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Original article

Effects of hemp (*Cannabis sativa* L. subsp. sativa) seed oil on cell viability and alkaline phosphatase activity in human osteoblast cells

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Abstract

***Backgrounds**:* Osteoporosis is the most common metabolic bone disease in the elderly, especially postmenopausal women. The disease is caused by the imbalance of bone remodeling, characterized by a decrease in number and impaired functionality of osteoblasts. Hemp seed oil contains high content of polyunsaturated fatty acids and bioactive phytochemicals including cannabidiol (CBD) and polyphenols. Hemp seed oil exhibits a wide range of pharmacological properties including antioxidants and anti-inflammatory.

***Objectives**:* This study examines the effect of hemp seed oil on cell viability and osteoblast differentiation by detecting alkaline phosphatase activity in the human fetal osteoblastic cell line (hFOB1.19).

***Methods**:* The cell viability on hFOB1.19 was tested by CCK-8 assay for 24 and 48 hours at 0.25-64 µg/ml concentration. Osteoblast differentiation was investigated after treatment with hemp seed oil for 5 and 7 days by both ALP enzymatic activity assay and ALP stanining using BCIP/NBT method.

***Results**:* Hemp seed oil had no cytotoxic effect with concentrations ranging from 0.5-8 µg/ml for 48 hours. The cell viability significantly increased with maximally at a concentration of 4 µg/ml at 48 hours. Following a treatment period of 5 and 7 days, hemp seed oil significantly increased in enzymatic ALP activity, with maximally at concentration of 8 µg/ml for 5 days. Hemp seed oil also increased blue purple color of positive ALP staining for 5 days.

***Conclusion**:* Hemp seed oil promotes cell viability and osteoblast differentiation by increasing the level of alkaline phosphatase. Therefore, it might be used for promoting bone formation and prevention of osteoporosis.

Keywords: Alkaline phosphatase, hemp seed oil, osteoblast, osteoblast differentiation, osteoporosis.

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Thailand has officially entered an aging society, with a continuously increasing elderly population. Naturally, as individuals age, their bodies undergo degenerative changes, which serve as risk factors for various diseases. These conditions may lead to chronic illnesses, disabilities, or even death. Osteoporosis is one such disease closely associated with aging, commonly found in elderly individuals of both genders. However, postmenopausal women are at the highest risk of developing osteoporosis. This condition has become a significant public health concern in the country. Given the rapid rise in the global and Thai elderly population, the incidence of osteoporosis and related fractures is also expected to increase. However, by implementing preventive measures before reaching old age, the likelihood of developing osteoporosis can be significantly reduced.

Osteoporosis is caused by an imbalance between osteoblasts and osteoclasts. It also results in a decrease of the density of bone minerals, making bones fragile and increasingly at risk for fractures. [1] Osteoporosis is a common disease among the elderly, affecting both men and women. However, it is significantly more prevalent in women than in men, with a ratio of 4 : 1. Bone loss occurs incrementally as a result of decreased osteoblast activity, resulting in decreases each year in bone mass in women. This process accelerates rapidly after menopause due to a decline in estrogen levels, which increases bone resorption and contributes to faster bone loss compared to men. [2] The causes of osteoporosis can be categorized into modifiable and non-modifiable factors. Non-modifiable factors include aging, as the risk of osteoporosis increases with age, with women being at a higher risk than men, particularly due to the decline in estrogen levels after menopause. Modifiable factors, on the other hand, are associated with lifestyle choices, such as smoking, excessive alcohol consumption, inadequate or improper physical activity, insufficient calcium and vitamin D intake, consumption of caffeine-containing beverages and carbonated drinks, and the use of certain medications that affect bone metabolism. [3] Hormone replacement therapy (HRT) is commonly used in the treatment of osteoporosis; however, long-term use has been associated with adverse effects, including an increased risk of breast cancer, ovarian cancer, and endometrial cancer. As a result, there is a growing focus on osteoporosis prevention through lifestyle modifications, particularly dietary adjustments. Consuming a diet rich in vegetables and fruits that contain polyphenolic compounds has been reported to promote bone formation and may contribute to osteoporosis prevention. [4] Therefore, natural products with bioactive properties can enhance bone function and support osteoporosis prevention.

