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The Effect of Ethyl Acetate Fraction of Marsilea Crenata Presl. LEAVEs in Increasing Osterix Expression in hFOB 1.19 cells

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bone formation;
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osterix;

Abstract

Marsilea crenata Presl. leaves contain phytoestrogens that are suspected to play a role in increasing bone formation. Bone formation activity is defined by the expression of Osterix, a transcription factor that plays a key role in bone development. This study aims to show the ethyl acetate fraction of *Marsilea crenata* Presl. leaves can increase the bone formation process in hFOB 1.19 cells in a TNF-α dependent manner, by measurement of Osterix. The hFOB 1.19 cells were grown in 24-well microplates and treated with 10 ng/ml TNF-α for 24 hours. The ethyl acetate fraction of *Marsilea crenata* Presl. leaves were also added in concentrations of 62.5, 125, and 250 ppm. Positive control was employed, at a concentration of 2.5 g/L of genistein. The expression of osterix was examined using an immunocytochemistry technique with CLSM to assess bone-forming activity. The results show that ethyl acetate fraction *Marsilea crenata* Presl. leaves can boost osterix expression in hFOB 1.19 cells, with an optimal concentration was 250 ppm at $p<0.005$. The ethyl acetate fraction of *Marsilea crenata* Presl. leaves can increase Osterix in osteoblast hFOB 1.19 cell, indicating improved bone-forming activity.

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1 Introduction

Bone is a specialized stiff tissue that supports the body's structure, facilitates movement through muscle attachment sites, protects organs, and stores calcium and growth hormones (Florencio-Silva et al., 2015; Wittkowske et al., 2016). Bones can heal and repair themselves throughout their lives. This process, known as bone remodeling, involves a variety of cell types and can be triggered by changes in biomechanical loads or the replacement of old, micro-damaged bone with newer, stronger bone (Kini & Nandeesh, 2012; Wittkowske et al., 2016). During the remodeling process of bone formation, bone cells work together in a coordinated manner to maintain a balance between osteoblasts that store new bone tissue, osteoclasts that break down the bone matrix, and osteocytes that regulate osteoblast and osteoclast activity in response to mechanical loads (Kim et al., 2020; Wittkowske et al., 2016).

The risk of unbalanced bone remodeling due to estrogen shortage grows with age, especially in menopausal women, and this will continue in osteoporosis (Compston et al., 2019). Long-term use of estrogen as a Hormone Replacement Therapy (HRT) may be carcinogenic, increasing the risk of cancer in the female reproductive organs (Bretler et al., 2012). To prevent bone mineral density loss due to menopause, researchers are focusing their efforts on natural components, which leads to the usage of phytoestrogen chemicals (T.-S. Yang et al., 2012).

People in the Surabaya area of East Java, Indonesia, grow *Marsilea crenata* Presl. as a plant. People in the area eat the leaves as a special cuisine (Sembiring et al., 2022). *Marsilea crenata* Presl leaves contain phytoestrogens, which have anti-osteoporosis activity in vitro, in silico, and in vivo (Aditama et al., 2020; Ma'arif et al., 2018; Yang et al., 2013). In vivo and in vitro, phytoestrogens in *Marsilea crenata* Presl. extract and fraction increased estrogen receptor activity and bone formation markers (Aditama et al., 2021; Laswati, 2011; Ma'arif et al., 2018).

Phytoestrogens are plant substances that have a molecular structure similar to estrogen in the body and can attach to the same estrogen receptors, effectively replacing estrogen's action. Estrogen has a crucial part in the development of bones (Aditama et al., 2022; Sirotkin & Harrath, 2014). One of the phytoestrogens' functions is to prevent bone loss caused by estrogen deficiency. Women, in particular, will endure menopause and estrogen shortage as they grow older, as well as increased tumor necrosis factor-alpha (TNF-α) release and bone loss activation. Phytoestrogens are found in a variety of plants, as Genistein, Kaempferol, Daidzen, Quercetin, and others. Genistein is a phytoestrogen that can boost osteoblast cell development and increase osterix expression (Gupta et al., 2016; Sirotkin & Harrath, 2014).

