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The Effects Of Piper Sarmentosum Aqueous Extract On MC3T3-E1 Differentiation

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ABSTRACT

Piper sarmentosumis an herbaceous plant under the Piperaceae family. It is also known as 'kaduk' among Malaysians. The plant extracts contain various compounds such as flavonoids and benzoic acid derivatives that are believed to repair and maintain bone strength. The aim of this study was to determine the capability of P. sarmentosum aqueous extract in inducing osteoblast differentiation of MC3T3-E1 cell line.The MC3T3-E1 cells were treated with specific concentrations of P. sarmentosumaqueous extract which were 1 mg/mL, 2 mg/mL, 3 mg/mL and 4 mg/mL and were cultured for 14 days. Three analyses were used to determine the differentiation potential of MC3T3-E1 cell into osteoblast, i.e., biochemical assay, gene expression and morphological analysis. Biochemical assay was carried out by determining the specific activity of alkaline phosphatase (ALP). The results showed an increase in ALP activity in cells treated with P. sarmentosumaqueous extract with the highest activity was on day 14. Analysis of molecular biology marker expression showed the expression of Alp, Runx2 and Col 1 genes which were osteoblast genes. The von Kossa staining showed formation of calcium nodules through brownish and black colour on day 14 of culture. In conclusion, P. sarmentosumaqueous extract through ALP activity, osteoblast genes expression and calcium mineralization were shown able to induce MC3T3-E1 cell differentiation into osteoblasts.

Keywords: Piper sarmentosum, MC3T3-E1 cell, osteoblast, differentiation

INTRODUCTION

Natural products such as plants and herbs were shown to have many phytochemicals with various bioactivities for medical benefit which globally been used in the prevention and treatment of various

diseases. Piper sarmentosum is an herbaceous plant commonly used in traditional medicine especially its root, leaves and fruit (Khalid et al. 2012). It is easily found in tropical countries such as Cambodia, South China, Indonesia, Laos, Malaysia and Philippines (Raman et al. 2012). Piper sarmentosum leaves or known as 'daunkaduk' in Malaysia were normally eaten raw as 'ulam' or salad. The water decoction of the leaves was also used for treating diabetes, hypertension, cough and joint aches by Malay folk (Subramaniam et al. 2003). Biological studies also shown the anti-cancer, fracture healing and tissue regeneration properties of P. sarmentosum aqueous extracts (Horcajada et al. 2008; Shahrul Hisham et al. 2009;Intan Zarina et al. 2020).

Osteoblast cell is a mononucleated cell that has a unique morphology, characteristic and biological activity which play important role in bone development (Kartsogiannis& Ng 2004; Florencio-Silva et al. 2015). Bone mineralization found on the bone surface is one of the osteoblast's functions, i.e., producing bone matrix in order to increase bone mass. On the other hand, MC3T3-E1 cell line derived from the calvaria bone tissue of normal mouse (Mus musculus) and widely used in the osteoblastdifferentiation study during bone development (Hwang & Horton 2019). The cell showed capability to differentiate into osteoblast cells which are responsible in synthesizing bone tissue (Yazid et al. 2010).

Establishment of primitive or progenitor cells differentiation towards osteoblasts has been done using combination of ascorbic acid and β-glycerophosphate as inducers. Ascorbic acid also known as vitamin C plays an important role in bone development. Ascorbic acid is one of the differentiation factors used in the in vitro bone cell culture to express the osteoblast phenotype (Coelho & Fernandes 2000).β-glycerophosphate is a synthetic chemical which acts as phosphate group donor that widely used to promote bone matrix mineralization. However, β-glycerophosphate has shown negative effect on the cells where it contributed to the decrease in cell proliferation (Coelho & Fernandes 2000).

The use of plant extracts has gain global attention due to their endless health benefits. Latterly, plants or herbs have been considered as inducers for stem cell proliferation and differentiation in vitro and in vivo tissue regeneration (Zhang et al. 2009; Gao et al. 2013; Saud et al. 2019; Intan Zarina et al. 2020). Studies have shown osteogenic differentiation in the presence of plant extract supplements (Zhang et al. 2009; Saud et al. 2019). Therefore, this study is aimed to determine the capability of P. sarmentosum aqueous extract in inducing in vitro osteoblast differentiation of MC3T3-E1 cell line.

MATERIALS & METHODS

Plant materials

Fresh leaves from P. sarmentosum were collected from the Forest Research Institute of Malaysia (FRIM), Kuala Lumpur, Malaysia. They were identified by a botanist from the Faculty of Applied Science, UniversitiTeknologi MARA (UiTM).

