RESEARCH ARTICLE

The Powdered Red Macroalgae (*Eucheuma spinosum*) Supplementation Potentially Enhanced Bone Structure in Osteoporotic Mice

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Abstract

BACKGROUND: Red macroalgae, such as *Eucheuma spinosum*, have been found to have potential benefits for bone health due to their unique bioactive compounds, including proteins, polyphenols, polysaccharides, vitamins, and minerals. Therefore, this study was conducted to evaluate the benefits of powdered *E. spinosum* supplementation in osteoporosis-induced mice.

METHODS: Thirty middle-aged mice were divided into 6 groups, namely: healthy control group (HC), negative control group (NC), positive control group (PC), and treatment groups supplemented with 1.25 mg/gBW (T1), 2.5 mg/gBW (T2), and 5 mg/gBW (T3) of *E. spinosum* powder for twenty days. Mice in NC, PC, T1, T2, and T3 groups were induced with 0.0029 mg/gBW of dexamethasone for 30 days to create osteoporosis mice models. Alkaline phosphatase (ALP) levels were measured by colorimetric methods before and after the intervention. Bone structures were evaluated using X-ray images and histological examination.

RESULTS: After the intervention, PC, T1, T2, and T3 groups showed a significant decrease ($p \leq 0.01$) in serum ALP levels compared to the NC group, which experienced an increase in ALP levels. The X-ray images revealed that the PC, T1, T2, and T3 showed radiopaque bone density. For bone histology, PC, T2, and T3 showed an improvement with thickened and intact trabeculae, but T1 still had visible osteoporosis cavities.

CONCLUSION: Supplementation of 2.5 and 5 mg/gBW of *E. spinosum* powder were able to improve bone density as well bone histology. Therefore, *E. spinosum* powder supplementation might potentially improve bone structure in osteoporosis.

KEYWORDS: *Eucheuma spinosum*, red macroalgae, alkaline phosphatase, osteoporosis

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Introduction

Osteoporosis is a disease of the bones characterized by a decrease in bone density and strength, deterioration of bone tissue microarchitecture, and fractures.(1) In postmenopausal osteoporosis, there is a decrease in levels of the hormone estrogen and calcium. Estrogen deficiency triggers an imbalance in bone remodeling between bone formation and resorption.(2) The prevalence in the 27 countries of the European Union is about 21% of women aged 50-84 years suffering from osteoporosis, which includes more than 22 million women in those countries. (3) In Indonesia, osteoporosis is 23% in women aged 50-80 years and 53% in women aged 80 years and over.(4)

Bone alkaline phosphatase (ALP) is responsible for bone remodeling process. The measurement of this enzyme is an indicator in the management of osteoporosis in premenopausal and postmenopausal women.(5,6) Bone remodeling is the process of bone formation and destruction

that is carried out by osteoblasts and osteoclasts. Osteoblast activity (bone formation cells) and osteoclasts (bone resorption cells) are unbalanced, causing the destruction of microarchitecture in the bone trabecular is associated with aging.(7,8) Bone ALP is a byproduct produced during bone remodeling. It can be measured in urine or serum and can indicate the rate of bone turnover.(5) Elevated ALP is often found in elderly patients.(9) Bone ALP is attached to osteoblast cell membranes, and only a small amount is released into the serum. Its concentration in serum elevates in cases of increased bone remodeling due to osteoblast activity. This parameter can help determine the loss of bone density in postmenopausal women.(10) The pharmacological therapy of osteoporosis is divided into two main categories: anti-resorptive and anabolic drugs.(11) Anti-resorptive drugs include bisphosphonates (alendronate, risedronate, and ibandronate), hormonal therapy with estrogen, calcitonin, denosumab, calcium supplements, and vitamin D. Anabolic drugs include teriparatide, romosozumab, and sodium fluoride. These medications have long-term side effects, including osteonecrosis, esophageal disorders, hypocalcemia, nausea, vomiting, diarrhea, cardiovascular disease, and musculoskeletal pain.(12,13) Meanwhile, estrogen hormone therapy long-term side effects increase the risk of heart disease, stroke, and breast cancer.(14) Challenges in the management of osteoporosis that cause the ineffectiveness of pharmacological therapy include side effects of treatment, digestive disorders in pre-existing patients, polypharmacy, and patient doubts about the potential benefits of taking drugs. In addition, inadequate compliance and perseverance with osteoporosis therapy are frequent and lead to a heightened chance of fractures, elevated healthcare expenses, and a rise in hospital stays. As an alternative to pharmacological therapy, supplementing functional nutrients in vulnerable groups to inhibit bone fragility is a rational approach to preventing and treating osteoporosis.(15,16) One form of functional nutrition is the algae group. Several previous studies have reported the potential benefits of algae supplementation as an anti-postmenopausal osteoporosis (PMOP).

