Effect of *Aquilaria crassna* crude extract on osteogenic activity of MC3T3-E1 cells

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Abstract

The present study aimed to investigate the effect of the *Aquilaria crassna* extract (AE) on osteogenic activity including cell viability, cell proliferation, cell attachment and osteogenic differentiation of osteoblast like cells (MC3T3-E1). These were evaluated in term of cell viability, cell proliferation and cell attachment by MTT assays. While the methods of alkaline phosphatase (ALP) staining and activity kits, quantitative real-time PCR of osteogenic gene expression and Alizarin Red-S staining were performed to evaluate the effect of the AE on osteogenic differentiation. The results showed that the concentration of AE at 10, 25 and 50 µg/ml had no cytotoxicity. The AE (50 µg/ml) effectively enhanced cell proliferation at 24 h and increased cell attachment at 4 h and 24 h in MC3T3-E1 cells. The AE (50 µg/ml) effectively promoted osteogenic differentiation of MC3T3-E1 cells by increasing an ALP activity, an expression of osteogenic gene markers (collagen type I, ALP, bone sialoprotein and osteocalcin) and a mineral deposition. In conclusion, the data presented in this study showed a potential of *Aquilaria crassna* extract to improve initial cell attachment and proliferation, and to stimulate osteogenic differentiation in MC3T3-E1 cells.

Clinical article (J Int Dent Med Res 2018; 11(2): pp. 510-519)

Keywords: *Aquilaria,* extract, osteogenic, osteoblast.

Received date: 12 March 2018 **Accept date:** 02 April 2018

Introduction

Currently, several regenerative procedures had been introduced to reconstitute alveolar bone loss such as guided tissue regeneration, bone replacement grafts and growth factors. However, there is still no ideal therapeutic approach to achieve predictable and optimal bone regeneration.[1,2] The one current method, using anti-resorptive agents inhibit osteoclast activity such as bisphosphonates.[3] Since the anti-resorptive agents still have some the adverse effects for example osteonecrosis of the jaw,[4] anabolic agents are considered as beneficial agents, which stimulate osteoblast activity and enhance bone formation. The current wildly anabolic agents, bone morphogenic proteins (BMPs) have been used in alveolar bone reconstruction or improving osseointegration of dental implant.[5,6] However, several studies reported that BMPs have some complications including severe gingival swelling and may associated with high cancer risk.[7, 8] Moreover, the clinical using of BMPs are still quite complex, costly and time consuming to produce.[9] Therefore, it is a great need to discover novel anabolic agents for bone regeneration.

Recently, natural plants used in traditional medicine have been accepted as one of the main sources of drug discovery and development. For traditional medicines, some natural plants have been used as an alternative drugs for bone diseases such as arthritis, gout and bone fracture. *Eurycomalongifolia* and *Labisiapumila* have been used as traditional medicines of Southeast Asian for bone fracture and osteoporosis treatment.[10] Some natural plant extract have been confirmed to have effect on osteogenic activity including *Rhizomadrynariae* and *Euodias-utchuenensis Dode* extract that enhanced the proliferation and osteoblast differentiation *in vitro* studies.[11,12] Thus, natural plant extract may be the good alternative choices

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of anabolic agents due to low adverse effects, obtainable, low cost and contain effective compounds.

Aquilaria crassna Pierre ex Lecomte or agarwood, the heartwood of tropical tree, belongs to the family *Thymelaeaceae* and class *Magnoliosida*. It can be found in many countries including Thailand, Indonesia and Malaysia. It has been used as traditional medicines for bone diseases including arthritis and gout.[13] Moreover, *Aquilaria crassna* extract was also reported other effects including anticancer, antioxidative and antibacterial activities.[14,15] However, there is still no published report describing the effect of the *Aquilaria crassna* extract on osteogenic activity until now. Therefore, this study was to investigate the effect of the *Aquilaria crassna* extract (AE) in various concentrations on cell viability, proliferation, morphology and attachment including osteogenic differentiation of osteoblast like cells (MC3T3-E1).

Material and Methods

Aquilaria crassna extraction

Aquilaria crassna Pierre ex Lecomte used in this experiment was obtained from Mr. Choosak Rerngrattanabhume, which originally cultivated in Thailand. Subsequently, it was identified by Dr. Pranee Nangngam, Department of Biology, Faculty of science, Naresuan University. The specimen voucher number 002540 was kept at Department of Biology Herbarium, Faculty of Science, Naresuan University. *Aquilaria crassna* was extracted with ethyl acetate. Briefly, the heartwood of *Aquilaria crassna* was sliced into small pieces and dried. Then, the dried *Aquilaria crassna* was extracted with ethyl acetate for two days. After that, the extract was concentrated with the rotary evaporator under reduced pressure.[16]

Cell culture

L929 cells, a mouse fibroblast-like cell line, were maintained in Dulbecco's Modified Eagle's Medium (DMEM). Whiles, MC3T3-E1 cells, a mouse osteoblast-like cell line, were maintained in alpha Minimal Essential Medium (alpha-MEM). The mediums were supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin and 5 µg/mL amphotericin B (Gibco, USA). The cells were maintained at 37 ^{o}C in humidified atmosphere of 5% CO_2.

