

## Osteoporosis-alleviating effects of unsaponifiable matter derived from *Chlorella* sp. Lipids on bone regeneration promotion and bone matrix enhancement in ovariectomized C57BL/6 mouse model

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### ABSTRACT

This study evaluated the osteoporosis-preventive effects of unsaponifiable matter extracted from microalgal lipids, specifically by inhibiting osteoclast-mediated bone resorption *In vitro* osteoclasts and in an *in vivo* ovariectomized (Ovx) mouse model. *In vitro* experiments using osteoclast cells revealed that treatment with *Chlorella* sp. unsaponifiable matter (CUM) significantly reduced expression of genes and proteins related to osteoclast differentiation (*DC-STAMP*, *NFATC1*, *MITF*, *OSCAR*) and bone matrix degradation (*TRAP*, *CATK*) by 23.5–52.2 %, thereby promoting bone health. In Ovx mice, dietary CUM increased femur weight by 20.3 %, as well as mineral content, particularly calcium and phosphorus levels, by 30.4 % and 25.6 %, respectively, thereby demonstrating the reproducibility of the *In vitro* results and indicating enhanced bone formation and mineralization in the animal model. Additionally, micro-computed tomography revealed a 110.5 % increase in bone mineral density, highlighting CUM's potential as a therapeutic agent for osteoporosis. Gas chromatography-mass spectrometry identified the main bioactive compounds in CUM, such as phytosterols and fatty acids, which contribute to its anti-osteoporotic effects. Therefore, CUM shows potential as a natural therapeutic agent for osteoporosis by promoting bone formation and enhancing mineralization.

### Introduction

Osteoporosis is a chronic metabolic bone disease primarily caused by hormonal changes, aging, and genetic variations, which result in decreased bone mass, bone tissue deterioration, and bone microstructure destruction. In turn, these decrease bone mineral density (BMD), thereby increasing the likelihood of fragility fractures (Ahire et al., 2024; Ralston and Uitterlinden, 2010). In menopausal women, decreased estrogen secretion leads to the continued production of sclerostin (*SOST*), which inhibits osteoblast activity, as well as the decreased secretion of insulin-like growth factor 1 (*IGF-1*), which promotes osteoblast differentiation and proliferation, thereby inhibiting calcium absorption and bone formation (Levin et al., 2018). In addition, excessive secretion of receptor activator of nuclear factor- $\kappa$ B ligand (*RANKL*) and osteoprotegerin (*OPG*), which both promote osteoclast activity, can

cause bone degradation and continued mineral loss, thus weakening bone strength (Zallone, 2006). According to the International Osteoporosis Foundation Annual Report in 2023, >53.6 million people in United States are affected by osteoporosis, which had an annual economic burden of \$16 billion in 2017 that is projected to increase 27 % by 2030 (James and Dennis, 2024). Osteoporosis is a serious health concern in Europe and the United States, seen in 30 % of menopausal women and, according to the *Fracture* study, in 15 % of women aged 50 years and older (Hsu et al., 2020; Tuzun et al., 2012). Estrogen modulators and osteoclast inhibitors such as raloxifene, alendronate, and risedronate are used forms of synthetic pharmaceutical therapy in the treatment of osteoporosis (Chen et al., 2019). Raloxifene (product name: Evista) is a widely used estrogen modulator that inhibits bone loss by modulating *SOST* and increases vertebral bone density by 2 %–3 % in postmenopausal women, promoting bone formation and reducing fracture

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risk (Liu, 2018). Meanwhile, alendronate (product name: Fosamax) is an osteoclast inhibitor that reduces bone degradation by inhibiting *RANKL* and increases bone density by 6 %–8 % over 3 years, thus reducing the risk of vertebral fracture by 50 % (Cannarella et al., 2019). However, side effects such as deep vein thrombosis, pulmonary embolism, and osteonecrosis of the jaw have been reported. Research is ongoing to find an alternative to these existing synthetic pharmaceutical materials.

Microalgae can address environmental issues in material production due to their rapid growth rate, minimal organic carbon source requirement, and high carbon fixation compared to terrestrial organisms. Microalgal lipids contain high amounts of bioactive substances such as tocopherol, triterpene, astaxanthin, lutein, and phycocyanin, as well as vitamins D and K and, minerals; various minerals; they are currently being studied for their use in food and medicine (Puspitadewi and Auerkari, 2018). Microalgal lipid-derived unsaponifiable matter is through the process of saponification, a chemical reaction wherein lipids react with alkali to produce glycerol and fatty acid salts. The soap and glycerol are then removed, and the remaining substances are called unsaponifiable matter. They have been gaining increasing interest because they contain high levels of bioactive substances which provide various health benefits, including antioxidants, anti-inflammatory, anti-obesity, and cholesterol-reducing activities (Marozik et al., 2018). Specifically, highly purified phycocyanin, a representative unsaponifiable matter, can inhibit the interaction between receptor activator of nuclear factor kB (*RANK*) and *RANKL*, which are two key transcription factors in osteoclasts, thereby inhibiting osteoclast differentiation and promoting the osteogenic process.

In recent years, various types of plant lipid-derived unsaponifiable matter from corn, avocado, soybean, and turnip, have been studied for their ability to reduce bone loss through the inhibition of *Cathepsin K* and tumor necrosis factor-alpha (*TNF-α*) signaling (Christiansen et al., 2015; El-makawy et al., 2020). Marine organisms contain abundant bioactive substances such as triterpenes, fucoidan, astaxanthin, caryophyllene, and fucoxanthin, which have potential for various health and medical applications. These substances are known to be effective in alleviating osteoporosis, but research on lipid-derived unsaponifiable matters from marine organisms remains limited (Peng et al., 2011). Therefore, this study aimed to evaluate the effects of lipid unsaponifiable matters derived from freshwater microalgae *Chlorella* sp. lipid unsaponifiable matter (CUM) on inhibition of osteoclasts, as well as enhancement of bone matrix and bone growth *In vitro* and *In vivo*. Additionally, to assess structural changes in bone and the increase in bone components resulting from regulation of osteocyte activity, we conducted an analysis of mineral content and bone matrix structure in femurs of ovariectomized (Ovx) mouse models following dietary intake of CUM. Through this, we sought to elucidate the mechanisms by which CUM inhibits bone resorption, promotes bone mineralization, and strengthens the bone matrix, establishing its potential as a functional ingredient in health supplements and pharmaceuticals aimed at improving bone health.

## Material and method

### Extraction of microalgal lipids and preparation of unsaponifiable matter

The microalgae *Chlorella* sp. (AG10105) was obtained from Korean Collection for Type Culture (KCTC, Jeongeup, Korea), and reagents used for preparation of lipid-derived unsaponifiable matter from *Chlorella* sp. were purchased from Sigma-Aldrich (St. Louis, MO, USA). For oil extraction, dried *Chlorella* sp. was mixed with hexane at a 1 : 5 (w/v) ratio and subjected to sonication at 40 kHz and 60 °C for 1 h using a tabletop ultrasonic extractor (SD-D250H, Sungdong Co., Hwaseong, Korea), followed by incubation in a shaking incubator at 150 rpm for 5 h to separate lipid. Hexane was removed by centrifugation at 3000 rpm (1036 x g) for 15 min using a centrifuge (Lobogene 1236R, Gyrozen Co., Daejeon, Korea), and by further evaporation at 80 °C for 9 h.

