Effects of *Sesbania javanica* Flower Extract on Cell Proliferation and Differentiation of Human Osteoblast Cell Line

ฤทธิ์ของสารสกัดดอกโสนต่อการเพิ่มจำนวนและการเจริญพัฒนาของเซลล์สร้างกระดูกมนุษย์

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ABSTRACT

Sesbania javanica Miq. or Sano flower contains large quantities of carotenoids including β-carotene, lutein, β-cryptoxanthin and zeaxanthin. The purpose of this study was to investigate the effects of *Sesbania javanica* extract on cell proliferation and differentiation of a human osteoblast cell line (hFOB1.19). *Sesbania javanica* flower extract had no cytotoxic effect and also significantly increased the cell proliferation at concentrations of 10 to 320 ng/ml for 48 hours. Positive BCIP/NBT staining, the alkaline phosphatase activity and the *ALP* mRNA expression using quantitative real-time PCR were significantly increased at concentration of 40-160 ng/ml in a dose-dependent manner ($p<0.05$). In conclusion, *Sesbania javanica* flower extract promotes the cell proliferation and differentiation of human osteoblasts.

บทคัดย่อ

ดอกโสน มีสารกลุ่มแคโรทีนอยด์สูง ได้แก่ เบต้า-แคโรทีน ลูทีน เบต้า-คริปโตแซนทีน และซีแซนทีน การศึกษานี้มีวัตถุประสงค์เพื่อทดสอบฤทธิ์ของสารสกัดดอกโสนต่อการเพิ่มจำนวนและการเจริญพัฒนาของเซลล์สร้างกระดูกของมนุษย์ชนิด hFOB1.19 ผลการทดลองพบว่า สารสกัดดอกโสนไม่มีความเป็นพิษต่อเซลล์ และสามารถกระตุ้นการเพิ่มจำนวนของเซลล์ที่ความเข้มข้น 10-320 ng/ml เป็นเวลา 48 ชั่วโมง ผลการติดตามการทำงานของเอนไซม์อัลคาไลน์ ฟอสฟาเตส ด้วยการย้อมสี BCIP/NBT กิจกรรมของเอนไซม์อัลคาไลน์ ฟอสฟาเตส และการแสดงออกของยีน *ALP* ด้วยเทคนิค quantitative real-time PCR พบว่า สารสกัดดอกโสนสามารถเพิ่มระดับการทำงานของเอนไซม์เอนไซม์อัลคาไลน์ ฟอสฟาเตสได้ ที่ความเข้มข้น 40-160 ng/ml อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) จึงสรุปได้ว่า สารสกัดดอกโสนส่งเสริมการเพิ่มจำนวนของเซลล์และการเจริญพัฒนาของเซลล์สร้างกระดูกของมนุษย์

Introduction

Nowadays, Thailand has changed the structure of population and become the ageing society. It was found that the number of reproductive age decreased (Weisenthal et al., 2018), whereas the number of the elderly population increased rapidly. The physical deterioration of the elderly age causes health problems and various chronic illnesses. Osteoporosis is a major public health problem in Thailand and over the world (Angwarawong et al., 2019). It is a degenerative skeletal disorder that is commonly found in the elderly people especially postmenopausal women due to the estrogen deficiency (Ji, Yu, 2015). The disease is characterized by low bone mass and microarchitectural deterioration of bone tissue that results in compromised bone strength and increased risk of fractures (NIH, 2001). The disease is caused by a change in the balance between activities of osteoblasts and osteoclasts, which the osteoblast number and function is decreased but the osteoclast function is increased (Feng, McDonald, 2011). Hormone replacement drugs are currently used in the treatment of osteoporosis. But it has the side effects after a long period of treatment, including breast cancer, ovarian cancer, and endometrial cancer.

Sesbania javanica Miq. or Sano flower is an edible vegetable flower which is commonly found in the central region of Thailand. It has also been used for its diverse medicinal properties. In ancient Thai traditional medicine, it was used as an anti-inflammatory for insect bites, detoxicification, intestinal abscess healing, stomach discomfort and to relieve internal fever and thirst. (Tangvarasittichai et al., 2005). The flower of *S. javanica* Miq. is nutritious with of carbohydrates, fiber, protein, calcium, phosphorus, iron, vitamin A, vitamin B1, vitamin B2, vitamin C, and high in carotenoids. The major carotenoids composition in Sano is β-carotene follows by lutein, β-cryptoxanthin, and zeaxanthin (Kijparkorn et al., 2010). β-carotene is a group of orange-yellow pigments in the carotenoid group, a precursor to vitamin A, and has antioxidant properties. Normally, the human body can convert beta-carotene to vitamin A which has the main function of vision in reducing the risk of cataract, and preventing skin from the harmful effects of ultraviolet rays. It also reduce the risk of cancer, slow down aging, and stimulate immune cells in the body. Previous reports showed that β-carotene suppressed both osteoclastogenesis and bone resorption (Wang et al., 2017). However, there is a little information available about the effect of *S. javanica* Miq. flower extract on the human osteoblast development *in vitro*.

