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Original Article

Activated Estrogen Receptor-β and Osteocalcin Improvement by Ethyl Acetate Fraction of Marsilea crenata C. Presl. in Human Fetal Osteoblast (hFOB 1.19) Cell Line

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ABSTRACT

Estrogen is important for increasing bone formation and preventing bone resorption. phytoestrogens are groups of chemicals that have structures or functions similar to estrogen. They can take the place of estrogen in the body and have a direct effect on bone density by activating estrogen-β receptors (ER-β*) and encouraging the expression of osteocalcin (Ocn). The leaves of *Marsilea crenata* C. Presl. are known to contain phytoestrogens. This research aimed to determine the effect of the ethyl acetate fraction of *M. crenata* leaves (EFM) on increasing the ER-β* by measuring free estrogen receptor-β (ER-β) expression as well as increasing osteocalcin (Ocn) expression in the human fetal osteoblastic (hFOB) 1.19 cell line. This research was carried out by giving the EFM at doses of 62.5, 125, and 250 ppm. Genistein 1μM is used as a positive control. Expression of ER-β and Ocn was analyzed using the immunocytochemistry (ICC) method and a confocal laser scanning microscopy (CLSM) instrument. The results showed that EFM optimally reduced ER-β expression at 250 ppm by 909.80 arbitrary unit (AU) and increased Ocn expression at 125 ppm by 734.94 AU. Further research in *M. crenata* is necessary because it suggests that the phytoestrogen content of the EFM may play a role in bone formation and have the potential to become an antiosteoporosis alternative drug.

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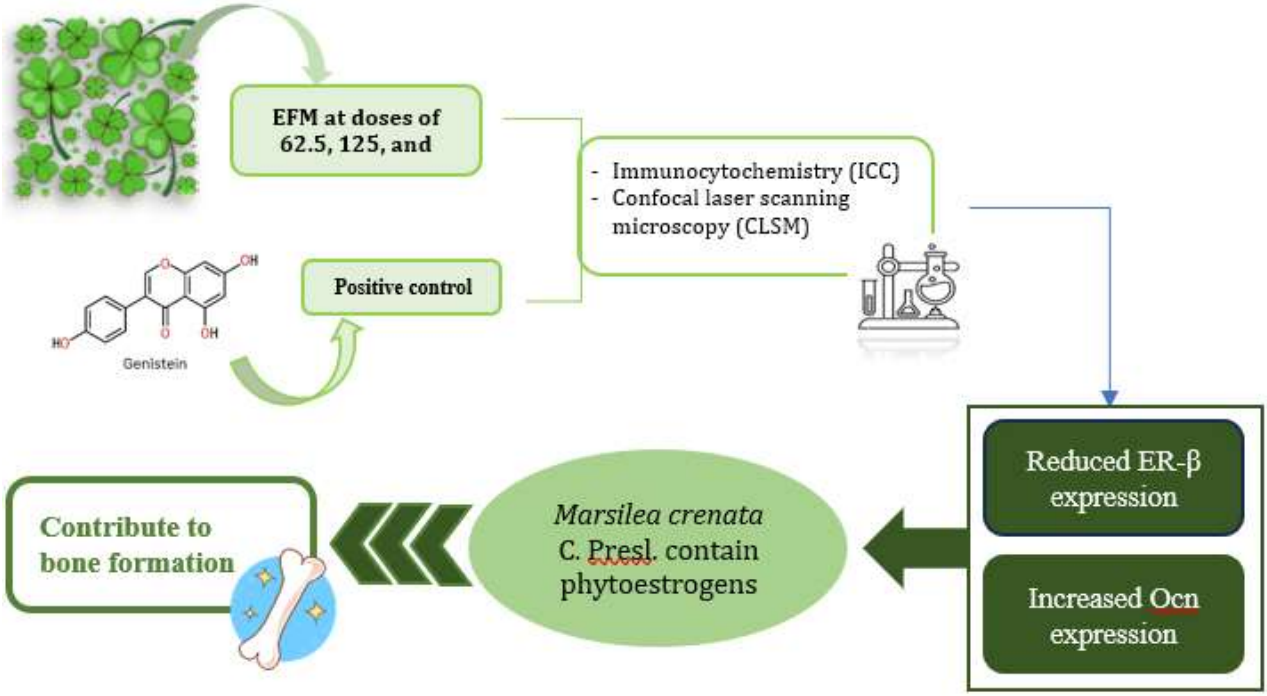
GRAPHICAL ABSTRACT



