mL). Five days later, TRAP activity was measured using the TRAP solution assay. The demonstrated data consists of the mean $\pm$ standard deviation (SD) from three different experiments. Statistical analysis revealed significant differences, denoted as **p<0.01, ***p<0.001 when compared with the indicated RANKL-treated group, and ###p<0.001 when compared with the normal treated group

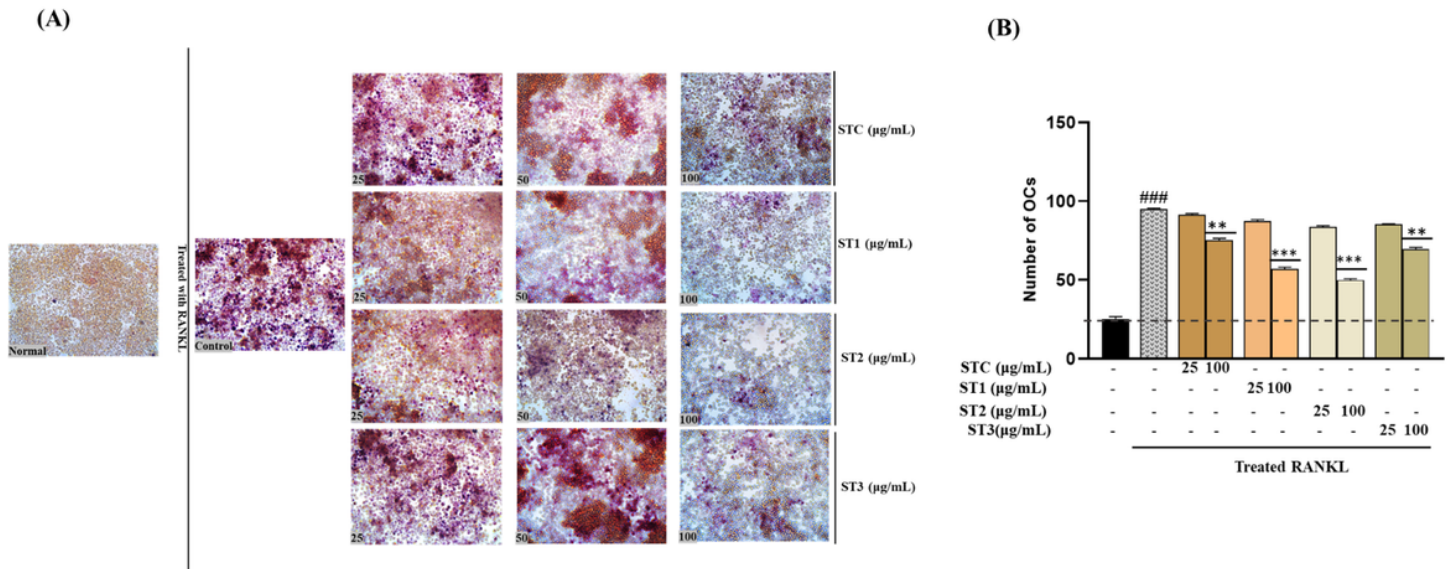


Figure 6

(A) RANKL-induced TRAP-positive osteoclast-like cell formation by SHF concentrations (25 to 100 µg/mL) for 7 days (A) Osteoclast differentiation: Multinucleated osteoclasts were visualized in 100× magnification under light microphotography. Scale bars, 100 µm. (B) TRAP-positive multinucleated cells were counted as osteoclasts. Statistical analysis revealed significant differences, denoted as ** $p < 0.01$, *** $p < 0.001$ when compared with the indicated RANKL-treated group, and ### $p < 0.001$ when compared with the normal treated group.