Hemp (*Cannabis sativa L. subsp. sativa*), which belongs to the *Cannabis sativa* species. Hemp contains cannabidiol (CBD), a compound known for its relaxing effects, and tetrahydrocannabinol (THC), which effects the neurological system and induces intoxication. However, hemp has a low THC concentration, therefore it doesn't induce psychoactive effects or formulate intoxication in humans. In addition, hemp seed oil is rich in essential omega-3, omega-6, and omega-9 fatty acids and also polyphenols and cannabidiol (CBD). Hemp seed oil contains bioactive substances with pharmacological activities including natural antioxidant, anti-inflammation, immunomodulatory effect and cardiovascular protection. [5] However, the effects of hemp seed oil on human osteoblast differentiation are yet to be elucidated. This study aims to examine the biological effects of hemp seed oil on stimulating the differentiation of osteoblast by detecting alkaline phosphatase level both ALP enzymatic activity and intracellular ALP staining in human osteoblasts.

Materials and methods

Preparation of hemp seed oil

Hemp seed oil was extracted from screw press oil extraction. The extract was prepared by Professor Dr. Kornkanok Ingkaninan, Department of Pharmaceutical Chemistry and Phamacognosy, Faculty of Pharmaceutical Sciences, Naresuan University. The extract was dissolved with 100.0% DMSO and filtered with 0.2 μm and stored at -20°C.

Culture of human fetal osteoblast cell line

The human fetal osteoblastic cell line (hFOB 1.19) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in a medium containing a 1:1 DMEM / Ham's F-12, $NaHCO_3$, 0.3 mg/ml of G418 and supplemented with 10.0% of fetal bovine serum (FBS) (Capricorn Scientific, USA) in a humidified 5.0% CO_2 atmosphere at a permissive temperature of 37°C. The cell growth was followed over time.

Cell viability

The hFOB 1.19 cells were seeded in a 96-well plate containing 10% FBS medium and incubated for 24 hours. After that, cells were washed with phosphate-buffered saline (PBS). Then, cells were treated with different concentrations of hemp seed oil and incubated in culture media containing 0.5% FBS for 24 and 48 hours, respectively. Subsequently, the cell viability was detected by adding of the CCK-8 (Cell Counting Kit-8) solution to each well and incubated for 1 hr at 37°C. The result of the dissolved orange-yellow formazan was measured by quantitative determination of absorbance at 450 nm using a microplate spectrophotometer. The percentage of cell viability was calculated in comparison with the untreated control group (100.0%).

Alkaline phosphatase activity

The hFOB 1.19 cells were seeded in 6-well plates with 10.0% FBS medium and incubated for 24 hours. Then, cells were treated with different concentrations of hemp seed oil and incubated in culture media containing 0.5% FBS for 5 and 7 days. The medium was changed every 4 days. After washing with PBS, cells were harvested, homogenized in a lysis buffer (50 mM Tris-HCl pH.7.5, 0.1 mM PMSF) and sonicated to break the cells. The cell lysate was then centrifuged, and the supernatant was collected for alkaline phosphatase (ALP) activity assay. ALP activity was performed by incubating the crude cell lysate in phosphatase buffer pH 10.3 containing *P*-nitrophenyl phosphate substrate at 37 °C for 30 minutes. The reaction was terminated by adding with NaOH and the amount of *p*-nitrophenol product (*p*-NP) was measured at 405 nm. The total cellular protein concentration was determined by Bradford protein assay. The percentage of ALP activity was calculated compared with the untreated control (100.0%).