Research on the red algae species *Lithothamnion calcareum* showed a beneficial positive impact as a dietary supplement for preventing bone mineral loss in osteoporotic female mice.(17) The effect of red algae (*Plocamium lyngbyanum* and *Ceramium secundatum*) can stimulate osteoblast activity and mineralization, as well as suppress osteoclast resorption.(18) The algae group, with its content of minerals, proteins, and vitamin C, has the potential to serve as an essential functional nutrient for bone health. Previous studies have demonstrated that the red algae powder from the *Gracilaria asiatica* species contains minerals such as calcium (19), while the red algae extracts from the *Gelidium chilense* species contains high protein (20), and red algae *Eucheuma cottonii* contains vitamin C.(21)

Eucheuma spinosum, one of red marcoalgae have been found to have potential benefits for bone health due to their unique bioactive compounds. The high calcium, phosphate, protein, and vitamin C levels in this *E. spinosum* might benefit osteoporosis management. However, due to the pro and contra, studies regarding *E. spinosum* as a functional nutrition for healthy bones still needs to be explored further. Therefore, this study was conducted to evaluate the benefit of supplementation with *E. spinosum* powder in an osteoporosis mice model.

Methods

E. spinosum Collection and Composition Analysis

This study was conducted in the Animal Laboratory, Faculty of Medicine, Universitas Hasanuddin during January until August 2023. *E. spinosum* was collected from the Laikang subtidal area, Takalar, Indonesia. Over three days, 2.5 kg of wet *E. spinosum* were dried in the sun from 9 AM to 3 PM in a container covered with black cloth. The resulting dried specimen was then blended until smooth. Subsequently, this fine specimen was dried in the sun for one day and sifted to separate the coarse components. The protein, vitamin C, calcium, and phosphate content in the powdered form of *E. spinosum* was analyzed. So did its ethanol extract. Spectrophotometric methods were used to determine vitamin C and phosphate content. The Kjehdal and AAS methods determined protein and calcium content, respectively. This composition analysis was carried out at the *Balai Besar Laboratorium Kesehatan* (BBLK) Makassar.

Acclimatization and Osteoporotic Mice Preparation

Thirty female mice, aged over six months and weighing between 20-25 g, were acclimatized for seven days under a 12-hour light and 12-hour darkness cycle. The mice were provided with *ad libitum* drinking water and rodent pellets. The mice were divided into six treatment groups: 1) healthy control (HC); 2) negative control (NC), consisting of osteoporotic mice without drug administration; 3) positive control (PC), consisting of osteoporotic mice given 0.026 mg/gBW alendronate orally for twenty days; and 4) treatment groups T1, T2, and T3, consisting of osteoporotic mice which were respectively given *E. spinosum* powder

with the dose of 1.25 mg/gBW, 2.5 mg/gBW, and 5 mg/gBW dissolved in 0.5% sodium carboxyl methylcellulose (Na-CMC) orally for twenty days. For the osteoporosis model, mice were administered dexamethasone at a dose of 0.0029 mg/gBW per day for 30 days orally using an orogastric gavage.

The sequence of research implementation was depicted in Figure 1. The experimental procedure of the animal study was reviewed and approved by the Animal Ethical Committee at Universitas Hasanuddin, Makassar, Indonesia (Approval No. 124/UN4.6.4.5.31/PP36/2023).