Objectives of the study

The aim of this study was to investigate the effects of *S. javanica* Miq. flower extract on cell viability and osteoblast differentiation process by detecting the alkaline phosphatase activity and studying the gene expression of *ALP* using quantitative real-time PCR in human fetal osteoblast cell line, hFOB1.19.

Methodology

Preparation of *S. javanica* flower extract

The flower of *S. javanica* was extracted from 95% ethanol and evaporated by a rotary evaporator. The extract was prepared by Assistant Professor Dr. Nungruthai Suprom. Department of Chemistry, Faculty of Science, Naresuan University. The extract was dissolved with 100% DMSO and filtered with 0.2 μm (syringe filter, sartorius) and stored at -20 ℃.

Cell Culture

Human fetal osteoblast cell line 1.19 (hFOB1.19) was purchased from American Type Culture Collection (ATCC, CRL No.11372) and was cultured in medium of 1:1 DMEM/Ham's F-12, 10% FCS, 1xPenStrep, 14.3mM $NaHCO_3$, 0.3 mg/ml G418 (completed medium). The culture was incubated at $37^{o}C$ with 5% CO_2 for 3-5 days until the approximately 80% confluence. Cells were subcultured by adding 0.05% trypsin-EDTA (CAPRICORN) to remove the cells from the flask prior to cell counting using hemocytometer and trypan blue reagent for measuring the viable cells using an inverted microscope.

Cell viability assay

The cell viability and proliferation effects after treatment with various concentrations of *S. javanica* flower extract was examined by PrestoBlue™ cell viability method using hFOB 1.19 cells as a cell model. The concentration of *S. javanica* flower extract at 10 to 320 ng/ml and without treatment were used for testing for 24-96 hours. Cells at a density of $5x10^3$ cells/well were seeded in 96-well black plates in completed media at 37℃ for 24 h. After that, the cells were washed with PBS and treated with various concentrations of *S. javanica* flower extract in 1:1 DMEM/Ham's F-12, 0.5% FBS, 0.3 mg/ml G418 and 14.3 mM $NaHCO_3$. The culture was incubated at $37^{o}C$, 5% CO_2 for 48 h. After treatment, the viable cells were determined by PrestoBlue™ cell viability reagent and further incubated for 30 min. The fluorescence signal was measured at excitation 530/25, emission 590/35 by using a microplate reader. The data was demonstrated as the percentage of cell viability compared with untreatment control.

Alkaline phosphatase (ALP) staining

The ALP activity was evaluated qualitatively and quantitatively during osteoblast differentiation. The qualitative analysis was detected intracellular ALP activity by ALP staining using BCIP/NBT method which used a convenient method to identify and monitor osteoblast cells by staining for alkaline phosphatase activity. The hFOB 1.19 cells ($3×10^4$ cells/well) was cultured in a 24-well plate at 37℃ for 24 h. Then, cells were treated with different concentrations of *S. javanica* flower extract in a media containing 0.5% FCS and incubated at $37^{o}C$ and 5% CO_2. After treatment for 5 and 7 days, the cells were fixed with 10% neutral buffered formalin (pH 6.8) and stained with BCIP/NBT substrate solution. The positive ALP staining cells were observed with a blue-violet precipitate and photographed by an inverted microscope.

Detection of ALP activity

The hFOB 1.19 cells were seeded into a 6-well plate of $1.2×10^5$ cells/well in the completed medium. The cells were then treated with *S. javanica* flower extract at different concentrations in a media containing

0.5% FCS and incubated for 5 and 7 days. Cells were collected using a cell scraper and added with lysis buffer (50mM Glycine-HCl, pH 7.5, containing 0.1 mM PMSF). The cells were lysed by using a sonicator and centrifuged at 11,000 rpm for 15 minutes at 4°C. The supernatant was collected and mixed with ALP substrate solution which consisted of p-nitrophenyl phosphate (pNPP) at pH 10.4. The ALP enzyme activity was performed at 37°C for 30 minutes and added with 0.1 NaOH to stop the reaction. The color development was measured at 405 nm using a microplate reader. The ALP specific activity was calculated by a comparison with p-nitrophenol standard curve and the protein was determined by Bradford assay.