Osterix is a transcription factor that plays a key role in osteoblast differentiation and maturation (Mizoguchi et al., 2014; Baek et al., 2010). TNF-α, which is released during estrogen deficiency due to menopause or arthritis, inhibits osteoblast cell development as well as osteoclast activity. Barriers to osteoblast development and maturation will lead to a reduction in bone-forming activity (Aditama et al., 2022; Liu et al., 2020). In some studies, administration of 10 ng/ml TNF-α to osteoblast cells can simulate the state of estrogen deficiency in osteoporosis patients and osteoblast cell lines (Aditama et al., 2022; Chen et al., 2020). In this study, the osteoblast cell line that was used was human fetal osteoblast (hFOB 1.19) cells, which are clonal human osteoprogenitor cells utilized as a model for osteoblast differentiation and maturation (Aditama et al., 2022).

The ethyl acetate fraction of *Marsilea crenata* Presl. was shown to be the most active in promoting bone formation in previous studies, so it was used (Setzer et al., 2009; Strzelecka-Kiliszek et al., 2017). To determine the activity of the ethyl acetate fraction of *Marsilea crenata* Presl. in the enhancement of Osterix expression in hFOB 1.19 cells, an advanced activity study of it is required (Marie, 2006; Simmons et al., 2004).

2 Materials and Methods

Materials

Marsilea crenata Presl. leaves obtained from the Benowo area, Surabaya, Indonesia were identified with the code 1a17b-18a-1. A total of 6 kg of leaves were dried and 1.6 kg of *Marsilea crenata* Presl. leaves powder was obtained. Cell line Human fetal osteoblasts (hFOB 1.19) were purchased from ATCC (USA). Anti-rabbit Osterix-FITC, Bovine serum albumin (BSA), Dimethyl sulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM), Ethanol 96%, Ethyl Acetate, fetal bovine serum (FBS), G418, n-Hexane, Osterix, paraformaldehyde (PFA), phosphate-buffered saline (PBS), penicillin-streptomycin 1%, Tween-80 were purchased from Sigma-Aldrich (Darmstadt, German)

Extraction and fractionation
Ethyl acetate fraction *Marsilea crenata* Presl. was extracted from 1.6 kilograms of *Marsilea crenata* Presl powder. Using an ultrasonic-assisted extraction method with 96% ethanol as the solvent, then liquid-liquid fractionation using n-hexane and ethyl acetate as the solvents in a sequential order portion of ethyl acetate. A mother liquor of 5000 ppm was created by mixing 50 mg of *Marsilea crenata* Presl. with 0.5% Tween-80 and 0.5% DMSO. The samples were then sterilized by filtering with a 0.22-millimeter millipore and prepared to achieve concentrations of 62.5, 125, and 250 parts per million (ppm).

Cell culture
hFOB 1.19 cells were cultured with the complete medium in a 75 ml flask. The composition of the Complete medium is DMEM, G418, Penicillin-streptomycin 1%, and FBS 10%. Incubation was carried out in an incubator with 5% CO_2 at 37^{o}C for 6 days and the culture medium was replaced every 24 hours. After the cell culture was 80-90% confluent, the cells were transferred to a 24-well microplate.

Osterix measurement
hFOB 1.19 cells that were 80-90% confluent were given TNF-α 10 ng/ml for 24 hours. Followed by washing using PBS and given ethyl acetate fraction *Marsilea crenata* Presl. leaves with a concentration of 62.5; 125; and 250 ppm were incubated for 48 hours. Cells were treated in stages, namely washing again with PBS and fixed with 4% PFA, given Triton-X-100, BSA, and primary antibody for osterix. Cells were incubated at 4^{0}C for 24 hours, then given anti-rabbit secondary antibody-FITC and analyzed for osterix expression in the form of immunofluorescence with (confocal laser scanning microscope) CLSM at 488 nm. Genistein with a concentration of 2.5 g/L was utilized as a positive control.

3 Results and Discussions

In vitro activity study was using immunocytochemistry methods and CLSM instrument to measure the Osterix expression in hfOB 1.19 cells (Canette & Briandet, 2014; Cardona et al., 2013; X. Yang et al., 2014). Osterix is involved in the maturation of osteoblasts into adult osteoblasts as well as bone production (Amarasekara et al., 2021; Liu et al., 2020; Sinha & Zhou, 2013).