Alkaline phosphatase staining

Cells were seeded in 24-well plates with 10.0% FBS complete medium and incubated for 24 hours. Subsequently, the cells were treated with varying concentrations of hemp seed oil and incubated for 5 and 7 days with culture media containing 0.5% FBS. The medium was changed every 4 days. After washing with PBS, cells were fixed with neutral buffered formalin (pH 6.8) at room temperature. Cells were rinsed with PBS and incubated for 1 hour at 37°C in dark condition with BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) substrate solution. The color development after ALP staining was observed and photographed using an inverted microscope.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) of at least three experiments. One-way analysis of variance (ANOVA) followed by Tukey's tests was performed in SPSS software version 27 to compare the different dose groups, where $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant.

Results

Cell viability on hFOB1.19 by CCK-8 assay

The viability of cells treated with hemp seed oil was tested by CCK-8 assay for 24 and 48 hours at 0.25-64 µg/ml concentration compared with the untreated control. In principle, water-soluble tetrazolium salt (WST-8) is reduced by dehydrogenase activities in cells to give a orange yellow-color formazan dye. The result showed that hemp seed oil had no cytotoxic effect with concentrations ranging from 0.25 - 64 µg/ml for 48 hours compared with the untreated group. The cell viability significantly increased with maximally at a concentration of 4 µg/ml hemp seed oil after treatment for 48 hours **(Figure 1).** The concentrations at 0.5-8 µg/ml of hemp seed oil were selected for ALP activity assay.

Alkaline phosphatase activity

Alkaline phosphatase is an initial biochemical marker of osteoblast differentiation. This enzyme catalyzes the *p*- nitrophenyl phosphate substrate into a dephosphorylated product, *p*-nitrophenol under alkaline condition. As shown in **Figure 2**, the results showed that hemp seed oil significantly increased the ALP activity of hFOB 1.19 cells with a maximum of 142.5% at 8 µg/ml on day 5 ($P < 0.001$) compared with the control group (100.0%). Additionally, hemp seed oil significantly increased the ALP activity at concentration of 2-8 µg/ml on day 7 ($P < 0.01$).

Alkaline phosphatase staining

Alkaline phosphatase (AP) staining is a convenient method for qualitative analysis the differentiation of osteoblasts by staining for alkaline phosphatase activity using BCIP/NBT substrate solution. As shown in **Figure 3**, the results found that hemp seed oil sequentially increased the blue-purple color of positive ALP staining after treatment with 0.5 to 8 mg/ml for 5 and 7 days. The highest color intensity appeared after treatment for 5 days compared with untreated control. Thus, our results suggest that hemp seed oil promotes osteoblast differentiation of hFOB 1.19 cells.