Plant aqueous extraction

Plant extraction was conducted at the Faculty of Science and Technology, UniversitiKebangsaan Malaysia (UKM). Leaves were prepared by cleaning with water and followed by drying in a 50°C oven for one week. The dried leaves were then ground to a powder using pestle and mortar at room temperature and stored in an air-tight container in the dark at 4°C prior to extraction.

Approximately 100 g powdered samples were boiled in distilled water (1:2 w/v) for 90 min before centrifugation at 6000 rpm (Mikro 22 R centrifuge machine, Hettich, Germany) for 10 min. Supernatant was recovered and centrifugation process was repeated until no pellet was observed. The supernatant was transferred to a new tube followed by freeze-drying using a freeze dryer Alpha 1-2 LD Plus (Christ, Germany). Once dried, the powder was stored in the dark at 4°C until required or weighed and dissolved in distilled water for further analysis.

Cell culture

MC3T3-E1 cell line (ATCC No: CRL-2596™) was used in this study. Cells were maintained in alpha minimum essential medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen, USA), 2% (v/v) penicillin/streptomycin and 1 mM sodium pyruvate (Sigma, USA). Cells were then incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Cell differentiation using P. sarmentosum aqueous extract

Differentiation of MC3T3-E1 cells was induced using different concentrations of P. sarmentosum aqueous extract, i.e., 1 mg/mL, 2 mg/mL, 3 mg/mL and 4 mg/mL. Positive control was a culture medium, α-MEM consists of 50 µg/mL ascorbic acid (Sigma, USA) and 10 mM β-glycerophosphate (Sigma, USA), while cells cultured in complete/culture medium only served as negative control. Cells were incubated for 14 days at 37°C in a humidified atmosphere containing 5% CO_2 and the medium was changed every 3-4 days.

Alkaline Phosphatase (ALP) Assay

The Alkaline Phosphatase (ALP) assay was conducted at days 0, 3, 5, 7, 10 and 14. The cells were washed with a culture medium followed by phosphate buffer saline (PBS) (Sigma, USA). The whole cells were treated at 37°C for 30 min in 0.1 M $NaHCO_3$-$NaCO_3$ buffer (pH 10), 0.1% (v/v) Triton X-100, 2 mM $MgSO_4$ and 6 mM p-nitrophenol phosphate (pNPP). The reaction was stopped with addition of 1 mL 1.5 M NaOH, and the absorbance was measured at 405 nm. One unit of ALP activity represents the hydrolysis of 1 µMpNPP per minute at 37°C. The ALP activity is represented as a specific activity, i.e., unit activity per total protein content (mg). The protein content was measured using Bradford method. The ALP specific activity was presented in percentage value, which ALP specific activity in control cells (cells culture in standard/culture medium) act as basal activity (100%).

Molecular Assay by RT-PCR

Total RNA was isolated from cells using TRI-Reagent (Sigma, USA) according to the manufacturer's instructions. Absorbance at 260 nm and 280 nm was used to measure the RNA purity; an A260:A280 ratio of 1.8-2.0 was acceptable. Reverse Transcriptase polymerase chain reaction (RT-PCR) was performed with AMV reverse transcriptase and Tfl DNA polymerase in the Access RT-PCR System (Promega Corporation, USA). The primers were designed by using Primer Premier 5.0 based on the sequences of Gapdh (accession number: NM 001289726.1), Col I (accession number: NM 007742.4), Alp (accession number: NM 001287172.1), and Dkk2 (accession number: NM 020265.4) obtained from NCBI. Table 1 shows the specific primer sequences that have been designed for RT-PCR reaction.

Table 1: Primer sequences used in RT-PCR reaction

Genes	Primers	Sequence	Annealing Temperature (°C)	Expected Size (bp)
Gapdh	Forward	5’ CAACGGCACAGTCAAGG 3’	65	717
	Reverse	5’ AAGGTGGAAGAGTGGGAGT 3’		
Col I	Forward	5’ TGAGACAGGCGAACAAGGT 3’	64	519
	Reverse	5’ TTCCAGTCAGAGTGGCACAT 3’		
Alp	Forward	5’ TGAGCGACACGGACAAGAA 3’	66	510

	Reverse	5’ GATACAGGCAAGGCAGATAGC 3’		
Dkk2	Forward	5’ GCAGCAGTGATAAGGAATGTGA 3’	66	517
	Reverse	5’ TTGGAAGAGTAGGTGGCATCT 3’		

The RT-PCR was carried out in a Mastercyler Gradient (Eppendorf, Germany). Reverse transcription (RT) was performed in a program of 45°C for 45 min and pre-denaturation at 94°C for 2 min. The amplification program was as follows: 30 cycles of three-step amplification (denaturation, 94°C for 30 s; annealing, 64-66°C for 60 s; and extension, 68°C for 60 s) followed by final extension step at 68°C for 7 min. Detection of the PCR amplicons was performed using 1.7% (w/v) agarose gel electrophoresis.