Cell viability and cell proliferation

Cell viability was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbro-Mide (MTT) assay[17] (followed ISO 10993-5 *In vitro* cytotoxicity test protocol). L292 cells were seeded at density 50,000 cells/well on 24-well-plate that added AE for different concentration including 10, 25, 50, 100, 500 and 1,000 µg/mL and without AE as control group (n=3 for each sample). After cultured for 24 h, the cells were incubated were incubated with 0.5 mg/mL MTT solution (USB Corporation, USA) for 30 min at 37^{o}C and the formation of formazan crystal was evaluated by dissolving in dimethylsulfoxide (DMSO, Sigma, USA). The optical density was evaluated at 570 nm.[18]

For the cell proliferation evaluation, MC3T3-E1 cells were seeded at density 50,000 cells/well on 24-well-plate which treated with AE at 10, 25, 50 µg/mL and without AE as control (n=3 for each sample). The cells were cultured for 24, 48 and 72 h. At the specified time-points, the cells were determined the proliferation by MTT assay based on the above instructions.[18]

Evaluation of cell attachment

Cell attachment was measured by MTT assay at 4 and 24 h.[19] MC3T3-E1 cells were seeded at density 50,000 cells/well on 24-well-plate which treated with AE at 10, 25 and 50 µg/mL and without of AE as control (n=3 for each sample). At the specified time-points, the non-attached cells were removed with PBS (pH 7.4). Then the remained cells were measured by MTT assay based on the above instructions.[18]

For morphology observation, the remained cells were fixed in 4% paraformaldehyde (Sigma, USA). After that, the samples were sequential dehydration in an ethanol and sputter-coated with gold. The morphological characteristics of the attached cells were examined using a scanning electron microscope (SEM)(Leo1455VP, USA).[19]

Evaluation of osteogenic differentiation

MC3T3-E1 cells were seeded at density 50,000 cells/well on 24-well-plate (n=3 for each

sample) for 3 days. To induce osteogenic differentiation, the cells were cultured in osteogenic medium (α-MEM medium supplemented 10% FBS (Gibco, USA), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/mL streptomycin, 5 µg/mL amphotericin B (Gibco, USA), ascorbic acid (50 µg/mL), dexamethasone (100 nM) and sodium phosphate (2 mM)for 7, 14 and 21 days. That osteogenic medium were treated with AE at 10, 25, 50 µg/mL and without AE as control (n=3 for each sample).

Alkaline phosphatase activity and staining assay

After 7, 14 and 21 days of cultured in osteogenic medium, the cells were measured ALP activity by colorimetric assay kit (K412–500, Biovision®, USA) as the manufacturer's protocol. The ALP enzyme activity was further normalized with total cellular protein concentration, which measured by BCA Protein Assay Kit (Pierce Biotechnology, USA). For the ALP staining assay, the cells were stained using the TRACP and ALP Double-Stain kit (Takara Bio Inc., Japan) according to the manufacturer's instructions. Images were visualized with a bright field optical microscope (Olympus Optical, Japan).[18]

Real-time polymerase chain reaction (PCR) analysis

After 7, 14 and 21 days of cultured in osteogenic medium, the cells were evaluated the osteogenic gene markers including collagen type I (Col I), ALP, bone sialoprotein (BSP) and osteocalcin (OCN). Briefly, total RNA from the cells of each group was extracted using NucleoSpin® RNA kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. The RNA samples were converted to cDNA by iScript™ Reaction (BIO-RAD, USA) following the manufacturer's instructions. The real-time PCR reactions were performed by Roche Light cycler 480 real-time PCR system machine (Roche Diagnostics, USA) with LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, USA). The primer sequences are listed in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for calculating fold differences in RNA levels of cells by the $2^{-\Delta\Delta CT}$ method.[20]

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product length (bp)
Col I	CTCCTGACGCAT GGCCAAGAA	TCAAGCATACCT CGGGTTTCCA	100
ALP	ACCCGGCTGGAG ATGGACAAAT	TTCACGCCACAC AAGTAGGCA	113
BSP	ACCGGCCACGCT ACTTTCTTTA	GGAACTATCGCC GTCTCCATTT	113
OCN	AGCAGGAGGGC AATAAGGTAGT	TCGTCACAAGCA GGGTTAAGC	118
GAPDH	AGCGAGACCCCA CTAACATCA	CTTTTGGCTCCA CCCTTCAAGT	118

Table 1. Primer sequences used for quantitative real-time PCR[20]

Alizarin Red-S staining and calcium quantification

After 7, 14 and 21 days of cultured in osteogenic medium, the cells were fixed with cold methanol for 10 min and stained with 1% Alizarin Red-S solution (Sigma, USA) for 3 min. The amount of calcium was quantified by destaining with by 10% cetylpyridinium chloride monohydrate (Sigma, USA) in 10 mM sodium phosphate for 15 min and then, the optical density was evaluated at 570 nm.[18]

Statistical analyses

The results were illustrated as mean ± standard deviation. Statistical significance was analyzed using a one-way analysis of variance (ANOVA), followed by Tukey HSD test for multiple group comparison. The differences at $P<0.05$ were considered to be statistically significant.

