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Enhancement of Osteoblastogenesis in the hFOB 1.19 Cell Line by the Induction of the n-Butanol Fraction Derived from *Marsilea crenata* C Presl. Leaves

Agnis Pondineka Ria Aditama[1], Siti Asiyah[2]*, Tintin Hariyani[2], Wahyu Nuraisya[2], Linda Andri Mustofa[2] and Anis Setyowati[2]

[1]Health Polytechnic of Jember, Jember, Indonesia.
[2]Department of Midwifery, STIKES Karya Husada Kediri, Kediri, Indonesia.
*Corresponding Author E-mail:aninkamila@gmail.com

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Osteoporosis is a disease characterized by bone loss, which can be treated by increasing the bone formation process. A marker of increased bone formation processes is the activity of the transcription factor Osx which stimulates the formation of Ocn. M. crenata leaves contains phytoestrogens which are thought to increase bone formation. The purpose of this research was to prove that the n-butanol fraction from the leaves of M. crenata can increase bone formation in hFOB 1.19 osteoblast cells by increasing the activity of the transcription factors Osx and Ocn. hFOB 1.19 cells that had reached confluence were given n-butanol fraction at a dose of 62.5, 125, and 250 μg/L, and Genistein 2.5 μg/ml as a positive control for phytoestrogens. Increased bone formation was identified through the expression of Osx and Ocn using the immunocytochemical method with CLSM. The findings indicate that the application of various dosages of the n-butanol fraction derived from the leaves of M. crenata has a positive impact on bone formation in hFOB 1.19 cells. The best dose was determined to be 250 μg/L, with a statistically significant difference of $p<0.005$. The n-butanol fraction obtained from M. crenata leaves was found to increase the expression of Osx and Ocn in hFOB 1.19 cells, indicating its potential to enhance bone production.

Keywords: hFOB 1.19; Marsilea crenata C Presl.; n-butanol fraction; Osteri; Osteocalcin.

Osteoporosis is a bone disease in humans characterized by reduced bone strength and density, leading to a higher risk of fractures. Bone density often decreases with age, showing variations based on the gender and race of individuals. Key irreversible factors contributing to osteoporosis development are age, menopause, and female gender[1]. Estrogen deficiency in women over 40 leads to reduced bone mass, raising the likelihood of osteoporosis[2,3].

The process of bone remodeling is characterized by its dynamic nature, wherein osteoclasts engage in the resorption of aged bone tissue and osteoblasts are responsible for the generation of new bone tissue. It is responsible for healing injured bones[4,5]. The formation stage involves the proliferation and differentiation of osteoblast precursors, followed by the mineralization of the new bone matrix[4].

The estrogen-receptor (E-ER) complex in the nucleus or cell membran can be influenced by

estrogen to affect the proliferation, differentiation, and maturation of osteoblast in different species[6,7]. One transcription factor that is important for osteoblast development is called Osterix (Osx). By upregulating the expression of proteins such as osteocalcin (Ocn), osteopontin (OPN), bone sialoprotein (BSP), and collagen type I (COL-I), it promotes the production of new bone[8,9]. As an adult, Ocn is a major protein in bones. It helps bones mineralize by interacting with ã-carboxyglutamic acid (Gla) residues and increasing the absorption of hydroxyapatite[10].

The use of estrogen in women with estrogen deficiency, such as long-term hormone replacement therapy (HRT), carries a carcinogenic risk to female reproductive organs and other side effects[11]. Therefore, research on estrogen alternatives prefers natural ingredients, especially phytoestrogen compounds, which provide bone protective effects by inhibiting bone resorption and increasing bone formation due to menopause[12,13]. Plant chemicals called phytoestrogens can act like estrogen by making osteoblast cells work harder by attaching to the estrogen receptor (ER) with low side effects[14].