Introduction

Women experience menopause as a permanent cessation of estrogen production due to the aging process's loss of ovarian follicular function [1,2]. The World Health Organization (WHO) estimated that the number of postmenopausal women who suffered from osteoporosis would increase by around 200 million people worldwide by 2050.

Estrogen plays an important role in maintaining bone homeostasis through osteoblast cell bone formation and osteoclast bone resorption. This type of homeostasis can prevent bone loss by stopping bone resorption caused by the osteoclastic estrogen receptor α (ERα), as well as increase bone formation by activating estrogen receptor β (ERβ) in osteoblasts. It was known that estrogen mediated such activities through ER-dependent and/or ER-independent signaling pathways [3].

In humans, not having enough estrogen raises the levels of inflammatory cytokines. These cytokines help bone cells differentiate directly through nuclear factor kappa-B (NF-κB) and indirectly by increasing osteoblasts' expression of Receptor activator of nuclear factor kappa-B ligand (RANKL). It indicates that estrogen deficiency reduces bone formation by activating NF-κB in osteoblast [4-6].

Postmenopausal women have used hormone replacement therapy (HRT) to treat estrogen deficiency. But long-term use of HRT causes undesirable side effects, such as an increased risk of cancer of the female reproductive organs. Such a risk encourages the use of natural ingredients to prevent menopause-induced bone mineral density reduction [7-9].

Researchers have conducted several studies on the use of natural ingredients to replace estrogen with minimal side effects, leading to the use of phytoestrogen compounds [10,11]. Phytoestrogens are compounds found in plants that have estrogenic activities and provide bone formation by stimulating osteoblasts via ERs and producing important bone formation proteins, such as osteocalcin (Ocn) [10-12]. Osteocalcin is a major non-collagen protein that is only found in osteoblasts. Increased expression of Ocn by osteoblasts is associated with increased bone formation and turnover [13]. In its carboxylated form, Ocn showed a high affinity for the hydroxyapatite that makes up bone minerals and gives bones their rigidity. During the mineralization process, it remains in the bone matrix [12]. However, in women who have gone

through menopause, the lack of calcium and phosphorus makes it harder for hydroxyapatite crystals to form. This means that free Ocn moves around in the blood instead of settling in the bone matrix. This will inhibit the mineralization stage and cause bone fragility [13,14].

Marsilea crenata C. Presl. is one of people's typical foods in Surabaya, East Java Province, Indonesia, and is known to contain phytoestrogens [15,16]. The solid-phase radioimmunoassay (RIA) method was used to determine phytoestrogen levels in dried *M. crenata* leaf infusion, resulting in 1068.0 ± 97.2 pg/g [17]. Scientists also discovered that the n-hexane part of *M. crenata* raised the activity of alkaline phosphatase (ALP) in MC3T3-E1 osteoblast cells. This could mean that more bone is being formed [18]. Working out and giving n-hexane fraction together also raises the density of trabecular bone in the backbones of female mice that have been given dexamethazone [19].

One of the largest chemical groups of phytoestrogens is flavonoids, such as daidzein and genistein [10,20]. Since it was found that *M. crenata* leaves contain phytoestrogens, the ethyl acetate fraction of *M. crenata* leaves (EFM) was used to further understand its activity in relation to bone formation [21]. The objective of this study was to discover how the EFM affected the growth of activated estrogen-β receptors (ERβ*) by measuring the amounts of free ERβ as well as measuring Ocn expression in the human fetal osteoblast (hFOB) 1.19 cell line. Phytoestrogens inside EFM can take the place of estrogen in the body and have a direct effect on bone density by activating ER-β* and encouraging the expression of Ocn.The findings of this study could aid in determining *M. crenata's* potential activity as a source for bone formatting agents.