Results

Cell viability and proliferation

The range of AE concentrations for investigation in this study were conducted using 10-1,000 µg/mL. After treated with AE for 24 h, the results show that there was no toxic effect on L292 cells when treated with AE concentrations less than 50 µg/mL. On the other hand, treated with AE concentrations above 100 µg/mL, the cellviability was decrease less than 50% when compared to control (Figure.1A). It was apparent that 50 µg/mL of AE concentration was the highest concentration in this range which had no toxicity. Therefore, the selected AE concentrations

were 10, 25 and 50 µg/mL for subsequent experiments.

For cell proliferation, MC3T3-E1cells were treated with AE at 10, 25 and 50 µg/mL for 24, 48 and 72 h. The results showed that the cell proliferation of cells treated with 50 µg/mL AE was statistically significant higher than those of other groups at 24 h. However, the proliferation rate was no statistically significant difference after treated for 48 and 72 h. (Figure. 1B).

Evaluation of cell attachment

After treated with AE for 4 and 24 h, MC3T3-E1 cells were analyst the cells attachment with MTT assay, the results of treated with 50 µg/mL AE group was significant enhanced when compared to the control group for both 4 and 24 h time points (Figure 2A).

Morphological observation of MC3T3-E1cells attached under phase contrast microscope (Figure. 2B). At 4 h, the most of cells appeared round shape cell attachment was obviously note of control group. In contrast, cell morphology of treated with 50 µg/mL AE group was appeared polygonal cells, which larger and flatter than those in control group. No difference in cell morphology was obviously detectable among at 4 h and 24 h. At high magnification, the SEM examination showed that the cells of treated with 50 µg/mL group appeared flat shape with a large and thin cytoplasmic layer and with numerous filopodia which extended from the cell body to the surface. While, the control group appeared round shape attached cell with short filopodia (Figure 2C).

Evaluation of osteogenic differentiation Alkaline phosphatase activity

The results of the alkaline phosphatase staining at 14 days time point was shown that the active ALP stained cells of the treated 50 µg/mL AE group appeared more than those of control groups (Figure. 3A). The quantitative examination of ALP activity indicated that the groups of treated AE with 50 µg/mL were exhibited the significantly highest ALP activities at every time point investigated (Figure. 3B).

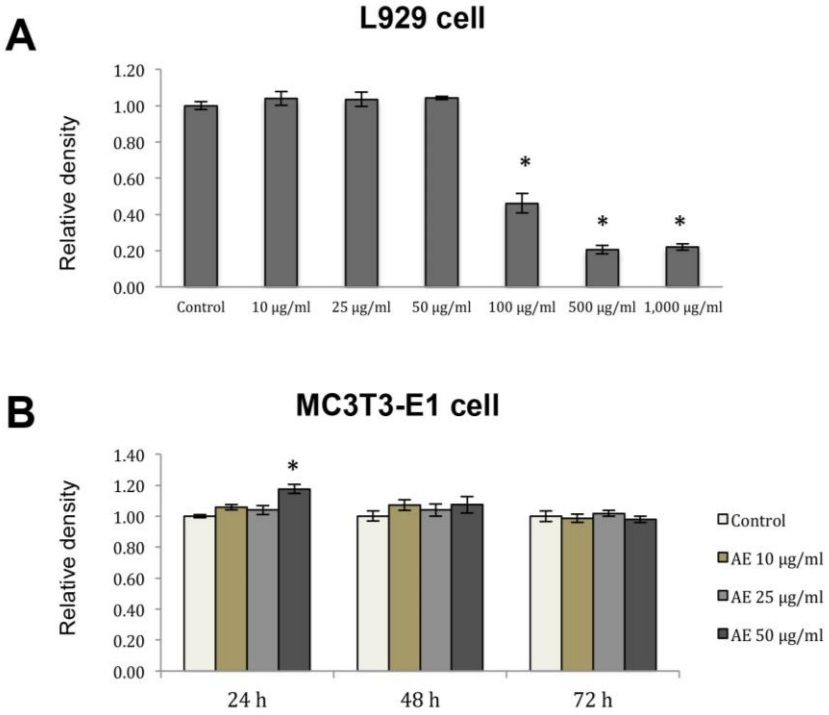

Figure 1. Dose-response effect of AE (10-1,000 µg/mL) on L929 cell viability, measured for 24 h by MTT assay. The AE concentrations over at 50 µg/mL were significantly decrease cell viability when compared to the control group (*: $P<0.05$)(A). Effect of AE (10, 25 and 50 µg/mL) on MC3T3-E1 cell proliferation was determined by MTT assay at 24 h, 48 h and 72 h time points. Cell proliferation was significantly enhanced only when treated with 50 µg/mL of AE at 24 h time point compared to the control group (*: $P<0.05$) (B).