Analysis of mRNA expression

The hFOB 1.19 cells were seeded into 6-well plate with 1.5×10^5 cells/well in a complete medium for 48 hours. Cells were treated with various concentrations of *S. javanica* flower extract in a media containing 0.5% FCS for 4 days. After treatment, cells were washed with PBS, scraped and centrifuged to collect the cells. Total RNA was extracted using the RNeasy mini kit (QIAGEN) and the genomic DNA contamination was digested using the DNase I enzyme. The concentration and purity of RNA were determined by measurement at 260 and 280 nm using a NanoDrop spectrophotometer (Thermo scientific). The complementary DNA was generated by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) by using oligo dT_{18} as a primer. A PCR reaction consisted of 1x qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems), 400nM of each PCR primers, 30 ng cDNA, and PCR grade water. The sequences of PCR primers for human genes were listed in Table 1. Real time PCR was performed using MyGo Pro Real-time PCR (iScience Technology) using an initial activation step at 95°C for 120 sec and followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec, and extension at 72°C for 20 sec. Melting curve analysis was used to validate the product. The relative mRNA expression of each gene was normalized to the level of Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The relative quantification of $2^{-\Delta\Delta Cq}$ method was used to calculate the fold change of target gene expression compared with treatment groups and untreatment control.

Table 1 Primer sequences used in Real-time PCR technique

Gene	Primer sequences	Product size (bp)
Alkaline phosphatase (*ALP*)	F: 5′ CATGGCTTGGGCAGAAGGA 3′ R: 5′ CTAGCCCCAAAAAGAGTTGCAA 3′	166
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	F: 5′ GAAGGTGAAGGTCGGAGTC 3′ R: 5′ GAAGATGGTGATGGGATTTC 3′	226

Statistical analysis

Data were expressed as the mean ± standard deviation. The differences between the mean values were evaluated by one-way ANOVA followed by Tukey's HSD test, when *p<0.05, **p<0.01), and ***p<0.005.

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Results

Effect of *S. javanica* flower extract on cell viability

The treatment of hFOB 1.19 cells with *S. javanica* flower extract at concentration of 10 to 320 ng/ml for 48 hours significantly increased the cell proliferation at approximated 120% ($p<0.005$), compared with 100% untreated control (Figure 1). In addition, no cytotoxic effect was observed.
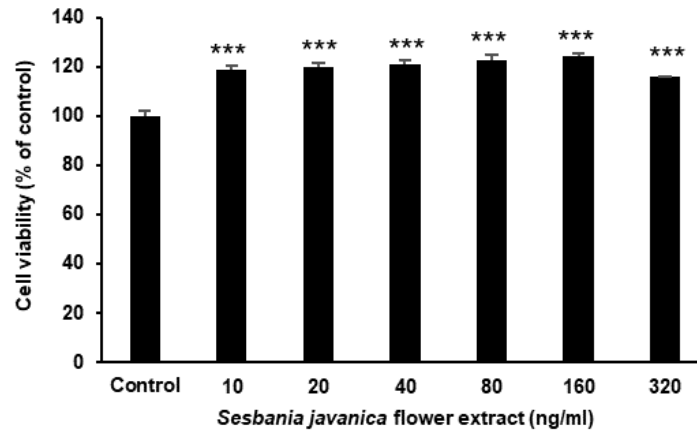

Figure 1 Effect of *S. javanica* flower extract on cell viability of hFOB 1.19. Cells were treated with the extract at concentrations of 10 to 320 ng/ml for 48 hours compared to untreated control (n=3), (***$p<0.005$).