**Table 1**

Genes sequences used in RT-PCR for analysis of genetic mechanisms inhibiting osteoclast differentiation.

| Gene                          | Forward (5'-3')       | Reverse (5'-3')        |
|-------------------------------|-----------------------|------------------------|
| <sup>1)</sup> <i>DC-STAMP</i> | TCTTCAATGGTGGGCTCAACA | TGAATGGAAGACCGAGCAGGAA |
| <sup>2)</sup> <i>TRAP</i>     | TGCTGCTGGGCCCTACAAATC | TCTCTTGCAATTGGCCATT    |
| <sup>3)</sup> <i>CATK</i>     | TGGGCCAGGATGAAAGTTG   | GAACCCGTTGATGTCCACITG  |
| <sup>4)</sup> <i>NFATc1</i>   | GACACCAGTTGCCTCCAGTA  | CCAAGCTTGATCGATGGAA    |
| <sup>5)</sup> <i>MITF</i>     | CCTCGTCTTAGTGCCTTCCT  | CCCTGACCACCTGGCTGGAT   |
| <sup>6)</sup> <i>OSCAR</i>    | CAAGTGTACCATCGGAAAT   | GACTCCATCCCGATGAAGGA   |
| <sup>7)</sup> <i>GAPDH</i>    | TGCCCCATGTTGTGATG     | GTGGTCATGAGCCCTTCCA    |

<sup>1)</sup> *DC-STAMP*: dendritic cell-specific transmembrane protein, <sup>2)</sup>*TRAP*: tartrate-resistant acid phosphatase, <sup>3)</sup>*CATK*: cathepsin K, <sup>4)</sup>*NFATc1*: nuclear factor of activated T-cells, cytoplasmic 1, <sup>5)</sup>*MITF*: microphthalmia-associated transcription factor, <sup>6)</sup>*OSCAR*: osteoclast-associated Ig-like receptor, <sup>7)</sup>*GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

To prepare unsaponifiable matter from lipids, the lipids were mixed with 2 N KOH at 1 : 25 (w/v) ratio and saponified at 80 °C and 300 rpm for 60 min. Then, 100 mL of hexane was added to recover upper phase. Mixture was re-extracted with another 100 mL of hexane to isolate unsaponifiable matter from saponified lower phase, followed by additional reaction at 80 °C for 14 h to purify by removing hexane. Residual hexane was evaporated in vacuum oven for 12 h and final product was stored at 4 °C for subsequent experiments.

### Osteoclast culture and differentiation

Murine macrophage cell line RAW264.7, derived from mouse ascites, was obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea), and cultured in α-minimum essential medium (α-MEM) supplemented with 10 % fetal bovine serum (FSB) and 1 % penicillin in animal cell incubator (MCO-5AC, Sanyo, Osaka, Japan) at 37 °C and 5 % CO<sub>2</sub>. Osteoclast differentiation was induced by culturing RAW264.7 cells at 1 × 10<sup>6</sup> cells/mL in 24-well cell culture plate for 48 h, then replacing the medium with α-MEM containing 100 ng/mL RANKL and 30 ng/mL M-CSF and changing medium every 3 days.

### Measurement of cell activity

Before evaluating effects of CUM on osteoclast differentiation inhibition, bone matrix enhancement, and bone growth, cell activity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to exclude potential effects of CUM on cell activity (Kang et al., 2024). Macrophage RAW264.7 cells were cultured at 1 × 10<sup>6</sup> cells/mL in 96-well cell culture plate for 24 h, followed by treatment with α-MEM diluted with CUM at concentrations ranging from 0.0 to 2.0 mg/mL. After 24 h, medium was removed, and cells were washed 2 times with phosphate-buffered saline (PBS). Then 0.25 mg/mL of MTT was added, and cells were incubated for additional 4 h. After removing the MTT solution, dimethyl sulfoxide was added to dissolve formazan crystals, and absorbance was measured at 520 nm using a microplate reader (AMR-100, Allsheng, Seoul, Korea). Cell activity was calculated as percentage using the following formula (Eq. 1)

$$\text{Cell activity (\%)} = \left( 1 - \frac{\text{Absorbance of CUM treated group}}{\text{Absorbance of } \alpha\text{-MEM treated group}} \right) \times 100 \quad (1)$$

### Evaluation of gene expression in osteoclast cell

To assess inhibitory effect of CUM on osteoclast differentiation, RAW264.7 cells were treated with CUM for 7 days. After removing medium and washing the cells 2 times with PBS, total mRNA was extracted using the AccuPrep® Universal RNA Extraction Kit. Complementary DNA (cDNA) was synthesized using the AmfiRivert cDNA

Synthesis Platinum Master Mix, and gene expression was amplified using specific primers (Table 1). The expression levels of gene products were analyzed using 1.5 % (w/v) agarose gel electrophoresis containing Gel Red® and quantified using the Davinch-Gel™ imaging system (Young In Labplus, Seoul, Korea) and CLIQS software (TotalLab, Newcastle upon Tyne, UK).

#### Evaluation of protein expression in osteoclast cell

To evaluate effects of CUM on key proteins related to osteoclast differentiation inhibition and bone strengthening, RAW264.7 cells were treated with CUM (0.0 ~ 0.06 mg/mL) and cultured for 7 days. After

$$\text{Mineral content (\%)} = \frac{(\text{Weight of crucible and ash} - \text{Weight of crucible})}{\text{Dry femur weight}} \times 100 \quad (2)$$

removing the medium and washing the cells 2 times with PBS, proteins were extracted using a mixture of radioimmunoprecipitation assay (RIPA) buffer and an inhibitor cocktail. Protein quantification was performed using the Bradford assay with 30 µg of protein, followed by electrophoresis on a 10 % (w/v) SDS-polyacrylamide gel and transfer onto a polyvinylidene difluoride (PVDF) membrane. To block nonspecific binding, the membrane was reacted in 5 % (w/v) skim milk for 1 h at 30 rpm, followed by washing with PBS-T (PBS containing 0.1 % (v/v) Tween-20) and reaction with primary antibodies (DC-STAMP, TRAP, CATK, NFATc1, MITF, OSCAR) diluted 1:100 ~ 1000 in 5 % skim milk for 12 h. After washing, membrane was incubated with secondary antibodies (diluted 1:500 ~ 1000) in 5 % skim milk for 4 h, and protein bands were visualized using ECL detection reagent and quantified using the Kwik quant Pro Multi-Image System (D1010, Kindle Bio, USA).

#### Evaluation of bone-promoting effects in mouse model

All animal procedures were approved by the Institutional Animal Care and Use Committee of Sunmoon University (approval no.: SM-2023-03-01, approval date: 10 December 2023). Ovariectomized (Ovx) mouse models were obtained from Daehan Biolink (DBL Co., Eumseong, Korea) and housed individually under controlled conditions (23 ± 1 °C, 50 ± 5 % relative humidity, and a 12 h light-dark cycle). After 1 week of acclimatization, mice were divided into groups with body weight-matched feeding of either a normal diet or a diet supplemented with CUM for 6 weeks. After fasting for 12 h, mice were euthanized by CO<sub>2</sub> inhalation (Table 2). Femurs were resected, and surrounding soft tissue was removed and washed with PBS. The right femurs were stored in PBS at 4 °C for mineral content analysis, while the left femurs were preserved

**Table 2**

Diet compositions for assessing the osteoporosis-alleviating effects of *Chlorella* sp. unsaponifiable matter in ovariectomized mice.

| Ingredient                | Control (g) | CUM diet (g) |
|---------------------------|-------------|--------------|
| CUM                       | 0.0         | 44.5         |
| Casein                    | 200.0       | 200.0        |
| L-Cystine                 | 3.0         | 3.0          |
| Corn starch               | 397.49      | 397.49       |
| Maltodextrin 10           | 132.0       | 132.0        |
| Sucrose                   | 100.0       | 100.0        |
| Cellulose                 | 50.0        | 50.0         |
| Soybena oil               | 70.0        | 25.5         |
| t-butylhydroquinone       | 0.01        | 0.01         |
| Mineral mix (S10022G)     | 35.0        | 35.0         |
| Vitamin mix (10x; V10037) | 10.0        | 10.0         |
| Choline bitartrate        | 2.5         | 2.5          |
| Total                     | 1000.0      | 1000.0       |

in 10 % formalin for bone mineral density (BMD) analysis using PerkinElmer (Quantum GX2 micro CT, MA, USA).