Measurement of ALP Levels

After dexamethasone induction, serum ALP levels were measured before and after intervention using blood obtained through periorbital puncture. ALP levels were measured spectrophotometrically on a 405 nm wavelength with Genesys UV-Vis following the DGKC colorimetric method using ALP kit protocol ®Glory Diagnostics (Cat. No. REF 1103005, Glory Biotechnologies Corporation, Seoul, Republic of Korea). Each measurement uses 20 µL of serum sample reacted with 1.0 mL of reagent then incubated at 37°C for 1 minute.

Bone X-ray Imaging

Before the X-ray was performed, mice were anesthetized using 50 mg/mL ketamine at a dose of 0.02 mL/mice. After the mice experienced a decline in consciousness, they were placed on a cassette X-ray with a right lateral position and photographed.(22) The bone X-ray image interpreted by a radiologist at Universitas Hasanuddin Veterinary Hospital.

Bone Histology Analysis

A harvested femur bone from each mice was fixed in 10% buffered formalin and decalcified in decalcification solution with 24.4% formic acid and 0.5 N sodium hydroxide. After decalcification, the femur area was excised longitudinally, 3-4 µm in size, and colored with hematoxylin and eosin (HE). Prepared bone histology samples then blindedly interpreted by an animal pathologist from Universitas Hasanuddin Veterinary Hospital. Observation parameters consisted of bone trabecular thickness and the presence of osteoblasts, osteoclasts, osteocytes, and osteoporotic cavities. Degree of bone damage: no abnormalities (score 0); osteoporotic cavities were found <25% (mild = score 1); thin and non-intact trabeculae were found 25-50% (moderate = score 2); thin, non-intact trabeculae were found >50% (severe = score 3).(23)

Statistical Analysis

Data processing was performed using IBM Statistical Package for Social Sciences for Windows (SPSS) version 21 (IBM Corporation, Armonk, NY, USA), with $p<0.05$ was considered as significant. Comparison between groups was carried out using the one-way ANOVA with *post hoc* least significant difference (LSD).

Results

Protein, Vitamin C and Mineral Content of *E. spinosum*

In the preliminary study, two preparations of *E. spinosum*, ethanol extract and powder, were tested for their protein, vitamin C, and mineral content. The results, shown in Table 1, indicated that the powder preparation had higher protein, vitamin C, and phosphate content than the ethanol extract. Although the calcium content in ethanol extract was higher than in powder, the nutritional studies suggest that the content of 2364.97 mg/kg was very high for the calcium content in food. For comparison, the calcium content in cow's milk

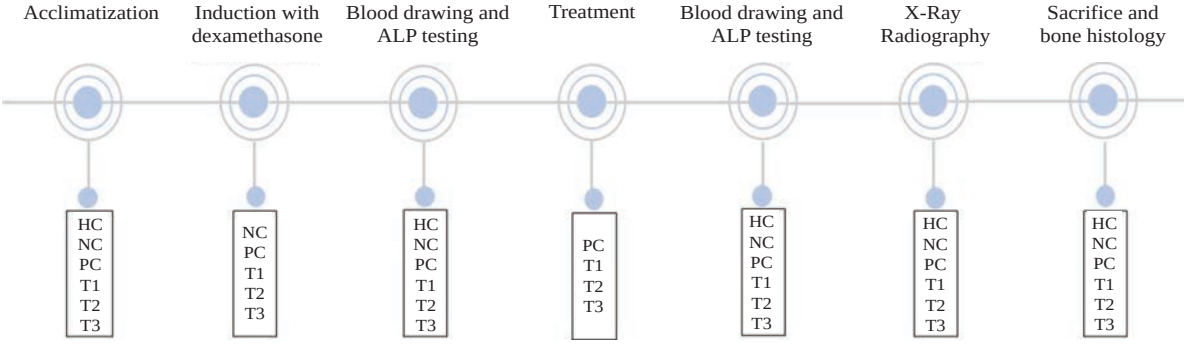

Figure 1. Timeline of the experimental treatments performed. HC: healthy control; NC: negative control; PC: positive control (treated with 0.026 mg/gBW alendronate); T1: treated with 1.25 mg/gBW *E. spinosum*; T2: treated with 2.5 mg/gBW *E. spinosum*; T3: treated with 5 mg/gBW *E. spinosum*.

Table 1. Content of *E. spinosum* powder and ethanol extract.