The aquatic plant *Marsilea crenata* C Presl. which is commonly consumed as a staple meal in Surabaya, East Java, Indonesia, has been identified as a source of phytoestrogens [15,16,17]. Numerous investigations conducted on *M. crenata* have revealed the presence of phytoestrogens in various forms, including triterpenoids, steroids, and isoflavones, within the ethanol extract, n-hexane, ethyl acetate, and the n-butanol fraction of *M. crenata* leaves. Additionally, it has the potential to stimulate the activation of ER-â and enhance the elevation of alkaline phosphatase (ALP) levels in the MC3T3-E1 preosteoblast cell line, hence promoting cellular proliferation and differentiation [18,19]. In vivo using male and female mice shows that *M. crenata* induces the proliferation of trabecular bone osteoblast cells [20,21].

The purpose of this research is to prove that the n-butanol fraction (BF) of *M. crenata* leaves can enchance bone formation in human fetal osteoblast (hFOB 1.19) by measuring the Osx and Ocn expressions using a confocal laser scanning microscop (CLSM) instrument. These cells are used to study how human osteoblasts differentiate, how osteoblasts work, and how cytokines affect osteoblast function[22,23].

MATERIAL AND METHODS

Materials

UPT Materia Medika, Batu, East Java, identified *M. crenata* leaves that were gathered from the Benowo region of Surabaya, East Java, Indonesia (code: 1a17b-18a-1)[24]. The hFOB 1.19 cell line (CRL-11372) was obtained from American Type Cell Culture (Virginia, USA). Quercetin, dimethyl sulfoxide (DMSO), paraformaldehyde (PFA), bovine serum albumin (BSA), and phosphate-buffered saline (PBS) were supplied by Sigma-Aldrich (Missouri, USA). Primary antibodies against mice osteocalcin and anti-rabbit osterix were acquired from Abcam (Cambridge, Britain). The Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS), and G418 were provided by Aretha Laboratory (Bandung, Indonesia). Anti-mouse secondary antibody Rhodamine, anti-rabbit secondary antibody fluorescein isothiocyanate (FITC), paraformaldehyde and Tween-80 were provided by the Central Laboratory of Life Science at Brawijaya University (Malang, Indonesia).

Method

Extraction, Fractionation, and Preparation of BF

A technique of extraction was performed using 1.5 kg of *M. crenata* leaf powder and a solvent consisting of 96% ethanol, with the assistance of ultrasonics wave. This procedure yielded a total of 70 g of extract[17]. Liquid-liquid extraction was done in a 1:1 ratio, the extract was combined with 600 mL of water and subsequently separated using n-Hexane. Ethyl acetate and n-butanol were employed to separate the aqueous phase. The isolation and drying of the n-butanol phase were conducted using a Heidolph Hei-VAP ML/GG3 rotary evaporator.

A stock solution with a concentration of 5000 ppm was prepared by combining a 50 mg BF with 0.5% Tween-80 in 0.5% DMSO (w/v). The solution is formulated with concentrations of 62.5, 125, and 250 µg/L.

Cell Culture

The hFOB 1.19 cells were cultured in a culture flask with a complete media including DMEM, FBS 10%, G418, and penicillin-streptomycin 1%. The flask was then placed in an incubator with a CO_2 5% and maintained at a temperature of 37°C for a period of 6 days. Cell proliferation was monitored at 24-hour intervals, and the media was routinely replenished until it reached a concentration of 80–90%. This was followed by the transfer of the cells to a microplate.

Osx Measurement

After reaching confluence, hFOB 1.19 cells were treated for 48 hours with BF at doses of 62.5, 125, and 250 µg/L and genistein 2.5 µg/ml as a positive control. After that, cells were fixed with 4% PFA and cleaned with PBS. Subsequently, Osx primary antibodies, BSA, and Triton X-100 were added, and the mixture was incubated for a full day at 4°C. Subsequently, Osx expression was examined by immunocytochemistry using an Olympus Fluoview Ver.4.2a CLSM at 488 nm, and anti-rabbit Osx (Secondary Antibody-FITC) was added.