#### Mineral content analysis

To assess femoral mineral content, the excised femurs were dried in a drying oven (VS-1202D4N, Vision Bionex, Bucheon, Korea) at 70 °C until no further weight change was observed. Crucibles for ash measurement were heated in a muffle furnace at 600 °C for 2 h, then cooled in a desiccator with silica gel for 2 h before weighing. The dried femurs were heated at 600 °C in the muffle furnace for 8 h, and the ash residue was cooled and weighed using the following formula (Eq. (2)).

#### Calcium and phosphorus content analysis

Calcium content in femurs was analyzed using a colorimetric method based on formation of a Calcium-OCPC complex with o-cresolphthalein complexone (OCPC) (Quang et al., 2024). Samples were washed with 1 N HCl to eliminate potential interference from calcium or magnesium. The OCPC solution was prepared by mixing 5 mL of 14.8 M aminoethanol-borate buffer (pH 10.0), 1.5 mL of 5 % (w/v) 8-quinolinol solution, and 5 mL of 0.1 % OCPC solution, then diluted to 100 mL with DW. Samples were reacted with 10 mL of OCPC solution for 10 min at room temperature, and absorbance was measured at 570 nm using a spectrophotometer (Optizen 2120UV, KLab, Daejeon, Korea). Calcium content was calculated in mg/g using CaCl<sub>2</sub> as positive control.

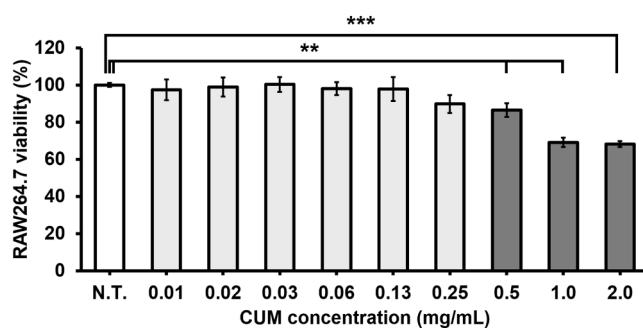
Phosphorus content was analyzed using a colorimetric method based on formation of a phosphovanadomolybdate complex (Altahan et al., 2021). Samples were mixed with vanadate-molybdate reagent in a 1:1 ratio and incubated at room temperature for 30 min. Absorbance was measured at 420 nm using a spectrophotometer, and sodium phosphate dibasic anhydrous (Na<sub>2</sub>HPO<sub>4</sub>) was used as a standard for phosphorus content calculation in mg/g.

#### Bone density analysis

Micro-CT imaging was performed using Quantum GX uCT scanning system (PerkinElmer, Hopkinton, MA, USA) at the Korea Basic Science Institute (Gwangju, Korea). Osteoporosis diagnosis was determined using dual-energy X-ray absorptiometry based on absorption data of bone to compare BMD (g/cm<sup>2</sup>) with control subjects of same age group via T-scores ( $\geq -1$  = normal) and Z-scores ( $> -2.0$  = normal) (Divittorio et al., 2006). The excised left femurs were wrapped in sterile gauze, then fixed in 10 % (v/v) formalin to prevent drying and preserve tissue structure for analysis. The scan was set to 90 kV, 88 µA, field of view (voxel size = 20 µm, 100 slides) of 10 mm, and scan time of 4 min. The three-dimensional (3D) images were visualized using the Quantum GX's 3D viewing software, and parameters such as bone volume to total volume (BV/TV), BMD, and cortical bone mineral density (Ct. BMD) were calculated (Meganck and Liu, 2017).

#### Unsaponifiable matter analysis

To perform qualitative and quantitative analysis of main lipid compounds in CUM responsible for inhibiting osteoclast differentiation, gas chromatography mass spectrometer (GC-MS, Agilent 8890/5977B,



**Fig. 1.** Changes in RAW264.7 cell viability based on the concentration of *Chlorella* sp. unsaponifiable matter treatment. Bars represent mean  $\pm$  standard deviation of three independent experiments, with results expressed as percentages compared to respective values obtained for non-treated group (N.T.). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

Agilent Technology Inc., CA, USA) equipped with an HP-5 ms column (0.25 mm, 0.25  $\mu$ m, Agilent Technology Inc.) was used. CUM was filtered through a 0.22  $\mu$ m syringe filter (Hyundai Micro, Gyeonggi-do, Korea) before analysis. Sample injection volume was set at 1  $\mu$ L, column temperature at 280 °C, and flow rate at 1 mL/min. The MS ion source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. Oven temperature program was initially set to 50 °C for 2 min, ramped to 150 °C at 10 °C/min, followed by 280 °C at 5 °C/min, and held at 280 °C for 5 min.

#### Statistical analysis

All experiments were performed in triplicate, and results were expressed as mean  $\pm$  standard deviation. Statistical significance was determined using analysis of variance (ANOVA) followed by Tukey's post hoc test, and a *t*-test was used to evaluate significant differences between experimental groups using GraphPad Prism software (San Diego, CA, USA). A *p*-value of  $<0.05$  was considered statistically significant.

## Results

### Evaluation of osteoclast viability

Osteoporosis is a condition wherein bone strength is weakened by an imbalance in bone formation and resorption. This is typically seen when the inhibition of osteoclast activity declines due to causes such as aging or decreased estrogen levels (Chen et al., 2023). Therefore, to demonstrate the osteoporosis-mitigating effect of CUM, we evaluated its effect on the regulation of differentiation factors of osteoclasts. Osteoclast activity was evaluated by determining the addition concentration that does not affect cell viability to exclude interference effects on subsequent gene and protein expression. There was no significant decrease in cell activity at 89.8% ( $p > 0.05$ ) seen with CUM concentrations of up to 0.25 mg/mL, but a significant inhibition of cell activity at 86.4% (\*\* $p < 0.01$ ) was seen at a concentration of 0.5 mg/mL. Thus 0.25 mg/mL was selected as the maximum addition concentration of CUM in osteoclasts (Fig. 1). Previous studies have shown that unsaponifiable matter derived from *Olea europaea*, *Glycine max*, and *Sword Bean* can inhibit osteoclast activity at concentrations of 0.13–0.25 mg/mL, which is greater than that of CUM (Campos-Vega et al., 2010; Chin and Pang, 2017; Valizadeh et al., 2021).

### Evaluation of osteoclast differentiation

Through the process of resorption, osteoclasts replace aged bone tissue and provide factors for the formation of new bone tissue. However, due to aging or decreased estrogen levels, their activity remains