Materials and Methods

Materials

Plant Materials

Marsilea crenata C. Presl. leaves were obtained from Sememi, Benowo sub-district, Surabaya, Indonesia, in September 2018. The Plant Determination Agency of Unit Pelaksana Teknis (UPT) Materia Medica, Batu, Indonesia, performed the identification using specimen number 1a-17b-18a-1. The leaves were dried and ground to obtain a powdered form.

Cell and Chemicals

American Type Cell Culture (Manassas, USA) provided the human fetal osteoblast cell line (hFOB 1.19 ATCC CRL-3602). Ethyl acetate pro-analysis was purchased from Merck (Darmstadt, Germany). Phosphate-buffered saline (PBS), anti-rabbit fluorescein isothiocyanate (FITC), paraformaldehyde (PFA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Anti-rabbit ER-β and anti-mouse osteocalcin were purchased from Abcam. Dulbecco's Modified Eagle's Medium (DMEM), G418, penicillin-streptomycin 1%, fetal bovine serum (FBS), and bovine serum albumin (BSA) were purchased from Aretha Laboratory, Bandung, Indonesia. Tween-80, paraformaldehyde, genistein, diaminobenzidine (DAB), H_2O_2, and trypsin were purchased from the Central Laboratory of Life Science, Universitas Brawijaya, Indonesia.

Methods

Extraction and Fractionation

An 80 g extract was prepared by extracting 1.9 kg of *M. crenata* leaf powder with 96% ethanol. The extract was further separated using the liquid-liquid extraction process, which involved suspending it in 800 ml of water and fractionating it with n-hexane at a 1:1 ratio. The resulting water phase was then fractionated with ethyl acetate, which was subsequently evaporated using a rotary evaporator (Heidolph Hei-VAP ML/GG3, Germany) to get 2.1 g of the EFM.

Cell Culture

The hFOB 1.19 cells were placed in a 75-ml flask and cultivated in a complete media made up of DMEM, G418, penicillin-streptomycin 1%, and FBS, and then incubated in a CO_2 incubator

(ThermoScientific Hera Cell 150i, USA) at 5% for 6 days at 37 °C. Cell growth was monitored for 24 hours, and the culture media was replenished if nutrient deficiency led to colour changes. Cells were sown in a 24-well microplate once they reached 80-90% confluence.

ERβ and Ocn Measurement

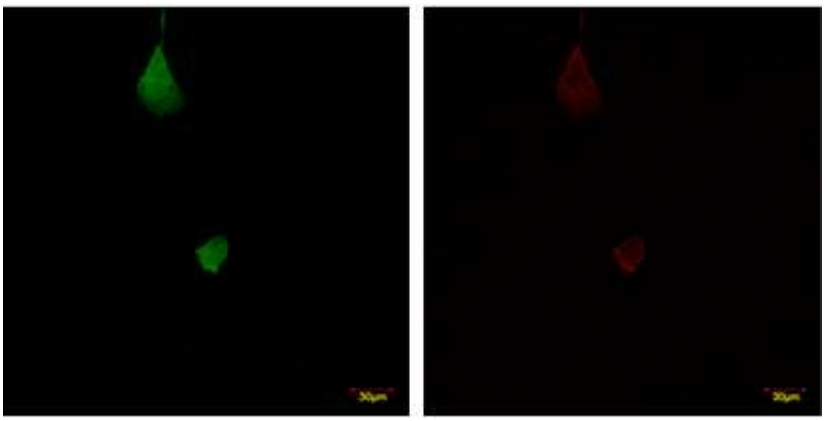
In a volumetric flask, 50 mg of EFM was combined with 0.5% Tween-80 and 0.5% (w/v) to create a 5000 ppm solution. A 0.22-micrometer millipore filtering technique sterilized the solution and diluted it to produce solutions of 62.5, 125, and 250 ppm [16,19,21]. All solutions were then combined with hFOB 1.19 cells in a 24-well microplate and cultured for 48 hours at 37 °C in a 5% CO_2 incubator. The combination was initially treated with 4% PFA, followed by 10% BSA. Every phase of the therapy included washing the cells with PBS. It was combined with the anti-rabbit primary antibodies ER-β, anti-mouse osteocalcin and allowed to rest at 4 °C for 24 hours. The microplates were then cleaned with PBS, and anti-rabbit FITC secondary antibodies and anti-mouse rhodamine were applied. After that, they were incubated in a dark environment at 37 °C for one hour. Confocal laser scanning microscope (Fluoview Olympus FV1000) was used for immunofluorescence investigation at 488 and 543 nm wavelengths. Marker immunofluorescence was evaluated with Olympus Fluoview Ver.4.2a software to quantify ERβ and Ocn expression levels. The negative control was a combination of 0.5% Tween-80 and 0.5% DMSO (w/v), whereas the positive control was 1 μM genistein.