Ocn Measurement

The hFOB 1.19 cells that had reached confluence were given BF at a dose of 62.5, 125, and 250 µg/L, and Genistein 2.5 µg/ml as a positive control, then incubated for 48 hours. Next, cells were washed with PBS and fixed with 4% PFA. After that, Triton Followed by the addition of anti-mouse Ocn (secondary antibody-Rhodamine) and analysis of Ocn expression using immunocytochemical techniques using the Olympus Fluoview Ver.4.2a CLSM instrument at a wavelength of 543 nm.

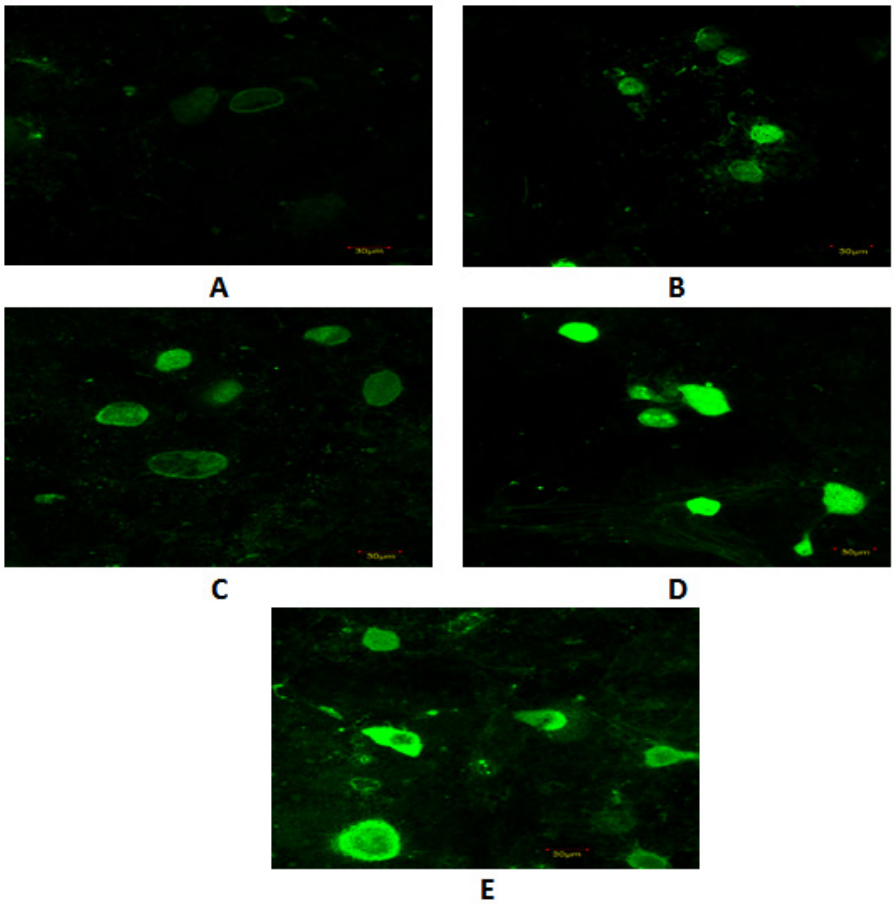

Fig. 1. Immunofluorescence Osx of hFOB 1.19 cell line. (A) Negative control, (B) dose 62.5 µg/ml, (C) dose 125 µg/ml, (D) dose 250 µg/ml, (E) genistein 2.5 µg/ml