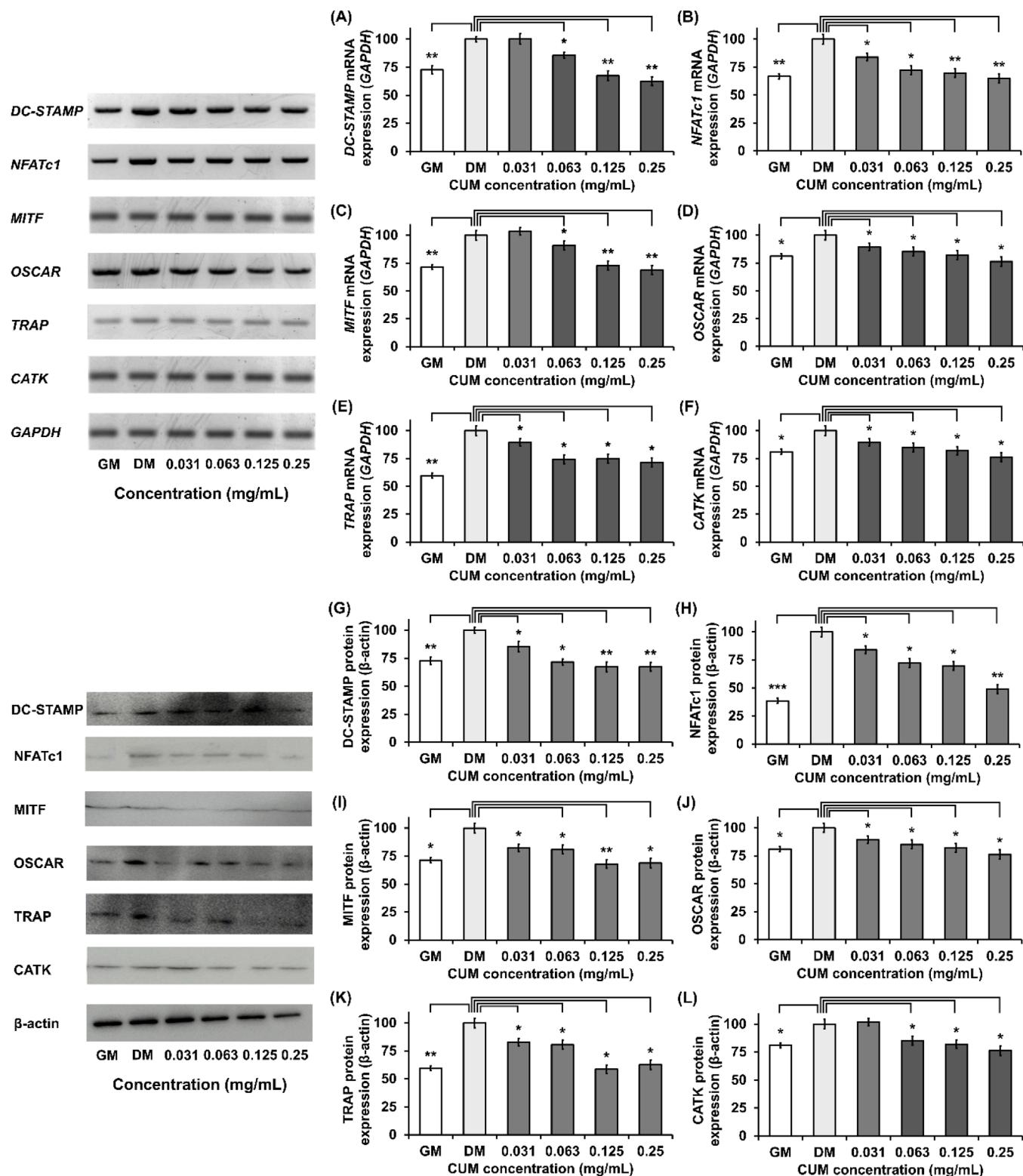
uninhibited, leading to continuous bone resorption and weakening of bone strength (Cheng et al., 2022). Inhibiting osteoclast differentiation is important in preventing bone weakening due to the decrease in bone mass and density, which are key symptoms of osteoporosis, as well as reducing the risk of fractures. This study evaluated the effects of CUM on osteoporosis, specifically its regulation of genes and proteins related to osteoclast differentiation, bone resorption, and reabsorption (Udagawa et al., 2021).

A significant increase was found in gene expression between the differentiation media (DM)-treated group versus the growth media (GM)-treated group, indicating that osteoclast differentiation was induced (\* $p < 0.05$ , Fig. 2). DC-STAMP, NFATc1, and MITF, which are essential transcription factors involved in osteoclast differentiation by mediating precursor cell fusion, activation of differentiation-specific genes, and regulation of bone-resorbing activity, had their expression levels significantly decreased by 37.6%, 32.4%, and 29.8%, respectively, at the highest concentration (0.25 mg/mL) of CUM treatment compared to DM (\* $p < 0.05$ ). This decrease in osteoclast activity was concentration dependent. Thus, CUM can inhibit osteoclast differentiation and bone resorption, thereby alleviating osteoporosis (Fig. 2A–C). Additionally, a decrease in protein expression due to the inhibition of gene expression was confirmed. The expression levels of DC-STAMP, NFATc1, and MITF proteins significantly decreased by 47.5%, 52.2%, and 39.8%, respectively, at the highest concentration of CUM treatment compared to DM (\* $p < 0.05$ ). Consistent with the gene expression results, this trend confirms that CUM inhibits osteoclast differentiation, continuously suppresses bone resorption, and promotes bone formation, thereby helping alleviate osteoporosis as a functional material (Fig. 2G–I).

OSCAR is a receptor that plays a critical role in osteoclast activity by mediating cell signaling essential for bone resorption and the regulation of osteoclast-specific gene expression; its expression was significantly decreased by 41.0% at the highest concentration of CUM treatment compared to DM (\* $p < 0.05$ ). Alternatively, TRAP and CATK are enzymes critical to osteoclast-mediated bone resorption that play distinct roles in bone matrix degradation and act as markers of osteoclastic activity. TRAP facilitates the breakdown of bone collagen and other matrix proteins through its phosphatase activity, whereas CATK is a lysosomal cysteine protease responsible for cleaving type I collagen, the primary organic component of the bone matrix. TRAP and CATK levels significantly decreased by 29.8% and 32.4%, respectively (\* $p < 0.05$ , Fig. 2D–F). A decrease in protein production was also confirmed. OSCAR, TRAP, and CATK significantly decreased by 23.5%, 48.3%, and 29.1%, respectively, at the highest concentration of CUM treatment compared with DM (\* $p < 0.05$ ), and this trend matched the inhibition of gene expression (Fig. 2J–L). Based on a similar trend seen with osteoclast differentiation genes, it was further demonstrated that CUM inhibits bone resorption by suppressing the production of factors necessary for bone resorption. This data supports its role in the alleviation of osteoporosis through the inhibition of osteoclast differentiation and bone resorption.

### Effects on mineral absorption enhancement

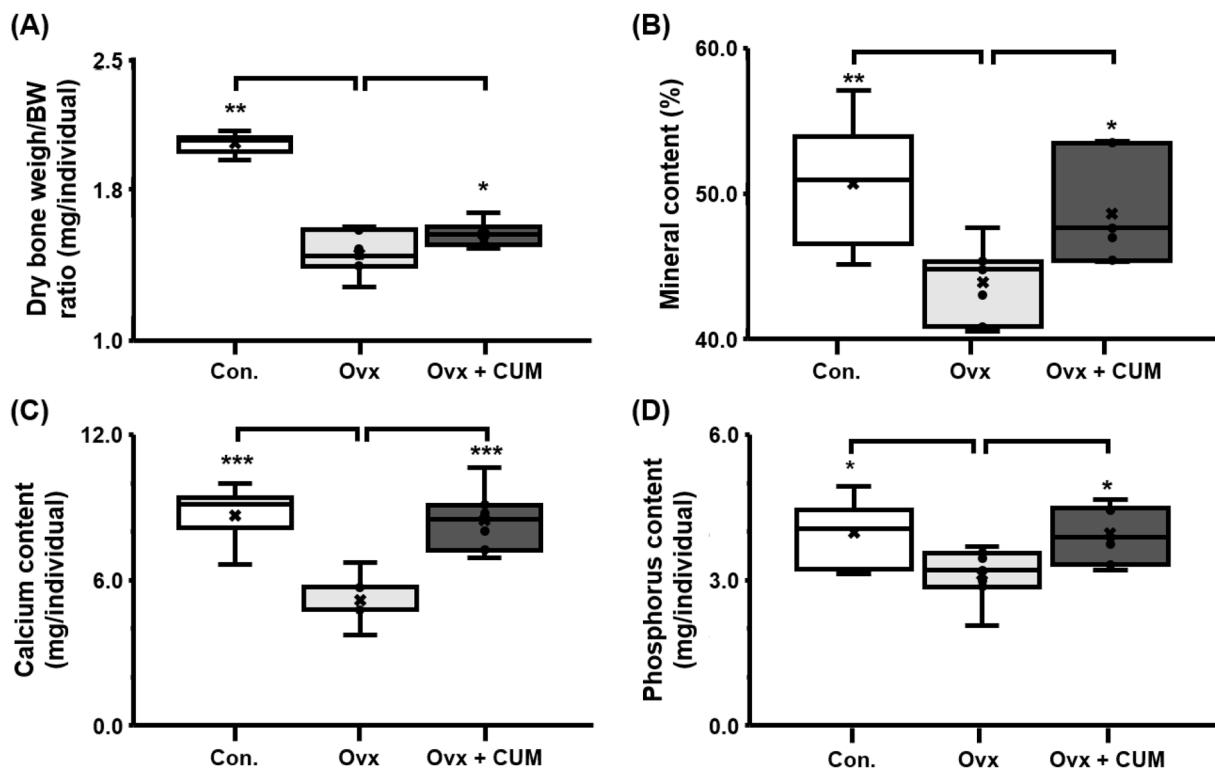
Patients with osteoporosis suffer from decreased bone mineralization, which weakens bone strength and disrupts calcium metabolism and absorption in response to decreased estrogen, resulting in abnormally weak structural strength of the bone (Liao et al., 2005). Estrogen is an important hormone for maintaining bone density, and its decrease can lead to an imbalance in calcium metabolism and absorption, resulting in weakened bone structural strength, increased bone resorption, and decreased bone formation, eventually leading to osteoporosis (Tenhuisen et al., 1995). This study aimed to evaluate the osteoporosis-mitigating effects of a CUM-containing diet in Ovx models, where estrogen production has ceased, specifically by examining changes in mineral, calcium, and phosphate content.