Data Analysis

Data analysis was performed by calculating the average of each treatment group, which aims to identify and compare the bone formation activity of each EFM group compared to the negative and positive control group. The results of the research data were tested using the One-way ANOVA test with a confidence level of 95%. Furthermore, to find out the significant differences between the tests, the LSD post-hoc test was carried out at $p < 0.05$.

Results and Discussion

Figure 1 displays data from immunofluorescence, showing ER expression in green fluorescence and Ocn expression in red fluorescence. Figure 2 indicates that ERβ expression went down after EFM was given, with 250 ppm being the best dose. Figure 3 shows that Ocn expression went up after EFM was given, with 125 ppm giving the highest Ocn expression



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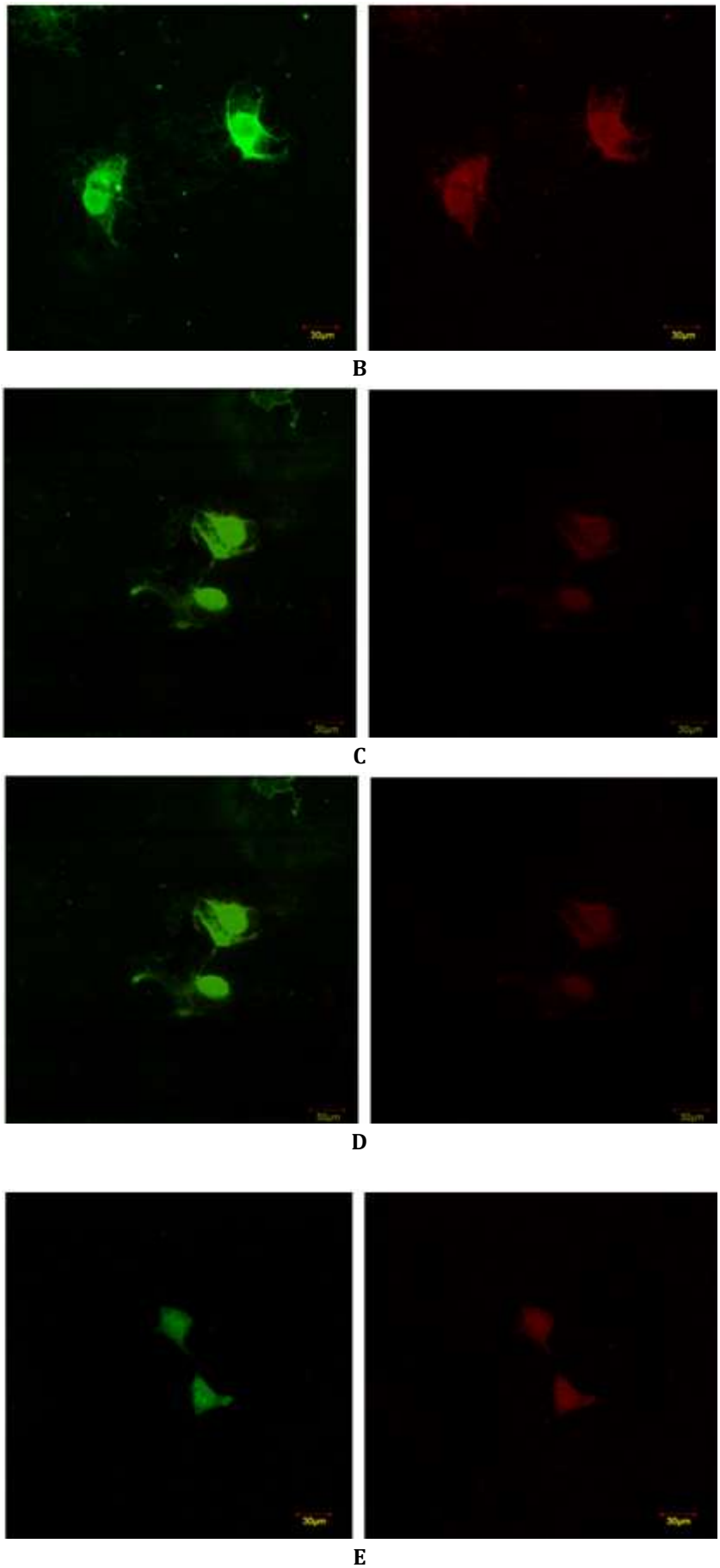