Von Kossa staining

Approximately 5×10^5 cells/mL were centrifuged at 75 × g for 5 min. The pellet was smeared onto a glass slide and left to air-dry for about 1 to 2 h. The cells were then labeled with von Kossa staining. For von Kossa staining, the cells were fixed with 10% (v/v) formalin in PBS for 30 min and washed with deionized water three times. Then, the cells were stained with freshly prepared 5% (v/v) silver nitrate solution for 30 min and washed well with deionized water three times. Next, the cells were developed with fresh 5% (v/v) sodium carbonate in 25% (v/v) formalin more than 5 min for mineral and matrix staining. After three washes with deionized water, the cells were finally fixed with 5% (v/v) sodium thiosulfate for 2 min to remove un-reacted silver nitrate. Finally, the cells were washed well with deionized water three times and air-dried. The von Kossa stained areas were viewed by light microscopy and analyzed using ImageJ software to quantify mineralization.

Statistical analyses

Data were statistically analyzed using Excel paired t-tests. Data were considered statistically significant at $P< 0.05$.

RESULTS

Biochemical Assay

ALP activity was carried out to measure osteoblast differentiation. ALP is a biochemical marker of osteoblasts as ALP enzyme is secreted during osteoblast differentiation. Figure 1 shows the percentage of ALP specific activity of P. sarmentosum aqueous extract induced MC3T3-E1 cells. ALP activity in MC3T3-E1 cells gradually increased from day 3 to day 14 of osteoblast differentiation by P.

sarmentosum extract for all concentrations. Similar observation was found for positive control (MC3T3-E1 cells cultured with supplementation of 50 µg/mL ascorbic acid and 10 mM β-glycerophosphate). Whilst, no increment of ALP was found for negative control. This is an indication that P. sarmentosum aqueous extract has the capability to induce osteoblast differentiation of MC3T3-E1 cells. Among four concentrations tested on MC3T3-E1 cells, 2 mg/mL extract showed highest ALP specific activity on day 14.

Molecular analysis during osteoblast differentiation

Molecular analysis was performed to determine the effectof P. sarmentosum aqueous extract supplementation towards in vitro differentiation of MC3T3-E1 cells. A number of genes such as Runx2, Opn, Col I, Alp and Dkk2 are expressed during osteoblastogenesis (Kartsogiannis& Ng 2004; Florencio-Silva et al. 2015; Rutkovskiy et al. 2016). All of these genes have been functionally implicated in osteoblast bone formation. Activation of Alp (~510 bp), Dkk2(~517 bp) and Col I(~517 bp)was observed by RT-PCR analysis in MC3T3-E1 cells cultured in culture medium, which contained P. sarmentosum extracts (Figure 2A-C). Similar genes activation was found for positive control, i.e., cells cultured in the standard differentiation medium (50 µg/mL ascorbic acid and 10 mM β-glycerophosphate).

In this study, we used the housekeeping gene Gapdh as a positive control for all types of treatments,i.e., P. sarmentosum extracts (1-4 mg/mL), positive control (ascorbic acid and β-glycerophosphate) and negative control (culture medium only) medium. Figure 2D shows that RT-PCR amplification produces a Gapdh band (~717 bp) from MC3T3-E1 cells in all types of treatments. The expression of the housekeeping gene is known to remain constant in the cells or in the tissues under inspection (Barber et al. 2005). The activation of the Gapdh gene in this study proves that the cell is performing the fundamental metabolic processes needed for cell survival.

Mineralization analysis of differentiated cells

Figure 3 shows the differentiated osteoblasts from MC3T3-E1 cells. After von Kossa staining of MC3T3-E1 cells cultured with 1-4 mg/mL of P. sarmentosum aqueous extract for 14 days, mineral nodules were found deposited at day 14. The similar findings found for MC3T3-E1 cells cultured in the culture medium consist of 50 µg/mL ascorbic acid and 10 mM β-glycerophosphate (positive control). Deposition of mineral modules by von Kossa staining was not indicated at day 0 until day 10 for all treatment groups (Figure 3A). The formation of brownish or black nodules after von Kossa

staining were observed only after 14 days of differentiation. On the other hand, negative control did not show any deposition of mineral nodules throughout 14 days differentiation (Figure 3A).

Analysis of mineralization intensity showed that 2 mg/mL extract has the highest percentage of mineralized cells as compared to three other concentrations (Figure 3B). Statistical analysis also showed that there was no significant difference ($P > 0.05$) of mineralization percentage when compared to the positive control (50 µg/mL ascorbic acid and 10 mM β-glycerophosphate). Whilst, the percentage of mineralization for the negative control cannot be counted as there was no mineralization detected.