**Fig. 2.** Inhibition of osteoclast differentiation and bone matrix degradation by Chlorella sp. unsaponifiable matter through downregulation of genes and proteins: DC-STAMP, NFATc1, MITF, OSCAR, TRAP, CATK. Bars represent mean ± standard deviation of three independent experiments, with results expressed as percentages compared to respective values obtained for differentiation media group (DM). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

Although the Ovx group had a mean mineral content of 43.9 %, the significant reduction in mineral content in the Ovx group ( $p < 0.01$ ) indicates that estrogen deficiency increases osteoclast activity and promotes bone resorption (Fig. 3A). With CUM diet supplementation, the Ovx group showed a significant increase in mineral content to a mean of

48.6 % ( $P < 0.05$ ). This suggests that CUM inhibited excessive osteoclast activity, thereby enhancing bone mineral content (Fig. 3B). The active ingredients in CUM may inhibit bone mineral loss by blocking the activation pathways of osteoclasts or reducing their survival. For instance, calcium content was significantly reduced from in the Ovx



**Fig. 3.** Bone-strengthening effects of *Chlorella* sp. unsaponifiable matter in ovariectomized (Ovx) mice via increased (A) dry bone weight, (B) mineral content, (C) calcium, and (D) phosphorus levels. Box plots represent mean  $\pm$  standard deviation of  $n = 8$ /group, with results expressed as percentages compared to respective values obtained for Ovx group. Control (Con.) Body weight (BW) \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

group versus the N.T. group (21.4 vs. 38.9 mg/individual \*\*\* $p < 0.001$ ), but this was significantly increased to 30.2 mg/individual in the Ovx group with CUM diet supplementation (\*\* $p < 0.001$ , Fig. 3C). The phosphorus content was also significantly reduced in the Ovx group versus the N.T. group (5.2 vs. 8.7 mg/individual, \* $p < 0.05$ ), but this was significantly increased to a mean of 8.5 mg/individual in the Ovx group with CUM diet supplementation (\* $p < 0.05$ , Fig. 3D). Therefore, CUM is effective in restoring both calcium and phosphorus content, suggesting that its main components positively affect bone formation and mineral metabolism by replenishing calcium and phosphorus, which are diminished in osteoporosis. Furthermore, the recovery of calcium and phosphorus content, alongside the increase in total mineral content, suggest the role of CUM in mitigating osteoporosis by promoting bone mineralization.

The total mineral content of the femur was significantly lower in the Ovx group versus the Con. group (43.9 % vs. 50.7 %, \*\* $p < 0.01$ ) suggesting that estrogen deficiency increased the activity of osteoclasts and promoted bone resorption (Fig. 3B). However, in the Ovx group with CUM diet supplementation, the mineral content was significantly increased to a mean of 48.6 % ( $p < 0.05$ ), suggesting that CUM reduced bone resorption by inhibiting the excessive activity of osteoclasts, which increased the bone mineral content (Fig. 3B). This indicates that the active ingredients in CUM may act to inhibit bone mineral loss by blocking the activation pathways of osteoclasts or reducing their survival.

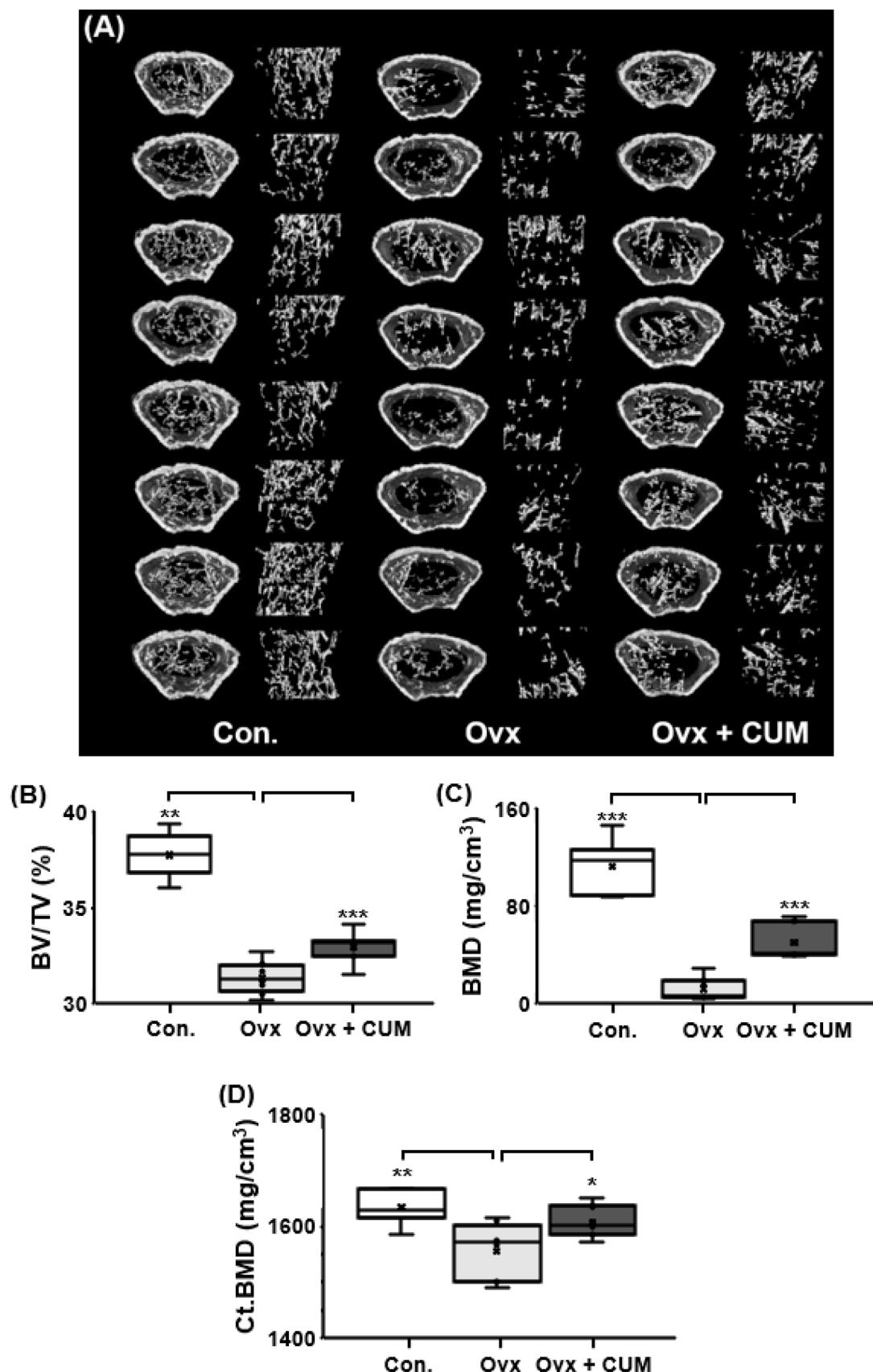
Meanwhile, Calcium and phosphorus levels significantly decreased in the Ovx group compared to the Con. group (21.4 vs. 38.9 mg and 5.2 vs. 8.7 mg/individual, respectively). However, CUM supplementation effectively restored calcium levels to 30.2 mg/individual (\*\* $p < 0.001$ ; Fig. 3C) and phosphorus levels to 8.5 mg/individual (\* $p < 0.05$ ; Fig. 3D), highlighting its role in enhancing bone mineral content by replenishing these essential minerals. This indicates that CUM is effective in restoring both calcium and phosphorus content, suggesting that the main components of CUM have a positive effect on bone formation and mineral

