

The Effect of Water Fraction Derived from Green Clover (*Marsilea crenata* C. Presl.) Leaves on Differentiation and Maturation of Human Osteoblast Cell

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Women over the age of 40 often experience a decrease in bone mass due to a lack of estrogen, which can increase the risk of osteoporosis. Previous studies have shown that the leaves of Green Clover (*Marsilea crenata* C. Presl.) contains phytoestrogen compounds which can potentially replace the function of estrogen in the body. In this context, this study aims to evaluate the ability of water fractions from *M. crenata* leaves in increasing the expression of osterix (Osx) and osteocalcin (Ocn), which are important indicators of increased bone formation, specifically on differentiation and maturation of osteoblast cell. In this study, the water fraction with a dose of 62.5; 125; and 250 $\mu\text{g/L}$, as well as Genistein 2.5 $\mu\text{g/ml}$, were given to human fetal osteoblast (hFOB) 1.19 cells that had reached the confluence point. The analysis method uses immunocytochemical techniques with confocal laser scanning microscopy (CLSM) to identify changes in the bone formation process. All dosages of water fraction considerably enhanced the bone formation in hFOB 1.19 cells, as indicated by the results. The results of this study provide confirmation that the water fraction derived from *M. crenata* leaves has promise as a possible agent for combating osteoporosis. This is achieved by enhancing differentiation and maturation of osteoblast cell through the upregulation of Osx and Ocn genes.

Keywords: Differentiation in vitro; Maturation; *Marsilea crenata* C. Presl.; Osteoblast; Osteocalcin; Osterix.

It is common for women over the age of 40 to suffer reduced bone mass as a result of estrogen shortage, which in turn increases the chance of developing osteoporosis. Data from the Central Bureau of Statistics of Indonesia 2020 indicate that the number of older individuals in Indonesia increased by 9.92% to around 26 million. The proportion of elderly women in Indonesia is higher than that of elderly males (10.43% vs 9.42 in Indonesia), 19.7% of the elderly population,

which accounts for 3.6 million people, suffer from osteoporosis. In 2005, there were 18 million elderly individuals in Indonesia, and this number is projected to increase to 33 million by 2020, with a life expectancy of 70 years. According to predictions, 30% of women who are 50 years old or older will have osteoporosis, while 37-54% are expected to have osteopenia, and 54% are at risk of experiencing issues related to fractures¹. It is projected that by the year 2050, Asia, including

Indonesia, would account for almost 50% of global osteoporotic fractures². According to Gallagher and Sai (2010)³, the incidence of osteoporosis is twice as commonly seen in females as it is in males.

Hormones including estrogen, testosterone, and parathyroid hormone (PTH) have a significant role in the creation and preservation of bones. During menopause in women and in older men, a drop in hormones causes a gradual decline in bone mass⁴. This phenomenon is observed in both types of individuals. It is common for women to experience menopause after the age of 40, which is defined by the cessation of menstruation and a decline in ovarian activity⁵. Menopause often occurs around the age of 40, and when women become older, estrogen levels drop, which raises the likelihood that we may develop osteoporosis^{5,6}.

Bones go through a process of ongoing production and repair while the remodeling process is taking place. During this process, estrogen plays a significant role by controlling the equilibrium between bone-forming cells (osteoblasts) and bone-destroying cells (osteoclasts)⁷. Additionally, estrogen has a function in the formation of bone. Activation of the transcription factor osterix (Osx) is necessary for osteoblast differentiation and maturation⁸. Bone formation is dependent on osteoblast differentiation and maturation⁹. Important proteins such as osteocalcin (Ocn), osteopontin (OPN), collagen type I (COL-I), and bone sialoprotein (BSP) are also altered by Osx¹⁰.

There are phytoestrogens present in *Marsilea crenata* C. Presl. as well¹¹. According to Putra and Laswati (2011)¹², the amounts of phytoestrogens that are present in these leaves are relatively high¹³. It has been demonstrated that the ethyl acetate fraction of *M. crenata* have antiosteoporosis activity, which in turn has been shown to enhance bone density in female osteoporotic mice¹⁴. This plant, belonging to the Marsileaceae group, is found in Laos, Kalimantan, Java, the Lesser Sunda Islands, New South Wales, Malaya, New Guinea, the Philippines, South Australia, Queensland, Thailand, the Northern Territory, Victoria, Vietnam, and Western Australia^{15,16}.