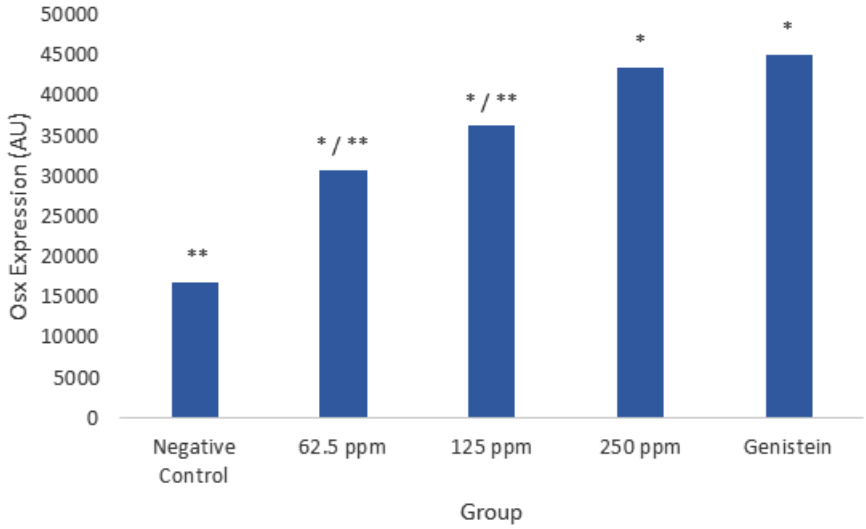

Fig. 2. Expression of Osx in hFOB 1.19 cells after administration of BF of *M. crenata* leaves. The "*" indicates significant difference to the negative control, while ** indicates significant difference to genistein 2.5 µg/ml

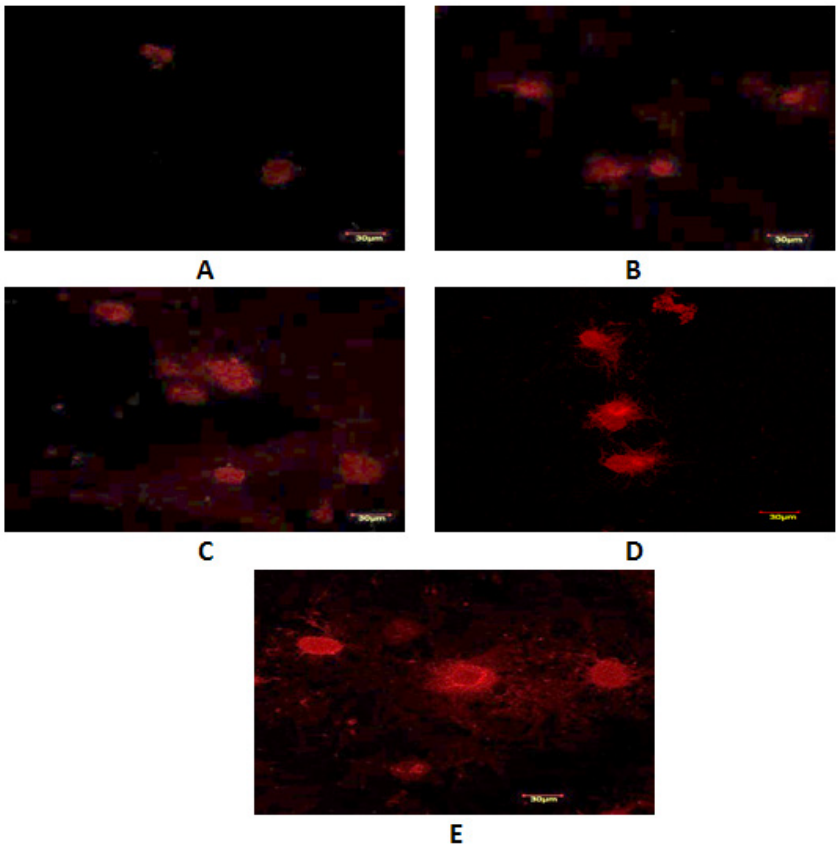

Fig. 3. Immunofluorescence Ocn of hFOB 1.19 cell line. (A) Negative control, (B) dose 62.5 µg/ml, (C) dose 125 µg/ml, (D) dose 250 µg/ml, (E) genistein 2.5 µg/ml

Data analysis

After obtaining data using CLSM, the next step is to carry out quantification to obtain numerical data, which will then be carried out using one-way ANOVA and post-hoc LSD analysis using the statistical software Statistical Product and Service Solutions (SPSS) with a significant difference of $p<0.05$.