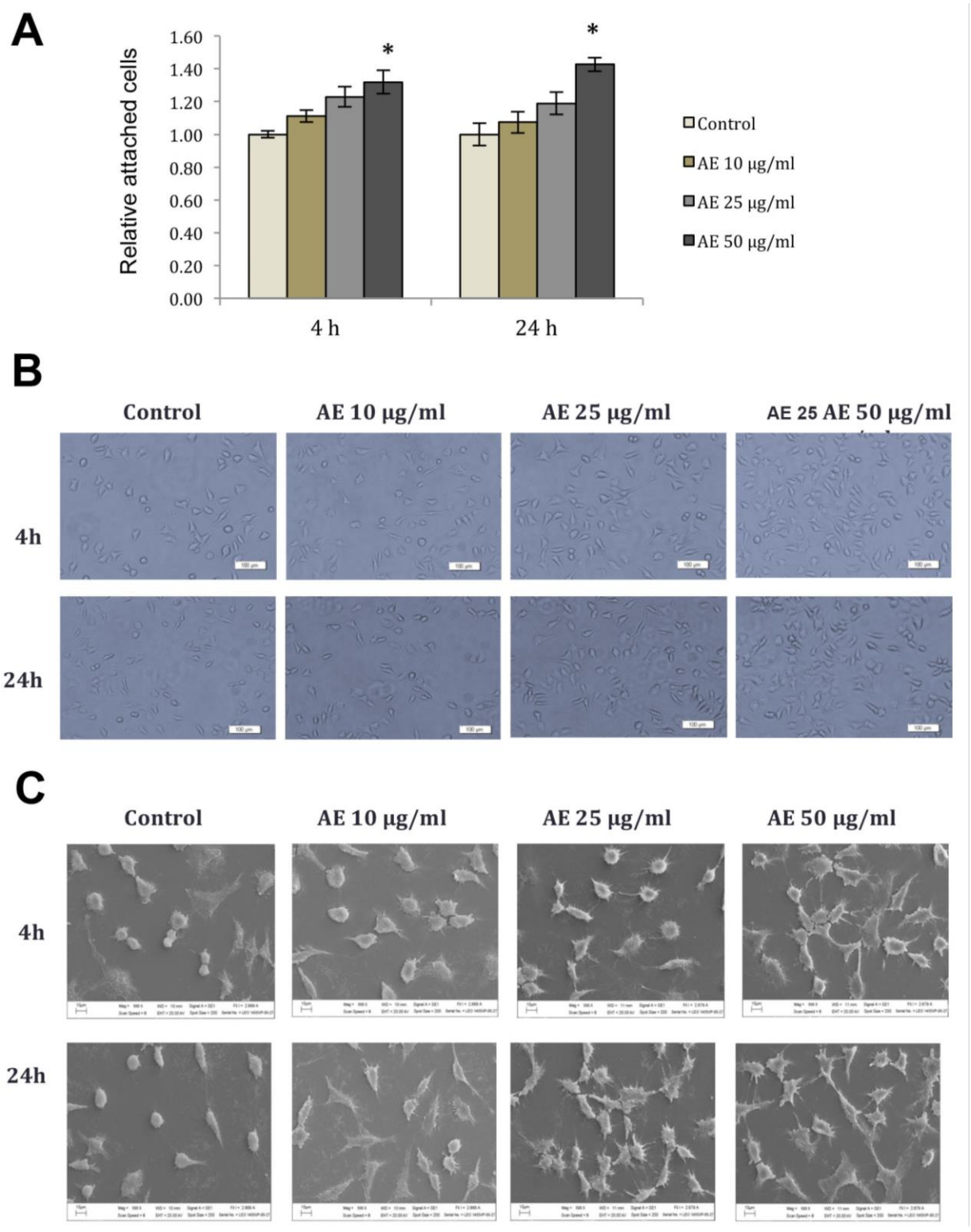

Figure 2. Effect of AE on MC3T3-E1 cell attachment was determined by MTT assay after treated with various concentration of AE (10, 25 and 50 µg/mL) for 4 h and 24 h. Cell attachment was significantly enhanced only when treated with 50 µg/mL of AE at both 4 h and 24 h time point compared to the control group (without AE) (*: $P<0.05$) (A). Morphology observation of attached cells after treated with AE using phase contrast microscopy (B) and using scanning electron micrographs for high magnification (C).