So, the purpose of this research is to determine the water fraction of *M. crenata* leaves could increase the expression of Osx and Ocn, which would indicate an increase in bone

production. hFOB 1.19 cells were given water fractions in varying dosages, with genistein serving as a positive control.

MATERIAL AND METHODS

Materials

The leaves of *M. crenata* were collected from Surabaya, East Java, Indonesia. The hFOB 1.19 cell line (CRL-11372) was purchased from ATCC, Virginia, USA. Genistein, paraformaldehyde (PFA), dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), and bovine serum albumin (BSA) were purchased from Sigma Aldrich, Missouri, USA. The anti-mice osteocalcin and anti-rabbit osterix antibodies were obtained from Abcam, Cambridge, UK. Penicillin-streptomycin antibiotics, and fetal bovine serum (FBS), G418, dulbecco's modified eagle's medium (DMEM), were supplied by Aretha, Bandung, Indonesia. Tween-80, paraformaldehyde, fluorescein isothiocyanate (FITC), rhodamine, and an anti-mouse and anti-rabbit secondary antibody were purchased from Universitas Brawijaya, Malang, Indonesia.

Method

Extraction, Fractionation, and Preparation of Water Fraction

Using ultrasonic-assisted extraction, 1.5 kg of *M. crenata* leaves was extracted with 96% ethanol solvent to get 60 g of extract¹⁷. Liquid-liquid extraction were used in the fractionation process. The extract was suspended in 700 ml of water and fractionated at a 1:1 ratio using n-hexane. After that, the resulting aqueous phase was separated and further fractionated using n-butanol and ethyl acetate. A Heidolph Hei-VAP ML/G3 rotary evaporator was then used to evaporate the water phase.

The samples were obtained by mixing 50 mg of water fraction with 0.5% Tween-80 in 0.5% DMSO (w/v), and prepared at doses of 62.5, 125, and 250 µg/L.

Cell Culture

Complete medium formulated from DMEM, penicillin and streptomycin 1%, G418, and FBS 10%, were used to cultivate hFOB 1.19 cells in a culture flask. The cells were then incubated for six days at 37°C in an incubator with 5% CO₂. Every 24 hours, the progress of the cells

was monitored, and the medium was changed out until it had 80–90% content. Following that, the cells were moved to a microplate with 24 wells.

Osx and Ocn Measurement

The hFOB 1.19 cells, which had reached confluence, were given a water fraction at doses of 62.5, 125, and 250 $\mu\text{g/L}$, as well as 2.5 $\mu\text{g/ml}$ genistein as a positive control. Following the cultivation of cells to 80% confluence in a 24-well microplate, the administration of 10 ng/ml TNF- α was performed. The cells were subjected to a 48-hour treatment with samples and genistein. After a subsequent washing with PBS, the cells were subjected to fixation using a 4% PFA solution, Triton X-100, BSA, and Osx and Ocn primary antibodies for immunochemistry. The cells were then incubated at 4 $^{\circ}\text{C}$ overnight. The cells then

received the secondary antibodies rhodamine and FITC, respectively. The samples were then subjected to analysis utilizing CLSM (Fluoview Olympus FV1000) at wavelengths of 488 nm and 543 nm. The immunofluorescence of the markers was analyzed using Olympus Fluoview Ver.4.2a software to determine the expression levels of Osx and Ocn¹⁸.

Data analysis

Following the acquisition of data through CLSM, the subsequent procedure involves quantification to derive numerical data. This quantification will be performed through one-way ANOVA and followed with post-hoc LSD analysis utilizing Statistical Product and Service Solutions (SPSS) statistical software, with a significance threshold set at $p < 0.05$.