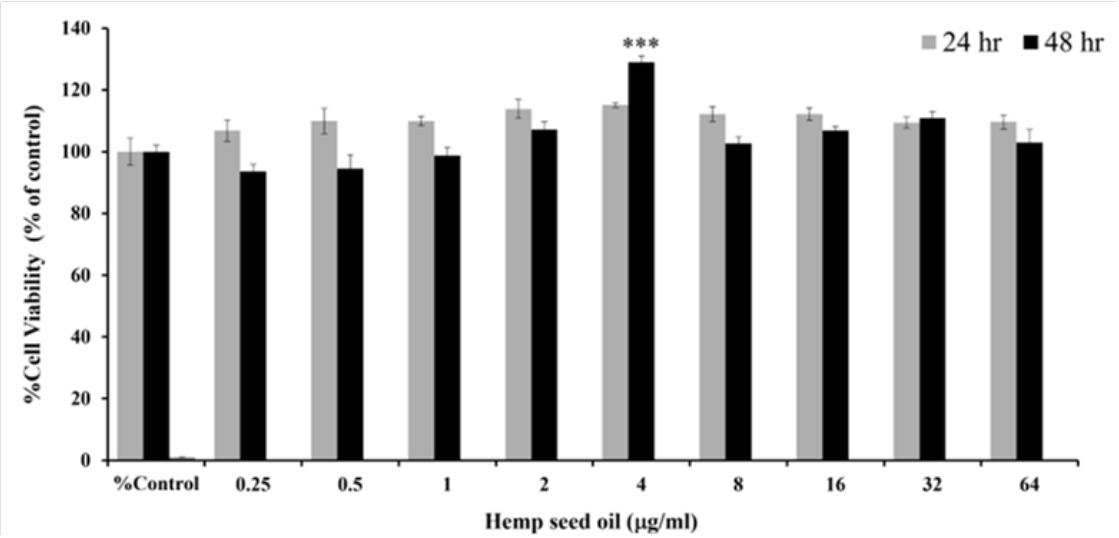

Figure 1. Effect of hemp seed oil on cell viability of hFOB 1.19 at a concentration of 0.25 to 64 mg/ml after treatment for 24 and 48 hours. Data represent means ± standard deviation compared with the untreated group (***$P < 0.001$), (n = 3).

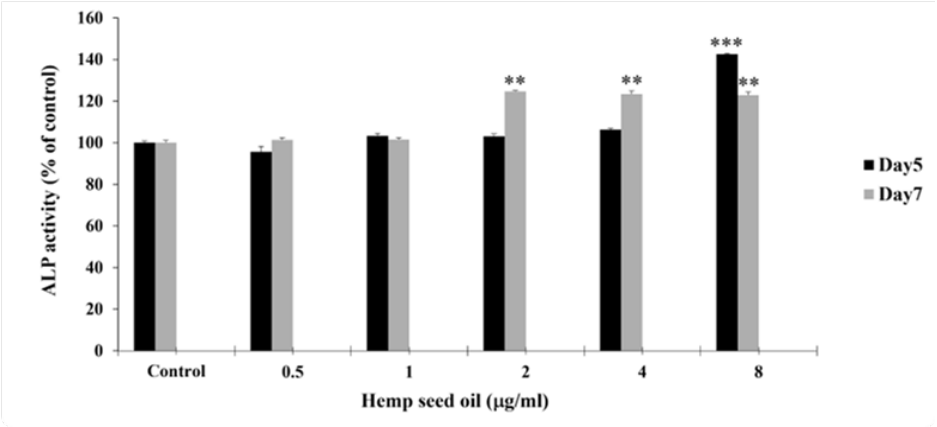

Figure 2. Effect of hemp seed oil on ALP activity of hFOB 1.19 after treatment at 0.5 to 8 µg/ml for 5 and 7 days Data represent mean ± standard deviation, compared with the untreated group (**$P < 0.01$, ***$P < 0.001$), (n = 3).