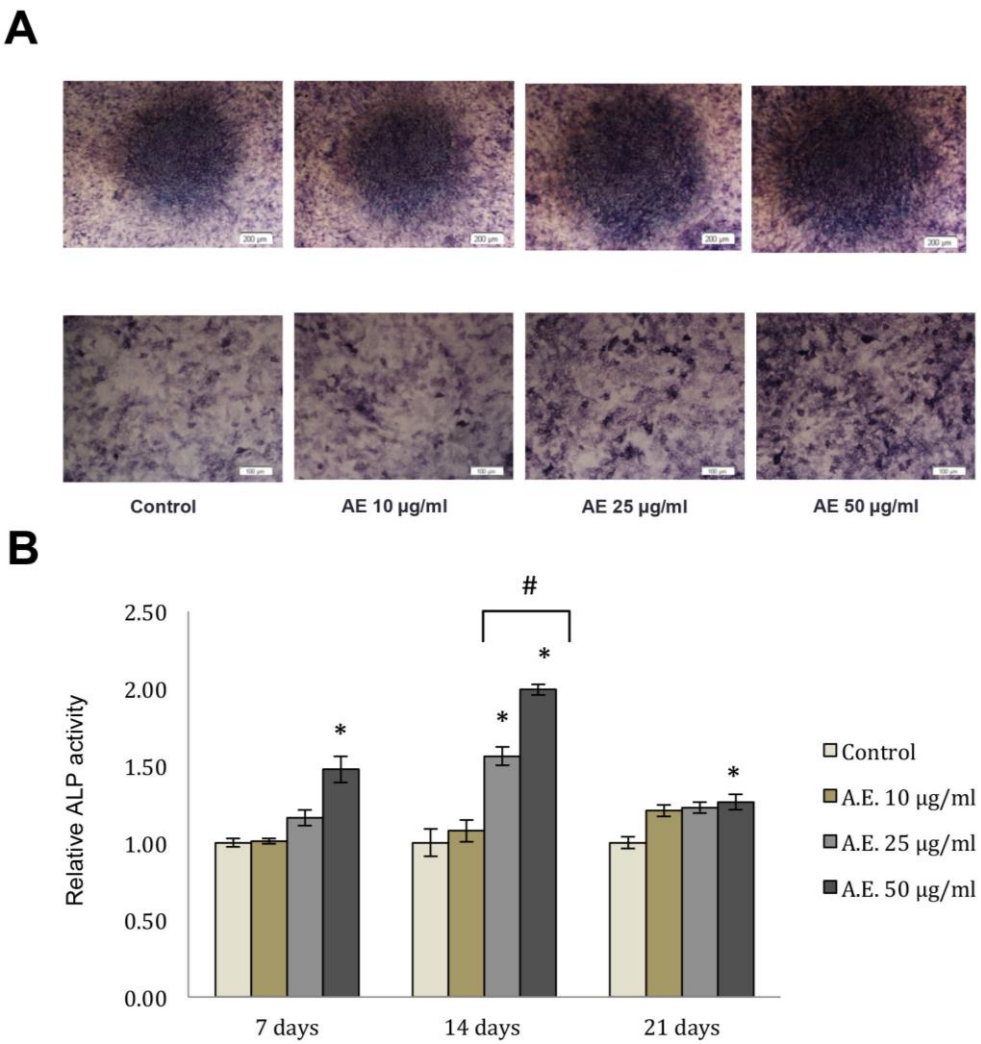

Figure 3. Effect of AE on the ALP staining and activity of MC3T3-E1 cells was evaluated after cultured in osteogenic medium. ALP staining of the cells at 14 days timepoint was shown (A). The ALP activity at 7, 14 and 21 days timepoints showed that the ALP activity of treated with 50 µg/mL AE groups was significantly higher than control groups at all time point (B). Significant differences (P<0.05) compared to control are marked with *. Significant differences between groups are marked with #.

Osteogenic genes expression

The expressions of osteogenic genes were evaluated using the real-time PCR. The results showed that Col I and ALP mRNA expression of the treated 50 µg/mL AE groups were exhibited the significantly highest compared to the other groups for all time points(Figure.4 A and 4B). In addition, BSP and OCN mRNA expression of the treated 50 µg/mL AE groups were exhibited the significantly highest compared to the other groups only in 14 and 21 days time points. There were no significantly differences of those between groups at 7 days time point (Figure. 4C and 4D).

Mineral deposition

The mineral deposition was performed by Alizarin Red S staining at 7, 14 and 21 days time points. The results showed that mineral deposition of 50 µg/mL AE treated group was significantly higher than control groups only at 21 days time point (Figure. 5).