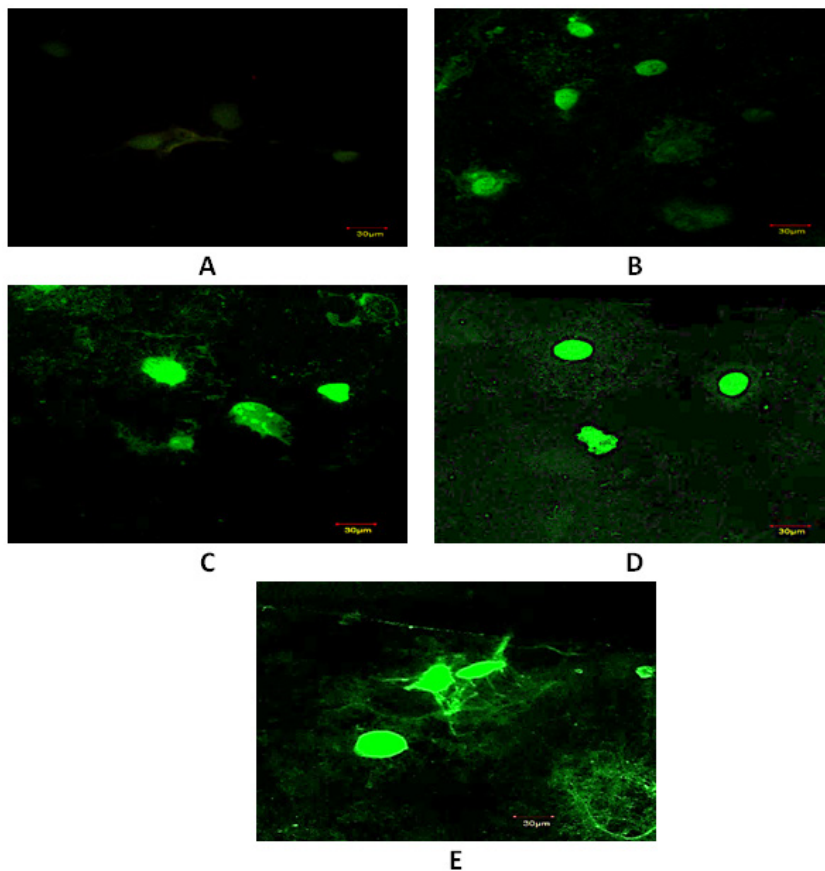


Fig. 1. Osx expression using FITC staining in a 488 nm wavelength. (A) Negative control, (B) dose 62.5 $\mu\text{g/ml}$, (C) dose 125 $\mu\text{g/ml}$, (D) dose 250 $\mu\text{g/ml}$, (E) genistein 2.5 $\mu\text{g/ml}$. Osx expression from groups B, C, D and E respectively showed an increase in green fluorescence intensity, while group A showed the lowest green fluorescence intensity

RESULTS AND DISCUSSION

A water fraction of 70 g was produced from the extraction process using 1.6 kg of *M. crenata* leaves powder using 96% ethanol solvent. After that, extract 96% ethanol from the leaves of *M. crenata* separated using liquid-liquid extraction and produced 27 g water fraction.

Figure 1 and Figure 2 show that after each dose was given, the amount of Osx immunofluorescence in hFOB 1.19 cells increased in all groups. The positive control group exhibited the highest intensity, followed by the treatment groups based on the dose level, while the negative control group displayed the lowest intensity. The 250 µg/ml dose group had the best results. They significantly increased Osx expression compared to the negative control ($p = 0.000$), but not significantly compared to the genistein 2.5 µg/ml dose group ($p = 0.125$). At the dose of 62.5 µg/ml, Osx expression was higher compared to the negative control ($p = 0.000$) and genistein 2.5 µg/ml ($p = 0.000$). It was also higher at the dose of 125 µg/ml compared to the negative control ($p = 0.000$) and genistein 2.5 µg/ml ($p = 0.000$).

A similar pattern was seen in Ocn observations, as depicted in Figure 3 and Figure 4, which show Ocn immunofluorescence in hFOB 1.19 cells in all groups. The positive control group showed the highest intensity, followed by the

treatment group based on the dose level, and the negative control group showed the lowest intensity. In terms of increasing Ocn expression, the 250 µg/ml dose group did the best. It did this significantly more than the negative control ($p = 0.000$) and also significantly more than genistein 2.5 µg/ml ($p = 0.000$). The increase in Ocn expression at a dose of 62.5 µg/ml was also significantly different from the negative control ($p = 0.000$) and genistein 2.5 µg/ml ($p = 0.000$), as well as at a dose of 125 µg/ml, which was significant compared to the negative control ($p = 0.000$) and genistein 2.5 µg/ml ($p = 0.000$).

The results showed that the water fraction from the leaves of *M. crenata* at a dose of 62.5; 125; and 250 µg/ml significantly and linearly increased the expression of Osx and Ocn in hFOB 1.19 cells compared with the negative control. Osx is a key transcription factor that plays an important role in the process of osteoblast cell differentiation, activating the SP7 promoter gene during the maturation of osteoblasts into mature osteoblasts and osteocytes. Inactivation of Osx can inhibit the expression of osteocalcin, bone sialoprotein, and osteopontin, thereby reducing the activity of forming new bone cells^{19,20}. The function of Osx is to regulate the production of bone formation proteins, including Ocn, which is the main non-collagen protein produced by mature osteoblast cells.