RESULTS AND DISCUSSION

To get 70 g of extract, 1.6 kg of dry powdered M. crenata leaves were extracted using 96% ethanol. The extract was then suspended in 1:1 aqua destillates. The suspension is then fractionated with the n-butanol solvent liquid extraction method using a separating funnel. From this process, the weight of the BF was 5.97 grams.

Improvement of the bone formation process in vitro using the BF from *M. crenata* leaves has been studied in hFOB 1.19 cells. The immunocytochemistry method using CLSM was carried out to measure the expression of Osx and Ocn. Measurements were carried out at a wavelength of 488 nm for Osx and 543 nm for Ocn. Osx expression was shown by green fluorescence intensity, while Ocn expression was shown by red fluorescence intensity in hFOB 1.19 cells (Figures 1 and Figure 3).

Figure 1 shows Osx immunofluorescence in hFOB 1.19 cells in all groups. The strongest intensity was shown in the genistein positive control group, followed by the treatment group according to concentration level, while the weakest intensity was shown in the negative control group. Figure 2 shows the 250 µg/ml dose group gave the best results by increasing Osx expression significantly compared to the negative control ($p = 0.000$) and not significantly compared to 2.5 µg/ml genistein ($p = 0.129$). The increase in Osx expression at the dose of 62.5 µg/ml was significantly different from the negative control ($p = 0.000$) and from genistein 2.5 µg/ml ($p = 0.000$), while the 125 µg/ml dose group was also significantly different from the control negative ($p = 0.000$) and from genistein 2.5 µg/ml ($p = 0.000$). This shows that genistein and the treatment group were able to increase Osx expression in hFOB 1.19 cells.

Figure 3 shows Ocn immunofluorescence in hFOB 1.19 cells in all groups. The strongest intensity was shown in the genistein positive control group, followed by the treatment group according to concentration level, while the weakest intensity was shown in the negative control group.