Administration of 10 ng/ml TNF-α over 24 hours can prepare hFOB 1.19 cells to mimic estrogen deficiency, where these conditions will inhibit the production of Osterix, as seen in Figure 1. TNF-α is a cytokine that is secreted when estrogen levels are low, especially in menopausal women, and will continue to rise as they become older (Compston et al., 2019; Noh et al., 2020; N. Yang et al., 2013). When TNF-α is administered to hFOB 1.19 osteoblast cells, it binds to its receptor, TNF-R1/p55, which is responsible for the majority of the biologic effects, one of which is the activation of inhibitors of kappa B kinase (IκK), which can subsequently phosphorylate inhibitors of kappa B (IκB). Phosphorylation of IκB causes the separation of IκB bonds from NF-κB, thereby causing activation of NF-κB. The activated NF-κB protein will translocate from the cytoplasm to the nucleus and bind to the promoter in DNA, then there is an upregulation of the p38 gene which is a gene complex that inhibits osterix activation. Osterix inactivation can cause inhibition of osteoblast

differentiation into mature osteoblasts, thereby reducing bone formation activity (Aggarwal et al., 2005; N. Yang et al., 2013).

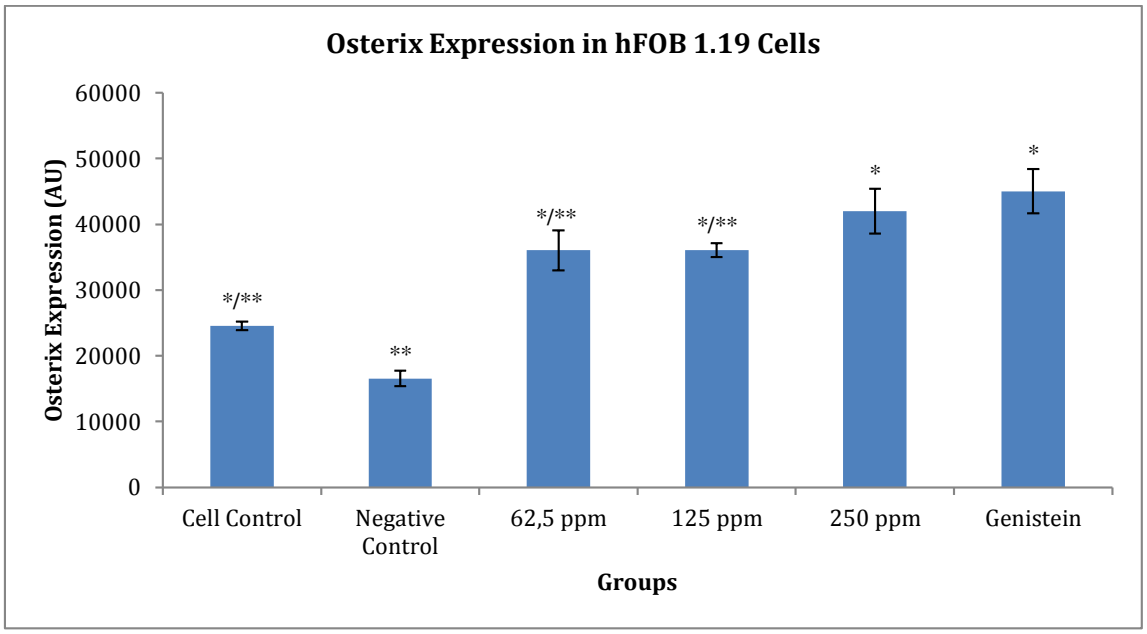
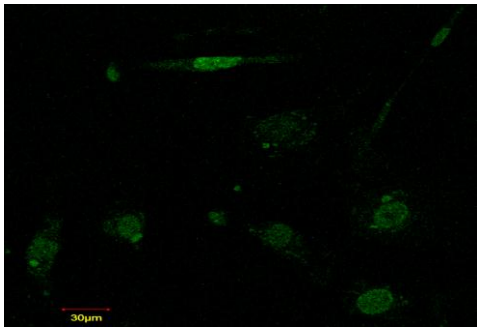
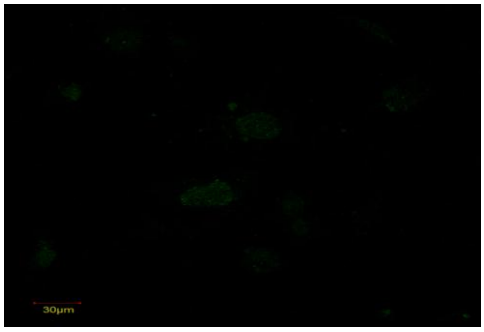


Figure 1. Osterix expression in hFOB 1.19 cells. (*) significantly different from negative control and (**) significantly different from Genistein