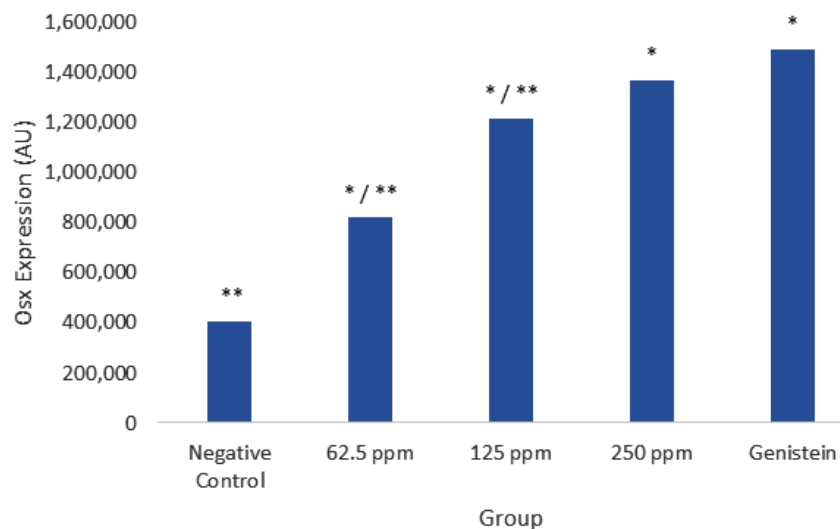


Fig. 2. Expression of Osx in hFOB 1.19 cells after administration of *M. crenata* leaves water fraction. The sign * is significantly different from the negative control, while ** is significantly different from genistein 2.5 µg/ml

The Ocn protein plays a crucial role in the bone mineralization process and maintaining calcium ion homeostasis. There are two forms of Ocn, the carboxylated form which has a high affinity for calcium and hydroxyapatite crystals, so they are in the bone matrix during the mineralization process with the help of α -carboxyglutamic acid (Gla) which helps absorption into hydroxyapatite, which is a crucial step in bone mineralization. Uncarboxylated osteocalcin has a low affinity for bone matrix and is likely to enter the bloodstream to reach other organs^{20,21}. The formation of the osteocalcin matrix plays an important role in increasing bone mineralization^{20,21,22}.

The administration of a water fraction derived from *M. crenata* leaves resulted in an

upregulation of Osx and Ocn expression. This observation serves as evidence supporting the involvement of this fraction in enhancing the process of bone growth. The observed phenomenon might perhaps be attributed to the regulatory mechanism of phytoestrogens present in the aqueous portion of *M. crenata* leaves, which has resemblance to the mode of action of estrogen. Phytoestrogens are botanical substances exhibiting a structural resemblance to 17 β -estradiol, hence enabling them to serve as substitutes for estrogen. Phytoestrogen substances encompass a variety of chemicals, including flavonoids like kaempferol and quercetin, isoflavonoids such as genistein and daidzein, as well as triterpenoids and steroids^{23,24,25}. Aditama *et al.*, 2021 also conducted similar