Discussions

Since current therapeutic approach for bone regeneration still has some limitations and adverse side effects.[2,7-9] We are interesting in

natural plants, which have been accepted as one of the main sources of drug discovery and often fewer side effects compared with synthetic compounds. *Aquilaria crassna* has been widely used in folk medicine for bone diseases such as arthritis and gout in Southeast Asian.[13]However, there was a lack of scientific evidence to support the application of *Aquilaria crassna* for bone repair.

The optimal concentration of AE determined non-toxic concentration. This study was used MTT assay[17] to determine cell viability of L929 cells[21]followed ISO 10993-5 *In vitro* cytotoxicity test protocol. The results showed that AE was no toxic effect on L929 cells when treated with AE concentrations less than 50 µg/ml (Figure.1A). These results indicated that the AE biologically safe concentration range between 10-50 µg/mL. Consistent with study of Dahham et al.[22] demonstrated the cytotoxic effect of AE on cancer cells including prostrate (PC3), colorectal (HCT 116) and breast (MCF-7) cancer cells. The cytotoxicity results showed50% cell death or 50 % inhibition concentration (IC50) with 72, 119 and 140 µg/mL respectively.

Bone formation is a process that depends upon a sequence of biological events including cell attachment, cell proliferation, osteogenic differentiation, organic matrix formation, and matrix mineralization.[23] Cell attachment is essential in cell communication and regulation, which is fundamental importance in the cell proliferation and differentiation.[24] In this study, the results show that treated with 50 µg/mL AE was significant increase cell attachment on both 4 and 24 h time points (Figure. 2A). The results were confirmed cells attached morphology with SEM (Figure. 2C). These results may indicate that AE stimulate cell attachment that may subsequently affect to promote cell proliferation and differentiation.

We determined the cell proliferation of MC3T3-E1cells after treated with AE for 24, 48 and 72 h. These results show the treated with 50 µg/mL AE group was significant higher cell proliferation than those of other groups at 24 h (Figure.1B). Previous studies have been reported that natural plants extract stimulate cell proliferation. Suh et al.[25] reported that 20 µg/mL of *Ulmusdavidiana* extract significant stimulate cell proliferation of MC3T3-E1 cells after culture for 48 h. While, Xiang et al.[26] demonstrated that *Polygonumorientale* extract significantly promoted the proliferation of MC3T3-E1 cells at 1-10 µg/mL concentration after culture for 24 h. However, the results of our study show no significant difference of proliferation rate after culture for 48 and 72 h (Figure.1B). Therefore, from this results may indicate that 50 µg/mL AE promoted MC3T3-E1 cells proliferation in first 24 h, after that the cells may lead to stage of differentiation without any subsequent proliferation.

ALP activity is a definitive early marker of osteoblast differentiation, while mineralized nodule formation is a marker of the late stage of osteoblast differentiation.[27] Some studies reported natural plant extract stimulate ALP activity including *Drynariae Rhizoma*,[11] *Ulmus davidiana*,[25] *Polygonum orientale.*[26] The results of our study showed that treated with AE at 50 µg/mL significant increased ALP activity for all time points. The ALP activity pattern was increased at 1-2 weeks and decreased at 3 week (Figure. 4B). This pattern correlated with mineralized nodule formation in that the latter was observed after 3 weeks of culture.

The expression patterns of transcription factors during osteoblast maturation reflect their roles as key determinants of osteoblast differentiation. The common osteoblast differentiation markers, early phase of differentiation are ALP and Col I, while BSP and OCN appears are the late phase markers. Also in this study, we evaluated the expressions of Col I, ALP, BSP and OCN to confirm osteogenic differentiation.[28, 29] While, the mineralized nodule formation is an important hallmark of complete osteogenic differentiation that the main composition of mineralized formation is calcium.[30] In this study, we used Alizarin Red-S staining to detect calcium deposition and quantify matrix mineralization.

Many previous studies repoted that natural plants extract exhibited osteogenic activities by promoting osteoblast differentiation and mineralization. Jeong et al.[31] reported that *Drynariae Rhizoma* extract has osteogenic effects through the promotion of osteoblastic differentiation in MC3T3-E1 cells that enhanced ALP activity and mineralization and increased mRNA expression of Col I, ALP and BMP-2. After that, they founded, main effective component of *Drhizomadrynariae* was Naringin.[32,33] Other study, Huh et al.[34] reports on the osteogenic effects of *Puerarin* that have potently induced

osteoblastic differentiation gene markers such as ALP, OCN, osteopontin (OPN), Col I, and mineralization in human osteoblast like SaOS-2 cells. While as, Muthusami et al.[35] reported *Cissus quadrangularis* positive regulation of on the proliferation, differentiation, and matrix mineralization of human osteoblast like SaOS-2 cells. Recently, Hwang et al.[12] reported that the extract from *Euodiasutchuenensis Dode* (ESD) leaf and young branch enhanced differentiation of murine primary osteoblasts.