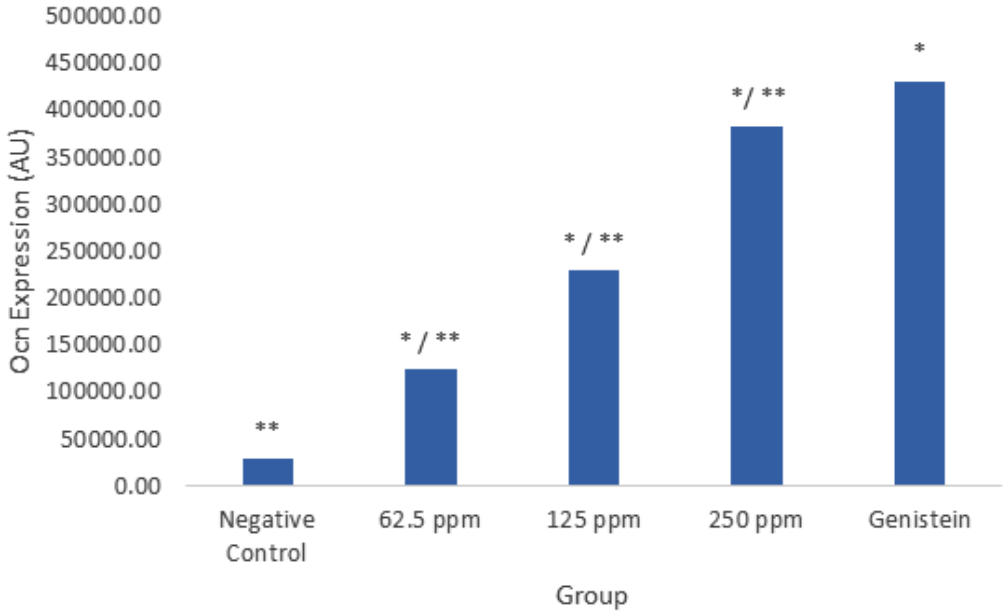

Fig. 4. Ocn expression in hFOB 1.19 cells after administration of BF from *M. crenata* leaves. The "*" indicates significant difference to the negative control, while ** indicates significant difference to genistein 2.5 µg/ml

Figure 4 shows the 250 µg/ml dose group gave the best results, with a significant increase in Ocn expression compared to the negative control ($p = 0.000$) and also significant compared to genistein 2.5 µg/ml ($p = 0.000$). The increase in Ocn expression at the dose of 62.5 µg/ml was also significantly different from the negative control ($p = 0.000$) and from genistein 2.5 µg/ml ($p = 0.000$), while the 125 µg/ml dose group also showed a significant difference compared to the negative control ($p = 0.000$) and genistein 2.5 µg/ml ($p = 0.000$). Meanwhile, the 250 ppm treatment group was only significantly different from the negative group, which shows that the ability to increase Osx was similar to the positive control group.

The BF from *M. crenata* leaves has been identified as a potential phytoestrogen that can function as an alternative to estrogen via the ER-â pathway[17]. Phytoestrogens are known to be able to stimulate ER-â activation, which plays a role in regulating transcriptional gene expression and triggering osteoblast cell proliferation and differentiation[8,9]. In the context of bone formation, osteoblast cell differentiation becomes a key factor, which is influenced by the activation of transcription factors such as Osx and runt-related transcription factor 2 (Runx-2), as well as the production of important proteins such as ALP, Ocn, type I collagen, and BSP, along with the mineralization process and increased bone density[8,9,10].

Because Osx plays a crucial role as a specific transcription factor for osteoblast cells, it regulates the process of osteoblast cell maturation and bone formation, including the expression of key proteins like Ocn[25]. On the other hand, Ocn was chosen as a marker factor because, as the main non-collagen protein produced by mature osteoblast cells, it is often used in the characterization of human bone cells. Ocn has a crucial role in bone formation and mineralization processes[10,26].

Genistein 2.5 µg/ml was chosen as a positive control because of its ability as a phytoestrogen, which can substitute the role of estrogen in preserving bone balance, and also because of its ability to increase the bone formation process in both in vivo and in vitro experiments[27]. Genistein is known to have high affinity for ER-â[28], inhibit nuclear factor-kappa B (NF-êB) activation[29], and increase Ocn expression[14,30]. Activity of the BF from *M. crenata* leaves. Similar to genistein, which was used as a positive control in this study, it is possible that it has activity similar to genistein. Dose 250 ppm of the of the BF from *M. crenata* leaves. This is the most optimal dose for increasing Osx and Ocn because the amount and intensity measured are similar to those of genistein.

Research data shows that the BF extracted from the leaves of *M. crenata* is able to increase the expression of Osx and Ocn. These findings indicate the presence of phytoestrogen compounds in the BF, which have a role in increasing the expression of Osx and Ocn, both of which are important indicators in the bone formation process.

CONCLUSION

The BF of *M. crenata* leaves at doses of 62.5, 125, and 250 µg/ml has been shown to enchance the expression of Osx and Ocn in hFOB 1.19 cells, with the optimal dose at 250 ppm. These results indicate that the BF from *M. crenata* has the potential to improve the bone formation process by inducing osteoblastogenesis. However, further preclinical and clinical research needs to be carried out to confirm the osteoblastogenesis effect of BF.

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Conflict of Interest

The authors declare that there are no possible conflicts of interest with respect to the research, authors, and/or publication of this article.

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