Figure 1: ERβ (green fluorosence) and Ocn (red fluorosence) expressions of hFOB 1.19 cell line after administration EFM; (A) Negative control, (B) 62.5 ppm, (C)125 ppm, (D) 250 ppm, and (E) Genistein

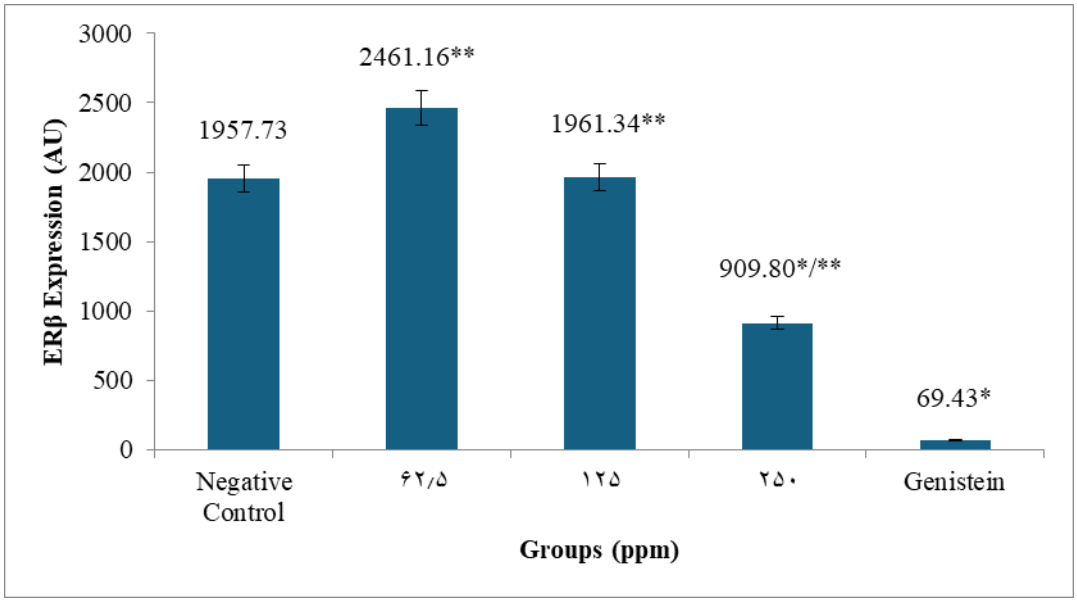

Figure 2: The ERβ expression value of hFOB 1.19 osteoblast cells treated with EFM is expressed as mean ± SD (AU) with p <0.05. The * indicates significant differences between the negative controls, while the ** indicates significant differences between Genistein