In this study, our results showed that cell treated with AE at 50 µg/mL was significantly increased expression of Col I, ALP, BSP and OCN for all time points. While, the mineralized formation results showed that 50 µg/mL AE treated groups was significantly increased mineral deposition at 21 days time point (Figure 5). Interestingly, cell treated with 50 µg/mL AE exhibited faster matrix mineralization than those of other groups. These data also indicated that 50 µg/mL of AE is a promising osteoinductive agent for preosteoblasts cells to stimulate differentiation and matrix mineralization.

Phytochemical constituents of AE might play a role in the induction of osteogenic differentiation and matrix mineralization. Previous phytochemical analysis of the crude extract of *Aquilaria crassna* showed the presence main compositions were triterpenes and flavonoid, which may have a stimulatory effect on bone formation.[22,36] Triterpenes reported to stimulate proliferation, protein synthesis, and ALP activity of periodontal ligament cell lineage *in vitro.*[37]

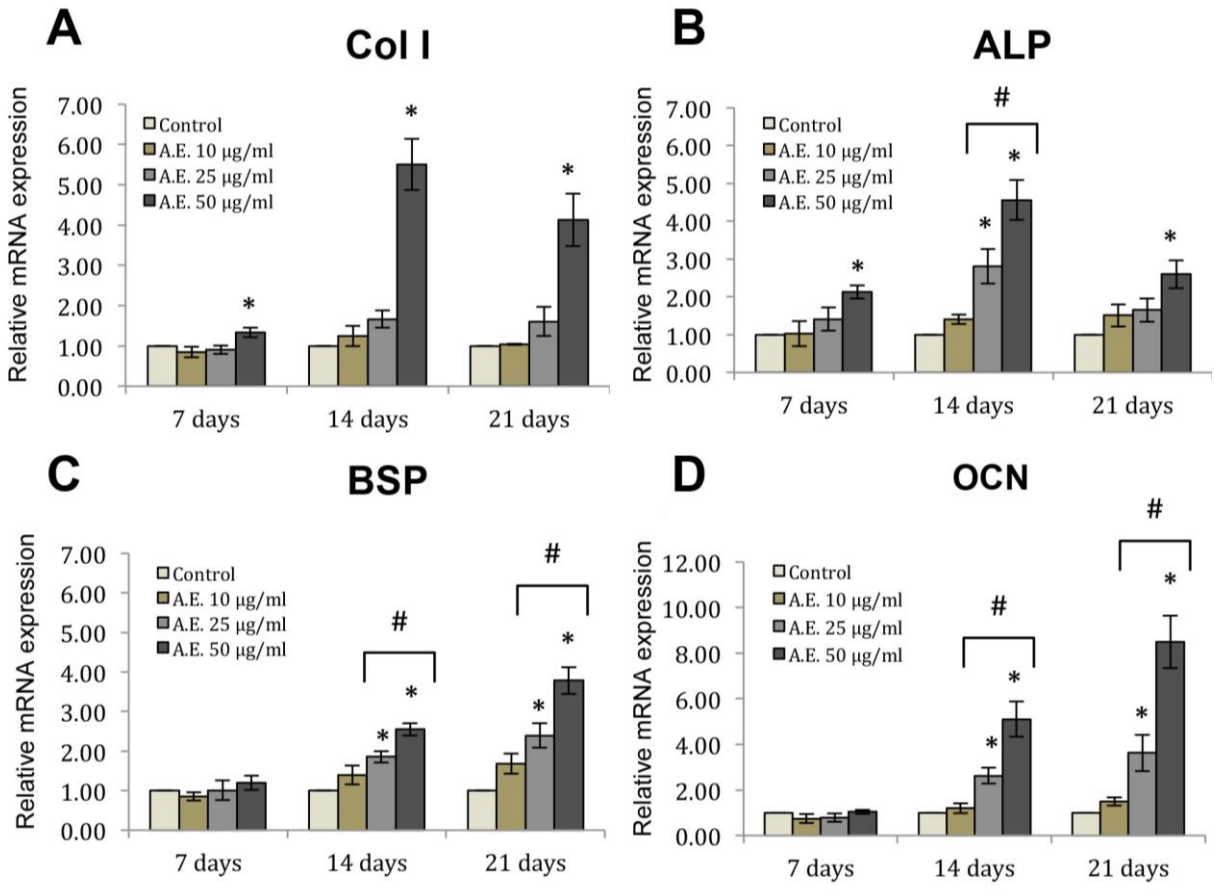

Figure 4. Effect of AE on the levels of expression of osteogenic differentiation related genes of MC3T3-E1 cells. Real-time PCR was used to analyze the expression of genes (Col I, ALP, BSP and OCN) after cultured cells in osteogenic medium at 7, 14 and 21 days (A-D). Significant differences ($p<0.05$) compared to control are marked with *. Significant differences between groups are marked with #.