metabolism, restoring the reduced calcium and phosphorus concentrations due to osteoporosis. Furthermore, the recovery of calcium and phosphorus content alongside the increase in total mineral content indicate that CUM helped mitigate osteoporosis by promoting bone mineralization.

#### Effect on femoral bone strength and density

Osteoporosis is a chronic disease characterized by decreased bone strength, which makes bones more susceptible to physical impact and increases the risk of fracture. Since the bone microstructure is impaired, osteoporosis is also marked by a decrease in BMD and an increase in the number and diameter of pores (Ammann and Rizzoli, 2003). In this study, a mouse model of osteoporosis induced with ovariectomy was given a diet supplemented with CUM. Afterward, BMD, a key marker for osteoporosis diagnosis, was analyzed using micro computed tomography (CT). We also evaluated bone volume fraction (BV/TV), which represents the bone matrix density and porosity ratio, and Ct. BMD, both of which are crucial in bone strength and stability (Raisz, 2005).

Compared to control, the Ovx group showed a significant decrease in BMD (112.6 vs. 12.1 mg/cm<sup>3</sup>), BV/TV (37.7 % vs. 31.3 %), and cortical BMD (1633.8 vs. 1554.8 mg/cm<sup>3</sup>) (all  $p < 0.05$ ), confirming the induction of osteoporosis (Fig. S2). When evaluating the effect of CUM supplementation, BMD was significantly decreased in the Ovx group compared to the Con. group (Avg. 112.6 mg/cm<sup>3</sup>,  $p < 0.001$ ) but significantly increased by approximately 67.0 % in the Ovx group fed a CUM diet (Avg. 50.1 mg/cm<sup>3</sup>,  $p < 0.001$ , Fig. 4B, C). Thus, CUM is effective in restoring BMD in osteoporosis, likely because its bioactive components contribute to mechanisms that promote bone formation and inhibit bone resorption. Meanwhile, BT/TV, which is the proportion of actual bone tissue in the total bone volume, is used as a critical measure of bone density and structural integrity. BV/TV was significantly reduced to 31.3 % in the Ovx group compared to the Con. group (Avg. 37.7 %,  $p < 0.01$ ), but significantly increased by approximately 1.6 % in



**Fig. 4.** Effects of CUM supplementation on bone density and microstructure in Ovx mice: (A) BV/TV, (B) BMD, and (C) Ct. BMD. Box plots represent mean  $\pm$  standard deviation of  $n = 8$ /group, with results expressed as percentages compared to respective values obtained for Ovx group. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

**Table 3**

Main bioactive substances identified in microalgae unsaponifiable matters using GC-MS.

| No. | Compound Name                         | Retention time (min) | Formula  | Component area |
|-----|---------------------------------------|----------------------|--|----------------|
| 1   | Ethyl 2-methylbutyrate                | 6.9662               | C <sub>7</sub> H <sub>14</sub> O <sub>2</sub>  | 26,944.3       |
| 2   | 1,4-Cyclohexanedione                  | 7.7991               | C <sub>6</sub> H <sub>8</sub> O <sub>2</sub>   | 27,901.3       |
| 3   | Ethyl hydrogen succinate              | 9.8875               | C <sub>6</sub> H <sub>10</sub> O <sub>4</sub>  | 12,168.4       |
| 4   | cis-3-Hexenyl 2-methylbutanoate       | 10.7628              | C <sub>11</sub> H <sub>20</sub> O <sub>2</sub> | 22,106.5       |
| 5   | 1,4-Cyclooctanedione                  | 13.3122              | C <sub>8</sub> H <sub>12</sub> O <sub>2</sub>  | 59,905.1       |
| 6   | 3-Ethyl-2,6,10-trimethylundecane      | 14.8479              | C <sub>16</sub> H <sub>34</sub>                | 17,460.5       |
| 7   | Butylated Hydroxytoluene              | 15.1897              | C <sub>15</sub> H <sub>24</sub> O              | 213,481.0      |
| 8   | 6-Cyano-1,2,3,4-tetrahydronaphthalene | 15.5702              | C <sub>11</sub> H <sub>11</sub> N              | 10,303.3       |
| 9   | Ethyl tetradecanoate                  | 20.0553              | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> | 65,606.8       |
| 10  | 6-Hepten-2-one, 7-phenyl-             | 22.3069              | C <sub>15</sub> H <sub>16</sub> O              | 19,637.0       |
| 11  | Palmitic Acid                         | 23.0227              | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> | 5556,113.2     |
| 12  | Ethyl palmitate                       | 23.7294              | C <sub>16</sub> H <sub>36</sub> O <sub>2</sub> | 847,624.6      |
| 13  | 1-Iododecane                          | 27.3064              | C <sub>10</sub> H <sub>21</sub> I              | 56,125.2       |
| 14  | Glycidyl Palmitate                    | 29.1434              | C <sub>19</sub> H <sub>36</sub> O <sub>3</sub> | 123,089.1      |
| 15  | Tetratriacontane                      | 35.1405              | C <sub>34</sub> H <sub>70</sub>                | 1952,282.4     |
| 16  | Cholesteryl laurate                   | 37.0883              | C <sub>39</sub> H <sub>68</sub> O <sub>2</sub> | 84,096.1       |
| 17  | Cholesteryl heptanoate                | 38.0159              | C <sub>34</sub> H <sub>58</sub> O <sub>2</sub> | 470,818.9      |
| 18  | Tetratriacontane                      | 39.0198              | C <sub>34</sub> H <sub>70</sub>                | 969,418.0      |
| 19  | Epicholesterol                        | 41.5439              | C <sub>27</sub> H <sub>46</sub> O              | 498,124.9      |