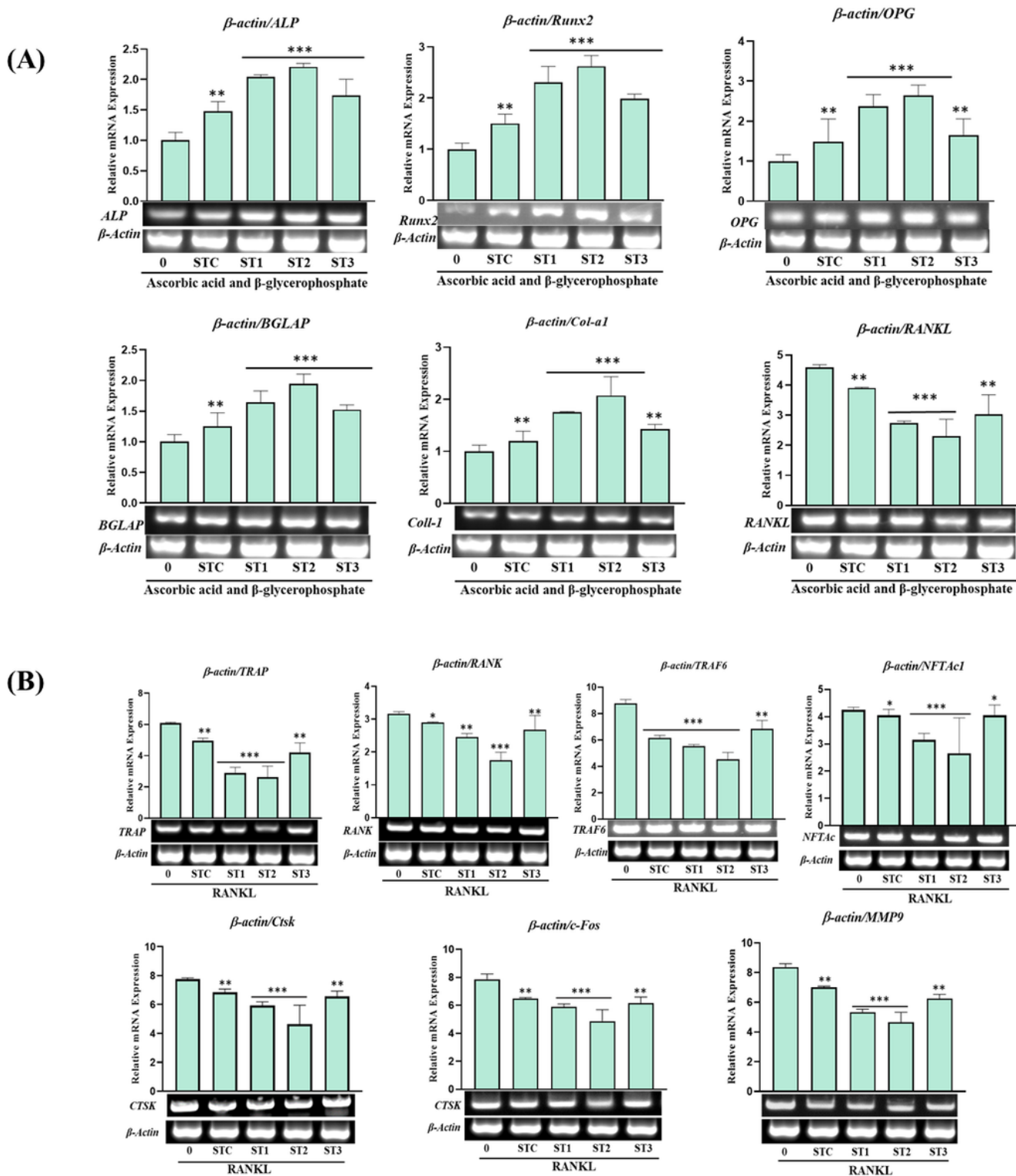


Figure 7

Effects of SHF extracts on osteoblast differentiation in MC3T3–E1 cells. Cells were treated with or without differentiation media, with the extracts, for 14 days. (A) The relative mRNA expression level of osteoblast markers (Alp, Runx2, Col–I, BGLAP, OPG, and RANKL) determined by real-time PCR. (B) mRNA expression of transcriptional factors of osteoclast differentiation. RAW 264.7 cells were treated with RANKL and SHF extracts for 7 days. The expression of osteoclast differentiation marker genes (TRAP,

RANK, TRAF6, MMP9, c-Fos, CtsK, NFTAc) was measured by real-time PCR. Statistical analysis revealed significant differences, denoted as $**p<0.01$, $***p<0.001$ when compared with the indicated ascorbic acid and β -glycerophosphate and RANKL-treated group, respectively.