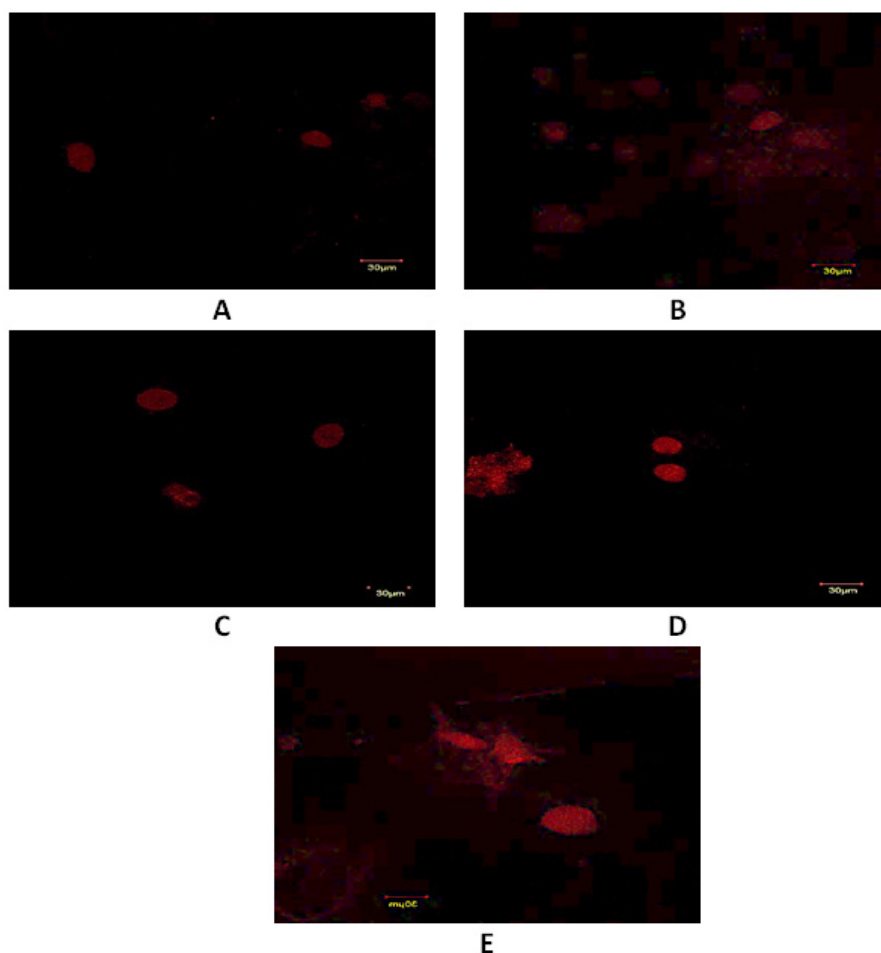


Fig. 3. Ocn expression using Rhodamine staining in a 543 nm wavelength. (A) Negative control, (B) dose 62.5 $\mu\text{g/ml}$, (C) dose 125 $\mu\text{g/ml}$, (D) dose 250 $\mu\text{g/ml}$, (E) genistein 2.5 $\mu\text{g/ml}$. Osx expression from groups B, C, D and E respectively showed an increase in red fluorescence intensity, while group A showed the lowest red fluorescence intensity

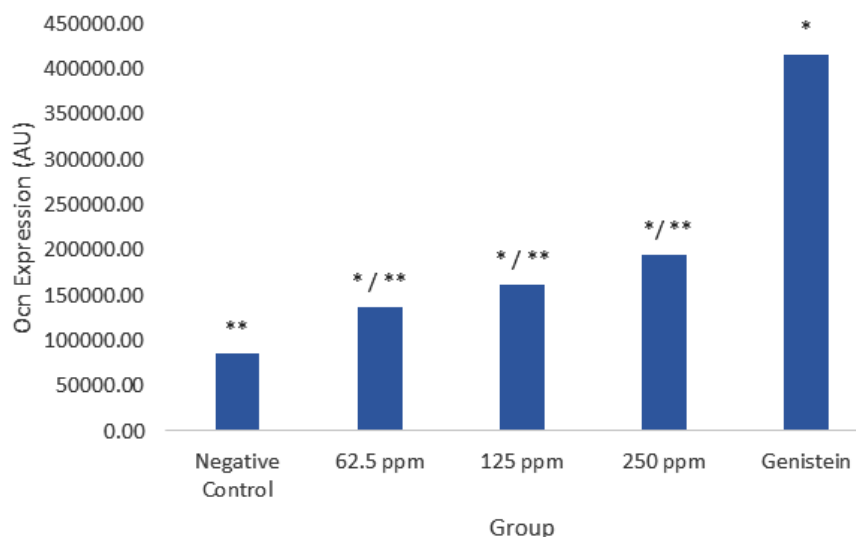


Fig. 4. Ocn expression in hFOB 1.19 cells after administration of *M. crenata* leaves water fraction. The sign * is significantly different from the negative control, while ** is significantly different from genistein 2.5 µg/ml

research. The ethyl acetate fraction of *M. crenata* leaves can increase osterix with an optimal dose of 250 ppm²⁶, and 96% ethanol extract of *M. crenata* leaves can increase osteocalcin expression with an optimal dose of 125 ppm²⁷.

CONCLUSION

The administration of water fractions derived from *M. crenata* leaves has the ability to enhance the expression of Osx and Ocn in hFOB 1.19 cells, the best dose that can increase Osx and Ocn is 250 µg/ml. This suggests that it has the potential to serve as a natural source for promoting differentiation and maturation of osteoblast through the upregulation of the Osx and Ocn genes.

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Conflict of Interest

The authors declare there is no conflict of interest.

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