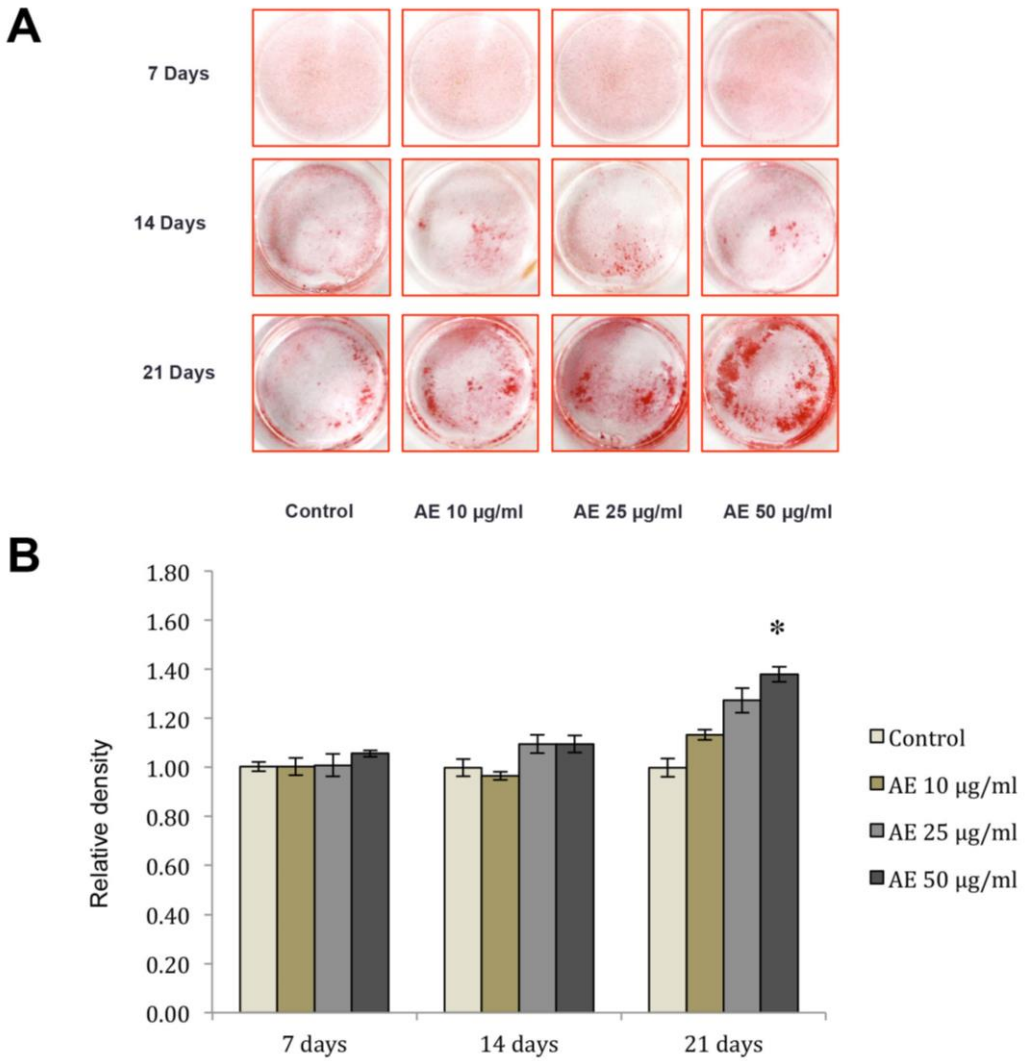

Figure 5. Effect of AE on the levels of mineral deposition of MC3T3-E1 cells. The mineral deposition was stained with alizarin red after cultured cells in osteogenic medium at 7, 14 and 21 days time points. The staining wells after treat with AE were shown (A). After staining, the dye was extracted using cetylpyridinium chloride and quantified (B). Significant differences ($p<0.05$) compared to control are marked with *

While, flavonoids were shown to promote the osteogenic differentiation of human bone marrow-derived mesenchymal stem cells.[38] However, it has not yet analyzed the chemical compositions of AE that used in this experiments. Therefore, in future studies need more in-depth analysis to identify and confirm the active ingredients that contribute to the osteogenic process.

Conclusions

Based on the results, it can be concluded that *Aquilaria crassna* extract was efficacious in inducing initial cell attachment and proliferation and promoted the osteogenic differentiation and matrix mineralization *in vitro*. Therefore, *Aquilaria crassna* are a promising anabolic agent for bone regeneration.

Acknowledgements

This work was supported by Naresuan University Research Grant (R2560C136) and Faculty of Dentistry, Naresuan University, Thailand. We would like to acknowledge Dr. Sarawut Kumphune, Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, for providing the *Aquilaria crassna* extract samples and helping with the extraction procedure.

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