Nutrition	Quantity (mg/kg)	
	E. spinosum Powder	Ethanol Extract
Proteins	40800	13000
Vitamin C	184.27	122.46
Phosphate	481.03	3.78
Calcium	2364.97	8140.77

was 1222 mg/kg. Therefore, the powder preparation was chosen as an intervention for this study.

Mice Condition After the Induction Phase

During the induction phase, mice in all groups exhibited no change in behavior and were healthy, active, and free of diarrhea and hair loss. However, at the beginning of the induction phase, mice experienced panic, allegedly due to oral induction. After entering week 2, the behavior of mice became natural. All groups of mice experienced weight gain (3-4 grams) after the induction phase with dexamethasone (Figure 2).

***E. spinosum* Powder Intervention Decreased ALP Levels**

Serum ALP levels in the osteoporosis mice model after intervention were presented in delta values, namely the difference between serum ALP levels after the intervention and induction phases (Table 2). The PC group and the intervention groups (T1, T2, and T3) experienced a significant decrease in serum ALP levels compared to NC, which experienced an increase in ALP levels. Administration of *E. spinosum* powder 2.5 mg (T2) and 5 mg (T3) per g of mice body weight showed a more significant reduction in ALP levels than PC.

***E. spinosum* Powder Intervention Increased Bone Density in Femur Bone**

Following a 20-day intervention period, X-ray images were captured and analyzed for mice positioned laterally on their right side. The findings from this analysis were depicted in Figure 3. The HC group exhibited no skeletal abnormalities and presented a radiopaque appearance. In contrast, the NC group displayed radiolucent areas along the femur bone. For the PC group, the femur bone appeared radiopaque, indicating an increase in bone density. Similarly, groups T1, T2, and T3 demonstrated an increase in bone density in the femur bone, which also appeared radiopaque.

***E. spinosum* Powder Intervention Thicken Trabeculae**

After a 20-day intervention, bone histology observations focused on the femoral bone with results as shown in Figure 4. The HC group showed that trabeculae appeared thick and intact, appearing to have osteoblasts, osteoclasts, and osteocytes with normal category damage (score 0). The NC group, showed that trabeculae looked very thin and severed, there were osteoblasts, osteoclasts, osteocytes and there were osteoporotic cavities with severe damage (>50%). The PC group showed thick and intact trabeculae, appearing to have osteoblasts, osteoclasts, and osteocytes with normal category damage (score 0). T1 showed that trabeculae appear thicker and intact, there were osteoblasts, osteoclasts, osteocytes, and osteoporotic cavities with a mild degree of damage (<25%). T2 showed that trabeculae appear thicker and intact, appearing to have osteoblasts, osteoclasts, and osteocytes with normal category damage (score 0). T3 showed trabeculae appear thicker and intact, also appeared the presence of osteoblasts, osteoclasts, and osteocytes with normal category damage (score 0).

Discussion

E. spinosum, a type of red macroalgae, has potential as a functional supplement. This algae is cultivated along the coast of Puntondo Hamlet, Laikang Village, Mangarabombong District, Takalar Regency, South Sulawesi, Indonesia. Locals process the wet algae into *dodol* and jam products,

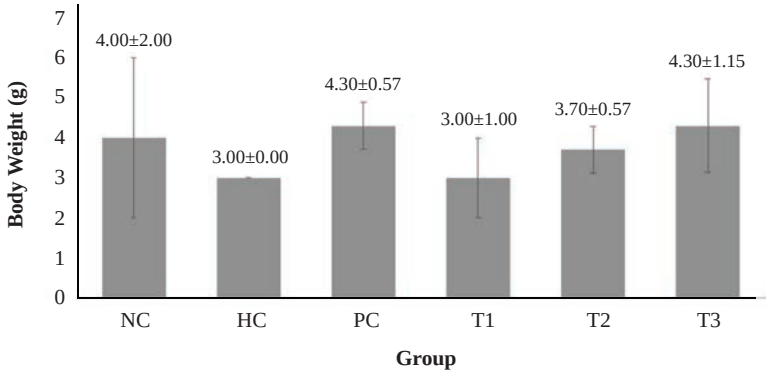

Figure 2. Body weight of mice in different groups after the dexamethasone induction.