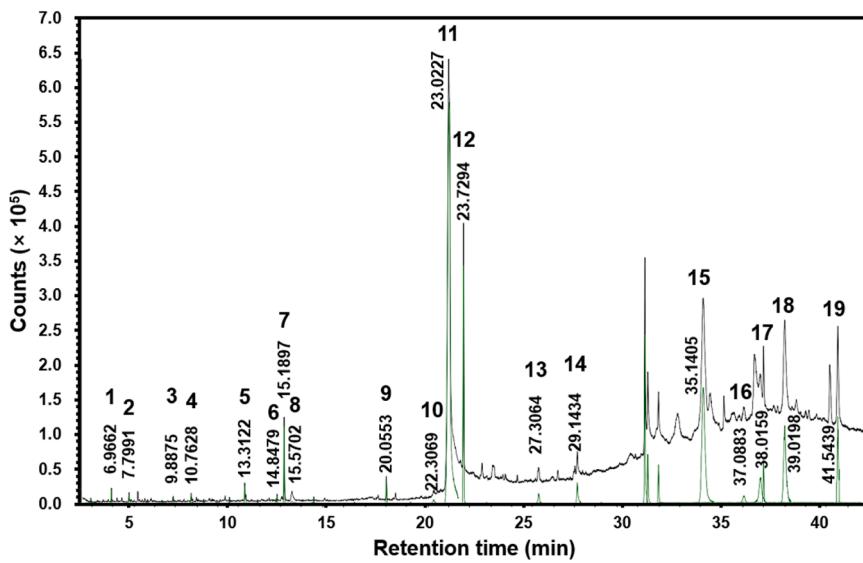
the Ovx group with CUM diet supplementation (Avg. 32.9 %,  $p < 0.001$ ). Thus, the CUM diet contributes to improving bone structural integrity, increasing overall bone density by reducing bone porosity caused by osteoporosis, which effectively restores bone strength. Lastly, Ct. BMD represents the mineral content per unit volume of cortical bone; it acts as an indicator of bone strength and density, with higher Ct. BMD suggesting stronger, more fracture-resistant bones. The average Ct. BMD was significantly lower by about 4.8 % in the Ovx group versus the Con. group (1554.8 vs. 1633.8 mg/cm<sup>3</sup>,  $p < 0.01$ , Fig. 4D). In the Ovx group with CUM diet supplementation, however, it averaged 1607.4 mg/cm<sup>3</sup>, which is a significant increase of about 3.4 % ( $p < 0.05$ ). This suggests that CUM can effectively restore mineral content in the cortical bone by reducing the number of pores in the bone matrix. By inhibiting bone resorption and promoting bone formation, CUM can effectively reverse osteoporosis-induced bone loss and improve bone density and strength.

### Bioactive substance analysis

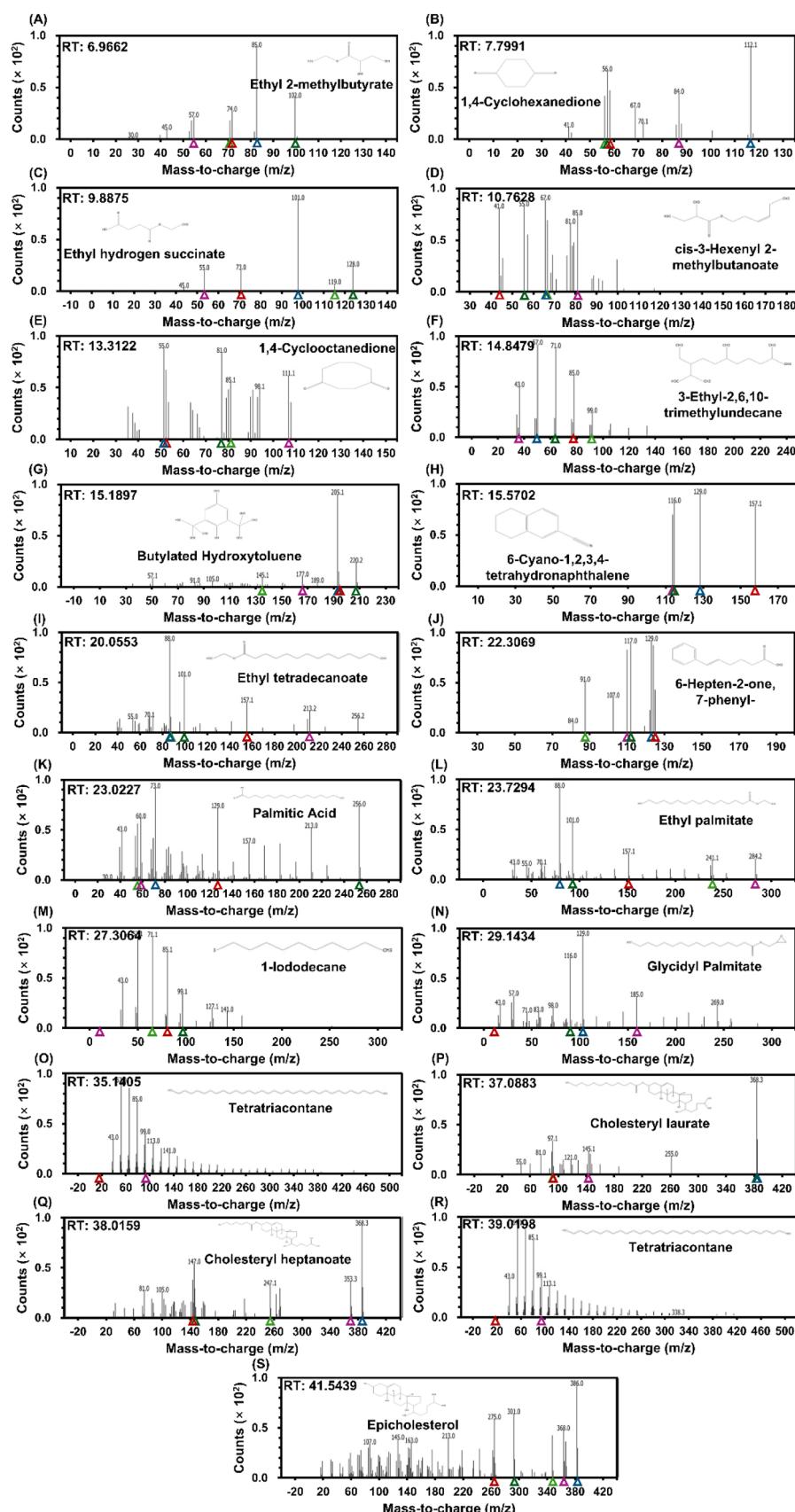
Unsaponifiable matters are the substances that remain after removing the products of saponification, a process wherein lipids react with bases to produce fatty acid salts and glycerol. They contain a variety of bioactive substances, including sterols, alcohols, hydrocarbons, vitamins, pigments, bioactive substrates, and hormone precursors, which can be enriched to high concentrations. In particular, microalgal lipids contain high amounts of bioactive substances such as docosahexaenoic acid (DHA); a fatty acid that has bone mineralization-enhancing, bone strengthening, and anti-inflammatory effects (methyl esters, alcohol, tocopherols, vitamin). These substances remain intact during the saponification process and can be concentrated to high purity (Kishikawa et al., 2019).

Gas chromatography-mass spectrometry analysis (GC-MS) was used to qualitatively and quantitatively analyze the bioactive substances in CUM that contribute to bone mineralization and matrix strengthening, revealing 19 major peaks, each confirmed as a different compound (Table 3, Fig. 5, Fig. S3). Upon examining the mass spectra and detection times of these compounds, various fatty acids, esters, hydrocarbons, and sterols were identified. The substances in CUM were identified as palmitic acid (retention time [RT], 23.02 min) tetratriacontane (RT 35.14, 39.02 min), and ethyl palmitate (RT 23.73 min), which inhibit osteoclast activity by modulating inflammatory and oxidative stress responses. When treated with vitamin D, it induces osterix activity, which is essential for osteoblast differentiation, and inhibits TNF- $\alpha$  and cathepsin k signaling pathway, which is known to be effective in the treatment of osteoporosis (Alsahli et al., 2016; Liang et al., 2022). These are expected to play important roles in the bone-strengthening effects of CUM.

The analysis of sterols, a major component of cell membranes, revealed the presence epicoprostanol (RT 41.54 min), cholesteryl heptanoate (RT 38.02 min), cholesteryl laurate (RT 37.09 min), and other sterols (Fig. 6). Sterols regulate osteoclast activity by promoting stability and fluidity in the cell membrane of osteoclasts and decreasing the reactivity of osteoclasts to external stimuli, thereby preventing overactivation and inhibiting bone resorption. Through these mechanisms, sterols are expected to contribute to bone strengthening by regulating the activity of osteoclasts and preventing bone loss. In addition, the antioxidants butylated hydroxytoluene (RT 15.19 min) and glycidyl palmitate (RT 29.14 min) were also identified. These can alleviate osteoporosis by preventing damage to osteoblasts and osteoclasts caused by free radicals, as well as inhibiting persistent osteoclast activity.