A



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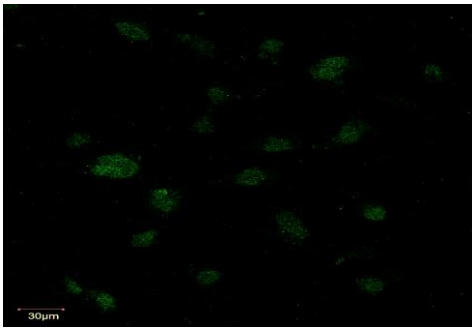

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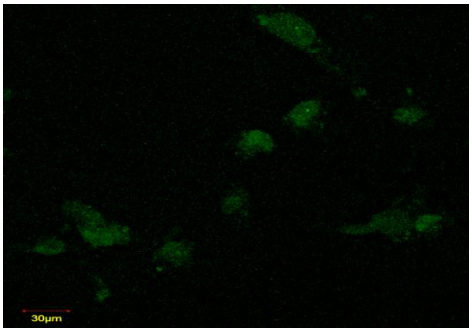

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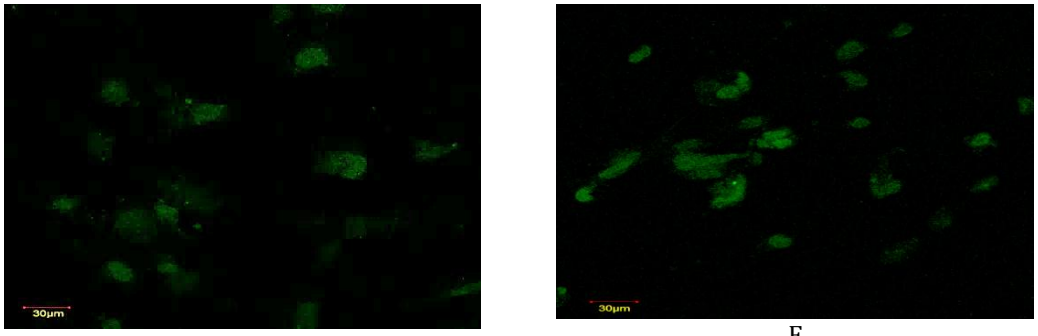

E F

Figure 2. Expression of Osterix (green fluorescence intensity) using FITC staining with a 488 nm wavelength laser beam CLSM instrument. (A) Control cells, (B) Negative control, (C) EA fraction of *Marsilea crenata* Presl. concentration of 62.5 g/ml, (D) concentration of 125 g/ml, (E) concentration of 250 g/ml, (F) Genistein.

The measured expression of osterix in the form of fluorescence intensity in Arbitrary Units (AU) indicated the activity of osterix transcription factors compared to negative controls. The results of the observation were that the expression of osterix increased along with the increase in the administration of the ethyl acetate fraction of *Marsilea crenata* Presl (Wu et al., 2009; Saravanakumar et al., 2021). leaves with a concentration of 62.5; 125; and 250 ppm, in this study the optimal concentration that can increase the expression of the osterix marker factor is a concentration of 250 ppm compared to the negative control was shown in figure 1, with the administration of this concentration it can increase the expression of the osterix marker factor which is close to the positive control (Genistein).

Genistein with a concentration of 2.5 µg/L is a phytoestrogen that can substitute estrogen in maintaining bone homeostasis and accelerating the process of bone formation in vivo and in vitro (Wang et al., 2019; Zhang et al., 2016), it was utilized as a positive control. The ER-dependent, ER-independent, G protein-coupled estrogen receptor 1 (GPER), and ER-X pathways are all involved in the activity of genistein as a phytoestrogen (Sirotkin & Harrath, 2014). When the estrogen receptor is activated, it binds to the estrogen response element (ERE), which can induce the transcription factors Runx2 and Osterix, as well as the production of specific proteins like osteocalcin (Aditama et al., 2021, 2022).

The findings of this study back with recent research indicating phytoestrogens in the ethyl acetate fraction of *Marsilea crenata* Presl. leaves can bind to estrogen receptors, effectively substituting estrogen as a mediating factor in bone production. This suggests that the extract and fraction of *Marsilea crenata* Presl. leaves can boost the production of indicators like alkaline phosphatase and osteocalcin, which are significant in bone formation (Aditama et al., 2020, 2021; Ma'arif et al., 2018; Yang et al., 2014).

4 Conclusion

The results of this study show the ethyl acetate fraction of *Marsilea crenata* Presl. leaves can increases Osterix in osteoblast hFOB 1.19 cell with an optimal concentration was 250 ppm at $p<0.005$, indicating improved bone-forming activity.

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