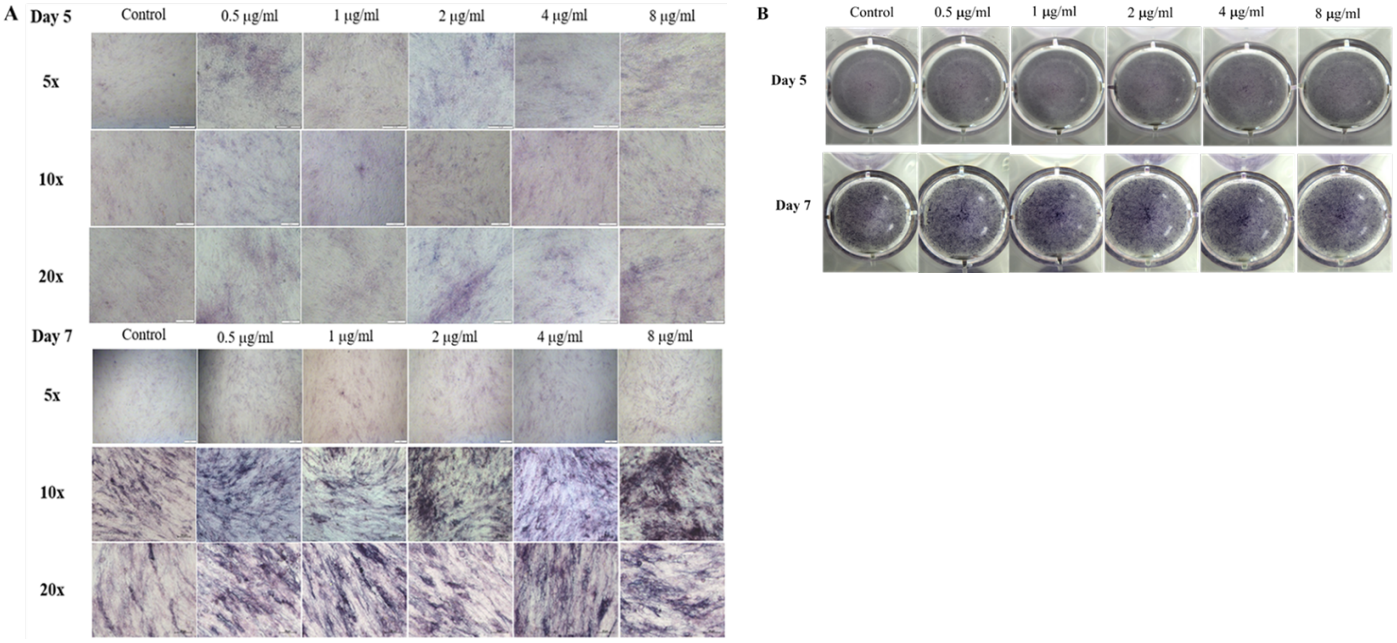

Figure 3. Effect of hemp seed oil on ALP staining of hFOB 1.19 after treatment at 0.5 to 8 mg/ml for 5 and 7 days. **(A)** Inverted microscope; **(B)** Top view.

Discussion

Osteoporosis involves an imbalance in the bone remodeling process due to the decreased activity of osteoblasts. Natural products are currently alternative sources for bone-targeting agents to promote osteoblast-mediated bone formation. In the present study, hemp seed oil actions on human osteoblast cell line, hFOB1.19, were investigated the cell viability and its ability to stimulate the osteogenic differentiation by alkaline phosphatase detection. Our results indicated that hemp seed oil enhances cell viability and stimulates the proliferation phase of human osteoblast hFOB 1.19 cells, with the highest CCK-8 assay activity observed at 48 hours of treatment. Alkaline phosphatase is a key enzyme indicative of osteogenic differentiation into mature osteoblasts. This study found that hemp seed oil stimulates the activity of the enzyme alkaline phosphatase both quantitatively (ALP activity) and qualitatively (ALP staining) , as evidenced by an increased blue – purple color staining within the cells. The highest stimulation was observed at 5 days of treatment. Therefore, our results suggest that hemp seed oil promotes the early stage of human osteoblast differentiation. These reasons might be the bioactive contents in hemp seed oil which contains a rich source of beneficial omega-3 and omega-6 polyunsaturated fatty acids (PUFAs). Accumulating studies have indicated that PUFAs could play an important role in maintaining bone health. It has been reported that omega-3 PUFAs could raise the level of superoxide dismutase (SOD) to reduce reactive oxygen species (ROS), thus suppressing oxidative stress in osteoblasts leading to inhibit osteoporosis. [6] Cannabidiol has been found to be present in hemp seed oil as well. CBD may interact with endocannabinoid receptors CB1, CB2, and TRPV1 are located on osteoblast cell membranes. [7] CB2 receptors a crucial role in bone metabolism and may promote osteoblast activity. Studies suggest that CB2 angonists can enhance bone mass and reduce the risk of osteoporosis. [7] CBD has been shown to stimulate osteoblast activity and suppress osteoclast differentiation in vitro, highlighting its potential utility in the treatment of bone-related disorders such as osteoporosis. [7] The limitation of this study was conducted only under in vitro conditions, which may not completely the intraction in organisms. Therefore, the reported results require more confirmation via in vivo model to confirm their biological affect osteoblasts.

Conclusion

Hemp seed oil can stimulate both cell viability and early differentiation in human osteoblastic cells. Therefore, this study may serve as preliminary data and a basis for further investigation into its molecular mechanism. Additionally, the findings could contribute to the development of alternative health-promoting

products for the prevention or treatment of osteoporosis in the elderly.

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Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for academic or non-commercial purposes from the corresponding author on reasonable request.

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