**Fig. 5.** Identification of major bioactive compounds in CUM using GC-MS based on retention time and signal intensity. Each numbered peak corresponds to a detected compound, with the relative abundance represented by the ion signal intensity.



**Fig. 6.** GC-MS-based quantitative and qualitative analysis of bioactive compounds in microalgae unsaponifiable matter: (A) Ethyl 2-methylbutyrate, (B) 1,4-Cyclohexanedione, (C) Ethyl hydrogen succinate, (D) cis-3-Hexenyl 2-methylbutanoate, (E) 1,4-Cyclooctanedione, (F) 3-Ethyl-2,6,10-trimethylundecane, (G) Butylated Hydroxytoluene, (H) 6-Cyano-1,2,3,4-tetrahydronaphthalene, (I) Ethyl tetradecanoate, (J) 6-Hepten-2-one, 7-phenyl-, (K) Palmitic Acid, (L) Ethyl palmitate, (M) 1-Iododecane, (N) Glycidyl Palmitate, (O) Tetratriactane, (P) Cholesteryl laurate, (Q) Cholesteryl heptanoate, (R) Tetratriactane, (S) Epicholesterol.

## Discussion

This study demonstrates the multifaceted anti-osteoporotic potential of unsaponifiable matter extracted from CUM, which exerts regulatory effects on osteoclast viability, differentiation, bone mineral content, and microarchitecture. Notably, CUM displayed minimal cytotoxicity up to 0.25 mg/mL, a concentration that allowed precise assessment of gene and protein expression without confounding effects from cell death. This safety profile is particularly valuable considering the inherent cytotoxicity of many microalgal lipids due to phytotoxins and oxidized derivatives, which are effectively removed during saponification. Mechanistically, CUM significantly suppressed the transcriptional and translational expression of key osteoclastogenic markers, including DC-STAMP, NFATc1, and MITF, as well as functional resorption markers such as TRAP, CATK, and OSCAR. These changes suggest potent inhibition of the RANKL–NFATc1 signaling pathway, consistent with previous reports on plant-derived unsaponifiables such as phytosterols and polyphenols (Liang et al., 2022; Valizadeh et al., 2021). The transcriptional suppression and downstream attenuation of resorptive enzyme activity provide strong evidence that CUM disrupts osteoclast maturation and function at multiple levels, contributing to bone preservation.

In Ovx models, which mimic postmenopausal osteoporosis, CUM supplementation restored total mineral content and improved calcium and phosphate levels, thereby reestablishing metabolic balance impaired by estrogen deficiency. These effects likely reflect reduced osteoclast activity as well as promoted osteoblastic mineralization via alkaline phosphatase-mediated hydroxyapatite formation. Enhanced mineral deposition is a hallmark of anabolic bone response and indicates that CUM not only inhibits bone loss but also facilitates bone regeneration. Micro-computed tomography (micro-CT) analysis further confirmed that CUM ameliorated bone microarchitecture, including significant improvements in BMD, bone volume fraction (BV/TV), and cortical bone parameters. Histomorphometric preservation of trabecular thickness, marrow cavity integrity, and Haversian systems suggests that CUM supports structural and functional recovery of osteoporotic bone. This is critical not only for mechanical strength but also for maintaining osteohematopoietic niches. Chemical characterization of CUM revealed bioactive compounds known to influence bone cell physiology. Fatty acids such as palmitic acid and ethyl palmitate have been shown to promote osteoblast differentiation and mitigate inflammation. Additionally, sterols including epicoprostanol and cholestryler esters may enhance membrane fluidity and suppress osteoclast hyperactivity, while antioxidants like butylated hydroxytoluene (BHT) protect bone cells from oxidative stress, a key contributor to osteoporosis progression. The synergistic action of these components positions CUM as a bioactive matrix with multifactorial efficacy.

Taken together, these findings support CUM as a promising functional material for osteoporosis management. Its broad-spectrum efficacy, low toxicity, and ability to modulate both osteoclast-driven resorption and osteoblast-driven formation offer a comprehensive therapeutic strategy. Future research should aim to isolate and characterize individual active fractions, evaluate long-term safety and efficacy in clinical models, and investigate bioavailability and pharmacodynamics to further elucidate its translational potential.

## Conclusion

The aim of this study was to evaluate the bone-strengthening effects of unsaponifiable matters isolated from microalgal lipids by inhibiting osteoclast differentiation and reducing bone absorption, and to demonstrate their anti-osteoporosis effects by analyzing their effects on bone formation, mineralization, and bone matrix formation in an Ovx model. The effect of inhibiting osteoclast differentiation as a result of suppressing osteoclast differentiation was evaluated, and both gene expression and protein expression was found to decrease in Dendritic cell-specific transmembrane protein DC-STAMP, NFATc1, MITF, OSCAR,

TRAP, and CATK, involved in osteoclast differentiation, bone degradation, and resorption. This demonstrates that the balance between bone formation and degradation is maintained by inhibiting bone matrix proteolysis and mineral dissolution processes, thereby proving excellent osteoporosis mitigation effect.

As the osteoporosis alleviation effect by regulating osteoclast cell activity and osteoclast differentiation factors has been verified, we evaluated the mineral content after CUM diet in the Ovx model to assess the effect of bone non-matrix enhancement and BMD enhancement *in vivo*. It was found that the total mineral, calcium, and phosphate content of femur were significantly increased, indicating that minerals, a major factor in bone formation, increased. Furthermore, improvements in BMD, BV/TV, and Ct. BMD were observed through micro-CT analysis, demonstrating bone structural stability and recovery. In conclusion, we found that CUM can effectively alleviate osteoporosis by regulating bone formation and resorption by promoting osteoblast differentiation and inhibiting osteoclast differentiation. This implies that CUM, as a safe natural product-based material, has the potential to be utilized as a functional food or medicine for the prevention and treatment of osteoporosis.

GC-MS analysis also confirmed that CUM contains high levels of various fatty acids, sterols, and other bioactive substances known to have anti-inflammatory and antioxidant effects. In particular, palmitic acid, sterols, and various hydrocarbon compounds have been found to be the main active ingredients, and they have been shown to play a crucial role in inhibiting osteoclast activity and strengthening the bone matrix. The combination of these compounds supports the osteoporosis-mitigating effects of CUM and provides a specific mechanism by which it regulates bone formation and resorption. Therefore, it was confirmed that CUM can effectively alleviate osteoporosis by regulating bone formation and resorption through the inhibition of osteoclast differentiation. Additionally, it was confirmed that the major bioactive compounds identified through GC-MS analysis support these effects. Thus, this suggests that CUM has the potential to be utilized as a safe, natural-based material for functional foods or pharmaceuticals for the prevention and treatment of osteoporosis.

## CRediT authorship contribution statement

**Min Ho Han:** Resources, Project administration, Methodology, Investigation, Formal analysis. **Sun Hye Lee:** Resources, Project administration, Methodology, Investigation, Formal analysis. **Min Ho Kang:** Writing – review & editing, Investigation, Conceptualization. **Youn Seon Hwang:** Software, Data curation, Conceptualization. **Ju Hwan Lee:** Visualization, Investigation, Formal analysis. **Jin Woo Kim:** Validation, Supervision, Resources, Project administration, Funding acquisition.

## Declaration of competing interest

No conflict.

## Ethics approval statement

All animal procedures were approved by the Institutional Animal Care and Use Committee of Sunmoon University (approval no.: SM-2023-03-01, approval date: 10 December 2023).

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## Data availability statement

The data underlying this article will be shared on reasonable request

to the corresponding author.

#### Funding statement

**Translational:** Any study involving predominantly animal models or where the laboratory and clinical aspects of *in-vitro* studies of cell or molecular biology are of approximately equal weight.

#### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.fufo.2025.100728](https://doi.org/10.1016/j.fufo.2025.100728).

#### Data availability

Data will be made available on request.

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