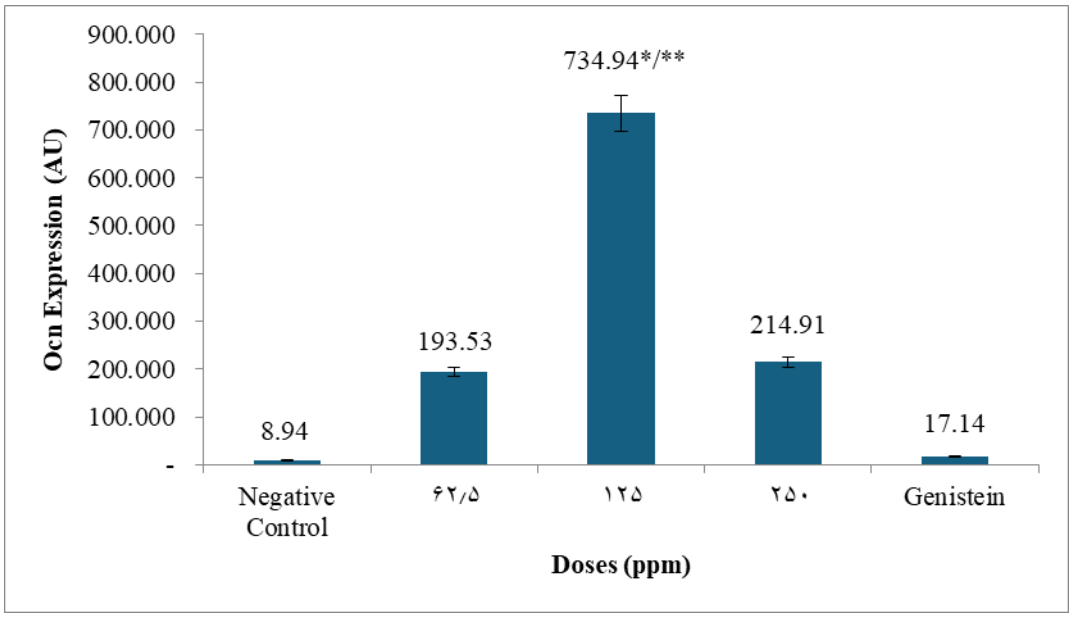

Figure 3: The Ocn expression value of hFOB 1.19 osteoblast cells treated with EFM is expressed as mean ± SD (AU) with p <0.05. The * indicates significant differences between the negative controls, while the ** indicates significant differences between Genistein

The increase in bone density during estrogen treatment, which helps prevent fractures from osteoporosis, demonstrates that reduced blood estrogen levels increase the rate of bone loss. Extragonadal tissues, such as bones and muscles, also synthesize estrogen, which, unlike ovarian-synthesized estrogen, acts locally at the site of synthesis to maintain important tissue-specific functions. Studies revealed high levels of aromatase activity in osteoclastic cell lines and primary human fetal osteoblasts, indicating local synthesis of estrogen in bones [3]. Estrogen can signal both ER and ER in human, rat, and mouse osteoblasts and osteocytes, which then activates

transcription by directly binding to DNA at the classical estrogen response element (ERE) [3]. Understanding the role of both ERs was conducted using ER-knockout mice. The findings showed that both ERs could control mechanical signaling in bones and affect how bones changed over time. For example, osteoblasts taken from mice lacking the beta estrogen receptor showed a rise in the number of cells after being put under mechanical stress [22].