Table 2. Delta values of serum ALP levels after the intervention phase.

Groups (n=6)	ALP (U/L)	
	Mean±SD	*p*- value
HC	-3.428±47.86	0.047
NC	35.195±49.24	-
PC	-21.406±49.68	0.007*
T1	-12.560±101.98	0.018*
T2	-66.919±78.31	0.000*
T3	-35.347±16.20	0.002*

Mean difference was compared to NC group, analyzed with one-way ANOVA test. *Significant if $p<0.05$.

which are soft in texture and rich in fiber. Meanwhile, dry algae are typically processed by the industry into ready-to-consume goods with high selling value, such as Alkali Tread Cottonii, Semi-Refined Carrageenan, and Refined Carrageenan.(24) This study evaluated the use of *E. spinosum* powder as a functional nutrient supplement to improve conditions of osteoporosis. *E. spinosum* powder contains nutrients that are important for bone health, such as protein, vitamin C, calcium, and phosphate, which are found in high concentrations (Table 1). The study used a model of female mice older than 6 months. The selection of the mice's age is another important factor to consider, as older mice have a fairly high risk of bone problems.(25)

ALP is a biomarker widely used to assess bone condition.(26) An increase in ALP indicates increased osteoblast activity, which can help determine the loss of bone density in a postmenopausal state.(27) In this study, dexamethasone induction could elevate ALP levels in mice models. Interestingly, the average ALP levels in the model receiving therapy in the PC group decreased compared to the NC group despite the statistical values of the two groups being significantly different. These results align with studies conducted on osteoporosis patients who were administered alendronate therapy, which showed a decrease in the bone ALP levels of these patients.(28) This suggests that ALP can be used as an indicator of osteoporosis treatment.(5)

Therapeutic intervention using red macroalgae powder at dosages of 1.25 mg, 2.5 mg, and 5 mg has been shown to significantly decrease ALP levels over a span of 30 days (Table 2). This reduction in ALP levels could indicate a decrease in bone turnover, a typical feature of osteoporosis. Moreover, research has shown a negative correlation between serum ALP and bone mineral density (BMD), implying that a decrease in ALP levels could be linked to an increase in BMD.(29) This suggests that the observed decrease in ALP levels in this study could potentially signal an improvement in osteoporosis. This is especially significant given the high concentrations of protein, calcium, phosphate, and vitamin C in red algae. These nutrients are recognized for their critical roles in maintaining bone health. For example, calcium, a primary constituent of bones, is crucial for preserving bone density.(30) It has been established that protein intake positively impacts bone health, particularly when combined with increased calcium intake.(31) Phosphate, another essential mineral in bones, contributes significantly to bone