Effect of *S. javanica* flower extract on cell differentiation

The effect of *S. javanica* flower extract on the process of osteoblast differentiation was observed the ALP activity both cell staining by BCIP/NBT method and determining the level of the alkaline phosphatase activity. For ALP staining, cells were treated with the *S. javanica* flower extract at concentration of 20 to 160 ng/ml for 5 and 7 days. Figure 2, showed that the blue-purple color of positive ALP staining sequentially increased after treatment with 20 to 160 ng/ml of *S. javanica* flower extract and appeared the highest color intensity at day 7 after treatment. In addition, *S. javanica* flower extract with concentration of 40-160 ng/ml on days 5 and 7 was able to stimulate the alkaline phosphatase activity in hFOB 1.19 cells with statistical significance ($p<0.05$) (Figure 3). The ALP activity of human osteoblasts after treatment for 7 days showed the highest level and significantly increased with 194.5%, 252.1% and 263.9% at 40, 80 and 160 ng/ml concentration, respectively ($p<0.05$). This result indicated that *S. javanica* flower extract could stimulate the pre-osteoblast stage into the early stage of osteoblast differentiation.
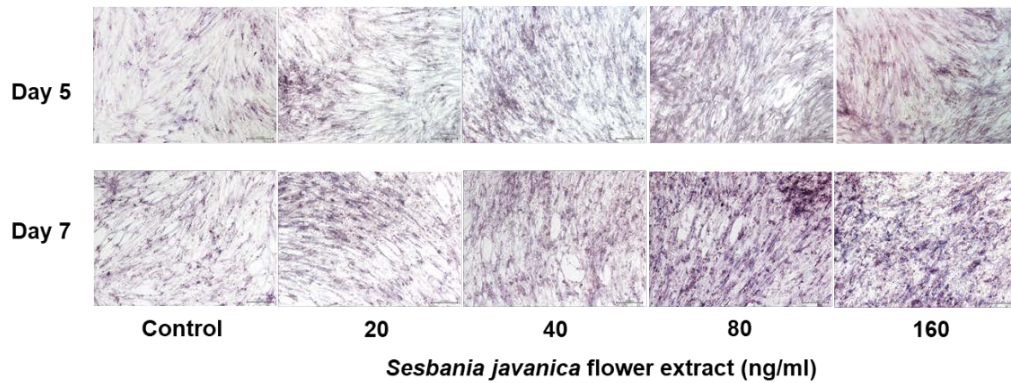
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Figure 2 Alkaline phosphatase staining of hFOB 1.19 cells after treatment with *S. javanica* flower extract using concentration of 20, 40, 80 and 160 ng/ml for 5 and 7 days. (Magnification = 20x, Bar = 100 µm.)
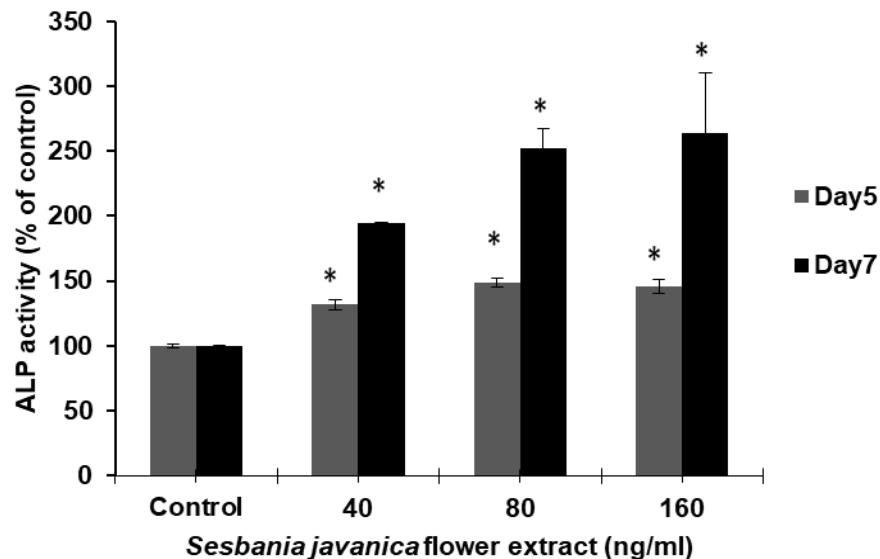

Figure 3 ALP activity of *S. javanica* flower extract on of hFOB1.19 cells on day 5 and 7 after treatment with 40, 80, 160 ng/ml concentration. Data represent mean±standard deviation (n=3), (*$p < 0.05$).