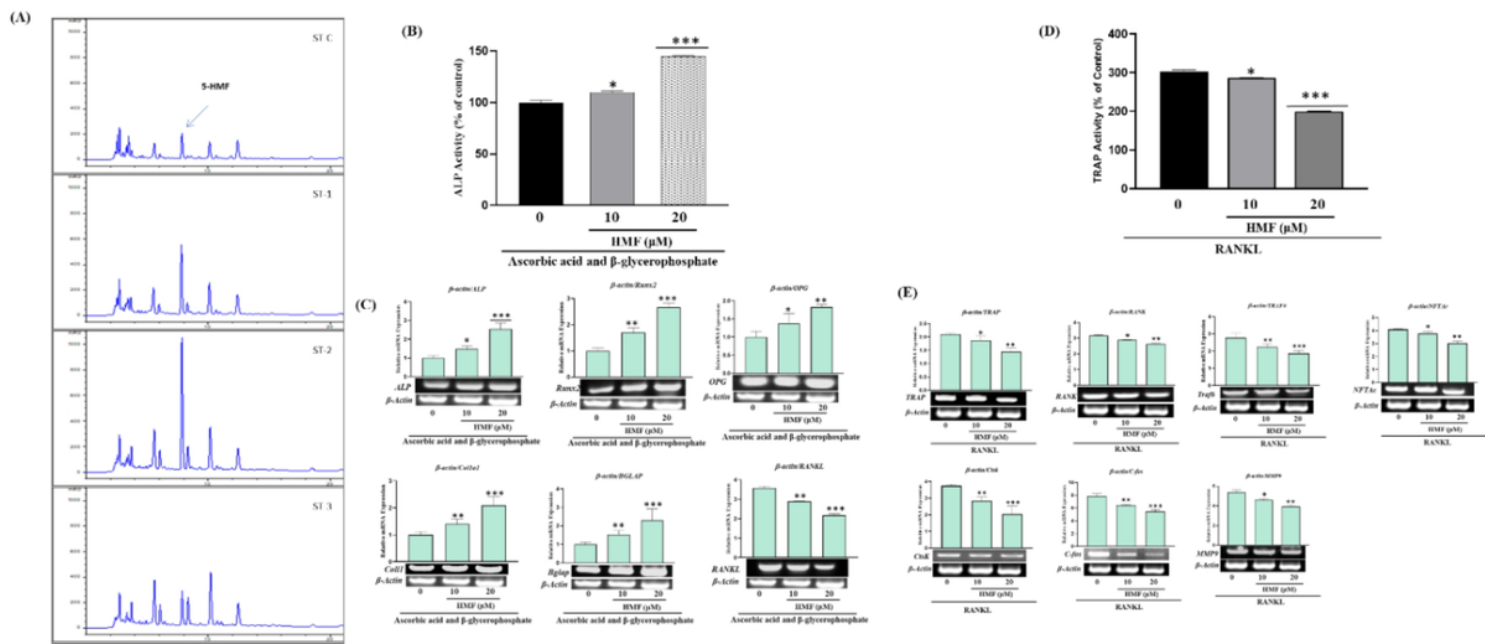


Figure 8

(A) Change in Content of HMF by steaming was determined by HPLC. (B) Effect of HMF on ALP activity and (C) its related gene expression, (D) on TRAP activity (E) and its related gene expression. Statistical analysis revealed significant differences, denoted as $**p<0.01$, $***p<0.001$ when compared with the indicated ascorbic acid and β -glycerophosphate and RANKL-treated group, respectively.

Supplementary Files

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