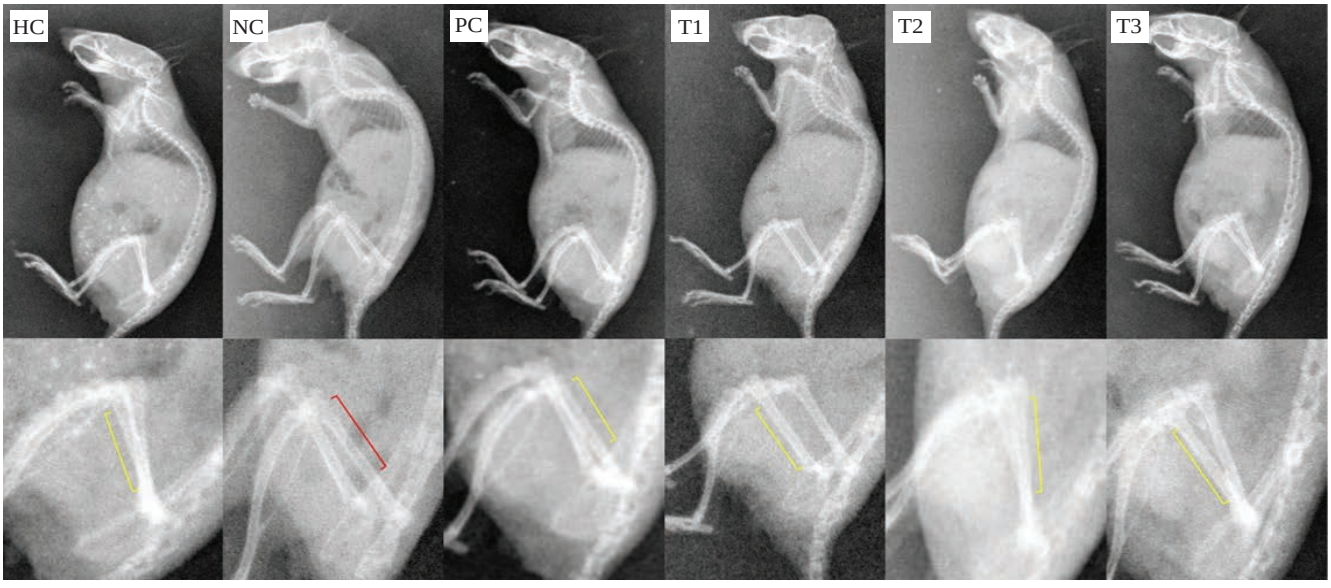

Figure 3. Bone x-ray of mice after the intervention. The red marks show a radiolucent area; the yellow marks show a radiopaque area.