Effect of *S. javanica* flower extract on mRNA gene expression

The mRNA gene expression level after treatment was evaluated by using quantitative real-time PCR technique. *ALP*, an osteogenic marker gene, was chosen for determining the degree of osteoblast development. Cells were treated with *S. javanica* flower extract at a concentration of 40-160 ng/ml for 4 days, using GAPDH as a housekeeping gene. *S. javanica* flower extract significantly stimulated the mRNA expression level of *ALP* with 1.58, 1.60 and 2.34 fold-change difference at concentration 40, 80 and 160 ng/ml, respectively ($p < 0.005$), compared with untreated control, figure 4. This result indicated that *S. javanica* flower extract had its ability to stimulate the *ALP* gene expression of osteoblast differentiation process.
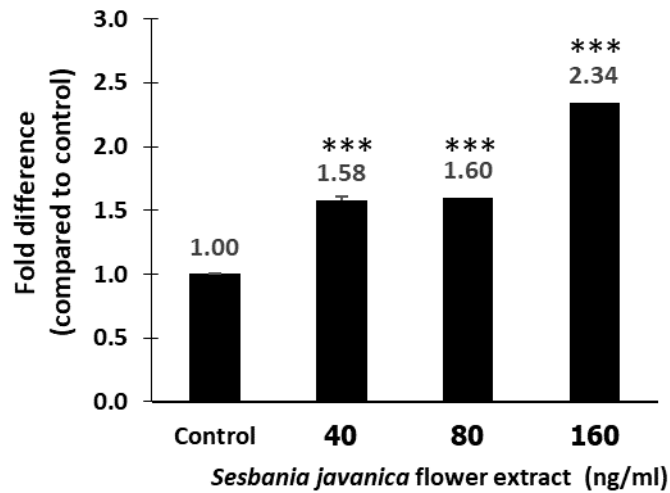
Figure 4 Effect of *S. javanica* flower extract on *ALP* mRNA expression in hFOB1.19 cells. Cells were treated with *S. javanica* flower extract at concentration of 40, 80, 160 ng/ml for 4 days. Data represent mean±standard deviation, compared to untreated control (***p< 0.005).

Discussion and Conclusions

In the present study, *S. javanica* flower extract actions on human osteoblast cell line, hFOB1.19, were investigated the cell viability and its ability to stimulate the osteogenic differentiation. Osteoblast function was measured by cell growth, alkaline phosphatase activity and *ALP* gene expression in a cellular level. The cell viability assay using PrestoBlue™ cell viability reagent was utilized for studying the cell viability and proliferation. This method is a sensitive assay which based on a quickly reduced by metabolically active cells into resazurin-based dye and detected using the fluorescence intensity. The data indicated that *S. javanica* flower extract could stimulate the osteoblast proliferation of hFOB1.1.9 cells during the early osteoblast proliferation phase.

Generally, alkaline phosphatase (ALP) is an important biochemical enzyme and is considered to be an early marker of osteoblast differentiation for bone formation. As ALP is observed on the cell surface and in matrix vesicles (Golub, Boesze-Battaglia, 2007) and is a byproduct of osteoblast activity, increased level of ALP refers to active bone formation. Here in, ALP-positive human osteoblast cells could be identified by blue/purple staining with the intense intensity on day 7 after incubation with *S. javanica* flower extract, compared with untreated control. In parallel, the ALP enzyme activity assay was determined by the quantitative analysis using *p*-nitrophenyl phosphate as substrate. About 2.5-fold higher ALP activity was observed in the hFOB cells exposed to *S. javanica* flower extract at 160 ng/ml concentration compared to the control group. Additionally, the ALP activity increased in a dose-dependent manner with highest ALP activity at 7 days of incubation. The results of the monitoring of alkaline phosphatase enzyme activity were consistent in both staining and activity base assay in hFOB1.19 cells, with the same concentration and maximal ALP activity at day 7. This data indicated that

S. javanica flower extract could stimulate the early stage of osteoblast differentiation of human osteoblast cells. In addition, the quantitative real-time PCR of *ALP* mRNA expression level was about 2.3-fold higher than the hFOB1.19 cells exposed to *S. javanica* flower extract compared to those in the control group. This data was correlated to those of ALP enzyme activity level.

In conclusion, *S. javanica* flower extract could stimulate the cell proliferation, alkaline phosphatase activity levels and *ALP* mRNA gene expression in human osteoblast cells (hFOB1.19). The findings of the present study supported our hypothesis that *S. javanica* flower extract increases the osteogenic effect in human osteoblast cells and these stimulated osteogenic effects are mediated by increasing osteoblast proliferation and differentiation. This suggested that *S. javanica* flower extract could be used for promoting the bone health and delay the degeneration of osteoporosis in the elderly.

Acknowledgements

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