The immunocytochemistry method is a laboratory technique able to visualize the localization of a protein or antigen in cells using a specific primary antibody that binds to it. A secondary antibody with a conjugated fluorophore should bind to the primary antibody to enable visualization and observation under a fluorescence microscope [23].

In this study, the fact that there were fewer free ERβ binding sites in the cells showed that the receptors were already attached to the phytoestrogen compounds in the fraction. The phytoestrogens might have a stronger bond and higher affinity for ERβ compared to the anti-rabbit ERβ. In this work, anti-rabbit ERβ could not bind the cells' ERβ receptors, which have already been bound to phytoestrogen compounds of the EFM. Activation of a receptor starts with binding a ligand to it, which could initiate some downstream signalling. Receptors can be active or inactive in their ligand-free or ligand-bound forms. A ligand-free receptor is generally in an inactive conformation, and the binding of an agonist shifts the equilibrium toward the active state [24]. Receptors that were not bound to phytoestrogens were considered free receptors, which could be analysed using the fluorescence strength (Figure 1). Weaker fluorescence revealed a reduced number of free ERβ and an increased number of activated ERβ bound to ligands.

The findings of this study demonstrated that an increase in the fractions' dose did not coincide with a rise in Ocn expression. The graph's slope values revealed the highest Ocn expression at 125 ppm, compared to 62.5 and 250 ppm, at several points within the given concentration range. The non-monotonic dose response (NMDR) effect, which frequently causes nonlinear activity responses at increased concentrations in *in vitro* activity tests and studies involving multiple hormones or hormone replacement compounds, could be one possible explanation for this. Differences in the level of affinities between hormones and hormone replacement compounds to target proteins, such as receptors, make responses difficult to predict despite increased doses of treatments [25].

Osteocalcin is a secreted factor that influences matrix mineralization and global metabolism. Osteoblasts release it during bone formation, where it binds with the mineralized bone matrix [14]. During bone resorption, the bone matrix releases osteocalcin into the blood, suggesting that osteocalcin is also a marker of bone turnover [13]. Genistein as a positive control showed its potency to reduce free ERβ expression but could not increase Ocn expression compared to the negative control. This may explain why Ocn expression is not solely caused by interactions between estrogen compounds and ER-β. Research by Liao *et al.* (2014) [26] proved that genistein showed its role in the process of bone formation through interaction with ERα, as could be seen from the increase in expression of Ocn or other markers. Both *in vivo* and *in vitro* study demonstrated the synergistic effects of genistein during the mineralization process.

The results of this study showed that Ocn expression depends on how ERβ interacts with estrogens or phytoestrogens, which is an important part of bone formation. The study also showed other ways that bone formation happens through genistein [3,10,26-27].

Based on ERβ and Ocn expressions, it is assumed to be possible that EFM binds to ERβ and causes Ocn to increase. According to how phytoestrogens work in osteoblastic cells, estrogen binds to ERβ to form phytoestrogen-ER complex bonds. These bonds then control genetic transcription to make Ocn and other proteins that are important for bone formation [4,26,28]. Ocn contributes to the proliferation and differentiation of osteoblasts, leading to the creation of a new bone matrix during the mineralization stage of bone formation [28]. Therefore, this study suggests that the phytoestrogen content of EFM could potentially

contribute to bone formation and serve as a bone formation agent.

This study is limited to the use of only two parameters in in vitro experiments. To achieve a comprehensive understanding, we need to conduct additional research by assessing additional factors and carrying out in vivo testing.

Conclusion

The results of this study showed that EFM reduced free ERβ expression optimally at 250 ppm by 909.80 AU and increased Ocn expression optimally at 125 ppm by 734.94 AU. Further research on *M. crenata* is necessary due to suggestions that its phytoestrogen content may contribute to bone formation and could potentially serve as an alternative treatment for osteoporosis.

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Authors' Contributions

All authors actively participated in the data analysis, drafting, and revising of the paper and willingly assumed responsibility for all elements of this process.

Conflict of Interest

The authors assert that this work is free from any conflict of interest.

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