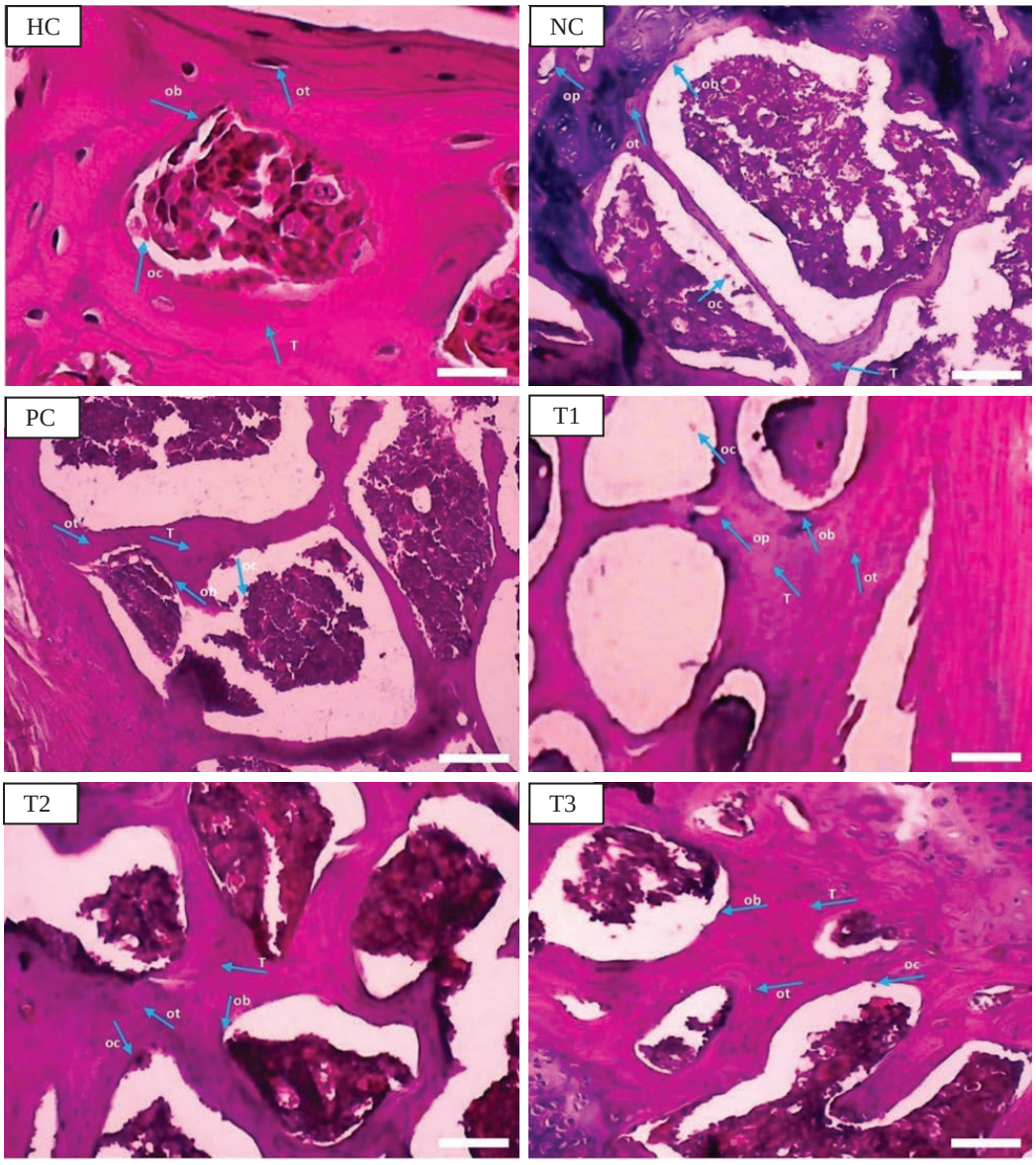

Figure 4. Histological representation of the femoral bone of the treated mice. Osteoblasts are reddish cells that are located at the edge of the lacuna. Osteoclasts are larger than osteoblasts and have a rounded, dome-like shape. T: trabeculae; ob: osteoblast; oc: osteoclast; ot: osteosit; op: osteoporosis cavity. White bar: 250 µm.

strength. Vitamin C has been identified to positively affect bone mineral density.(32)

X-ray images of mice bones are presented in Figure 3. A decrease in bone density, characterized by the presence of radiolucent areas, is evident in the osteoporosis bone model.(33) The administration of alendronates has been shown to increase average bone mineral density. This result is consistent with the known role of alendronates as the current standard for preventing and treating glucocorticoid-induced osteoporosis, such as that caused by dexamethasone and osteoporosis in postmenopausal women.(34-36) The histological analysis of mice bones, shown in Figure 4, indicated the successful development of an osteoporosis mice model. This outcome aligns with previous research on dexamethasone induction in mice, which reported that a 30-day induction period with a dose of 0.0029 mg/g body weight per day decreased the average percentage of trabecular bone density, leading to bone loss.(37) In our study, supplementation of *E. spinosum* flour had an improved effect on the bone histology of osteoporotic mice. Although research on the effects of this type of red macroalgae, as far as the authors know, has no previous report, other types of red macroalgae have similar results. Research on other species of red macroalgae has shown its efficacy in

improving the loss of bone trabeculae structure, suggesting its potential use as a therapeutic agent in osteoporosis.(38) Similar research has investigated the potential of red algae against osteoporosis, explicitly examining the osteogenic potential of *P. lyngbyanum* and *C. secundatum* for treating osteoporosis in zebrafish larvae.(18)

Our research evaluating the use of red macroalgae (*E. spinosum*) powder for treating osteoporosis in mice undoubtedly has numerous facets that require additional exploration to realize its potential fully. Future studies should incorporate additional testing parameters that aim to elucidate the beneficial effects of red macroalgae powder supplementation on health. In light of recent research, future investigations could focus on several key areas. Firstly, studies could examine the specific mechanisms by which *E. spinosum* influences bone health, particularly in the context of postmenopausal osteoporosis. Secondly, given the promising results of *E. spinosum* in animal models, clinical trials in postmenopausal women could be conducted to validate these findings. Thirdly, research could also investigate the potential synergistic effects of *E. spinosum* with current osteoporosis treatments, such as alendronates. Lastly, further studies could also explore the long-term effects of *E. spinosum* supplementation on bone health and fracture risk in postmenopausal women. This would provide valuable information on the sustainability and long-term safety of *E. spinosum* as a supplement for osteoporosis.

Conclusion

Since the supplementation of 2.5 and 5 mg/gBW *E. spinosum* powder has the effect in improving the condition of bone fragility in mice by improving the bone density and bone histology; therefore, *E. spinosum* powder might have the potential to be a functional supplement for the management of osteoporosis.

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Authors Contribution

JP and IY were involved in planning, compiling manuscripts, processing data, conducting analysis, designing drawings, interpreting results, revising manuscripts, and preparing manuscript drafts. RN, GVS, LH, and S were involved in reviewing the implementation of research and writing research manuscripts. All authors gave critical revisions and have agreed with the final revision of the manuscript.

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