DISCUSSION

MC3T3-E1 cell has been widely used to study osteoblast differentiation (Hwang & Horton 2019). In this study, we used MC3T3-E1 cell as a host cell to study the potential of P. sarmentosum aqueous extract in inducing osteoblast cells. ALP is known as an early biomarker for osteoblast differentiation (Martino et al. 2008) and important in assembling the matrix for mineralization (Chang et al. 2004). Our study showed that ALP activity in MC3T3-E1 cells cultured in the supplementation of P. sarmentosum aqueous (1 - 4 mg/mL) were increased gradually for 14 days, similar to the positive control. This is an indication that P. sarmentosum has the capability to induce osteoblast differentiation by expressing ALP as a biomarker.

MC3T3-E1 is fully differentiated into osteoblast cells after 14 days in differentiation medium (Wang et al. 1999; Shahrul Hisham et al. 2010). Therefore, RT-PCR analysis was done on day 14 to observe the activation of osteoblast genes after P. sarmentosum induction. The RT-PCR analysis of the transcripts from MC3T3-E1 cells cultured in culture medium consisting of P. sarmentosum extracts (1-4 mg/mL) showed the activation of Alp (~510 bp), Dkk2 (~517 bp) and Col I (~519 bp) genes. Similar bands were observed for positive control, i.e., cells cultured in standard differentiation medium (50 µg/mL ascorbic acid and 10 mM β-glycerophosphate). However, no band was found from the MC3T3-E1 cells that only cultured in culture medium, which served as the negative control (Figure 2). Whilst, the housekeeping gene, i.e., Gapdh used in this study remained activated in all conditions, indicating that the cells are viable and undergo survival processes (Tarze et al. 2007). During osteoblast differentiation, a number of genes such as Runx2, Opn, Col I, Alp and Dkk2 are expressed which are functionally implicated in the osteoblast formation (Kartsogiannis& Ng 2004; Florencio-Silva et al. 2015; Rutkovskiy et al. 2016). Activation of Alp, Dkk2 and Col I after 14 days induced by P. sarmentosum extracts showed that the extracts have similar potential with the

positive control to induce the differentiation of osteoblast cells. The activation of osteoblast marker transcriptions suggests that MC3T3-E1 cells differentiated into osteoblast in the existence of P. sarmentosum extracts.

The von Kossa staining is widely used to reveal the mineral nodules in osteogenic cells differentiated from mesenchymal stem cells (Alhadlaq& Mao 2003; Jeon et al. 2018). The effect of various stimulants is also observed using this staining by quantifying the formation of mineralization nodules in osteoblasts cultures (Tsuang et al. 2006). Mineralized cells can be easily seen as brownish or black nodules due to precipitation reaction. We observed the formation of black or dark-brown nodules after von Kossa staining after 14 days of MC3T3-E1 cells cultured in the supplementation of P. sarmentosum extracts (1 - 4 mg/mL). MC3T3-E1 cells cultured in the standard differentiation medium (culture medium with addition of 50 µg/mL ascorbic acid and 10 mM β-glycerophosphate) also showed the deposition of black or dark-brown nodules after 14 days of differentiation.

The increased expression of ALP enzyme activity and formation of mineralization nodules after 14 days differentiation are in line with osteoblast differentiation characteristics (Chang et al. 2004; Martino et al. 2008). Mineralization is considered as the final stage of osteoblast differentiation (Rutkovskiy et al. 2016). In addition, the activation of osteoblast marker transcripts, i.e., Alp,Dkk2 and Col I showed that MC3T3-E1 cells are induced by theaqueous extracts to differentiate into osteoblast cells. Previously, P. sarmentosum extracts have been reported to reduce bone loss and increase bone strength in rats (Horcajada et al. 2008). This extract also found to accelerate tissue regeneration in zebrafish (Intan Zarina et al. 2020).

Standard osteoblast differentiation has been established using ascorbic acid and β-glycerophosphate as differentiation factors or inducers. The capability of P. sarmentosum in producing similar osteoblast properties during differentiation of MC3T3-E1 cells showed that this extract can be used to induce osteoblast cells instead of using the standard differentiation factors such as ascorbic acid and β-glycerophosphate. The potential of using natural products would benefit the cellular therapy as an alternative inducer is safer as compared to the synthetic chemicals currently used.

CONCLUSION

MC3T3-E1 cells after 14 days induction by P. sarmentosum aqueous extracts, produced increment in ALP specific activity, osteoblast genes as well as mineralized cells as indicated by von Kossa staining. Therefore, P. sarmentosumaqueous extract is able to induce MC3T3-E1 cell differentiation into osteoblasts. Hence, P. sarmentosum have potential to be considered as alternative differentiation inducer to the current standard osteoblast differentiation factors/inducers.

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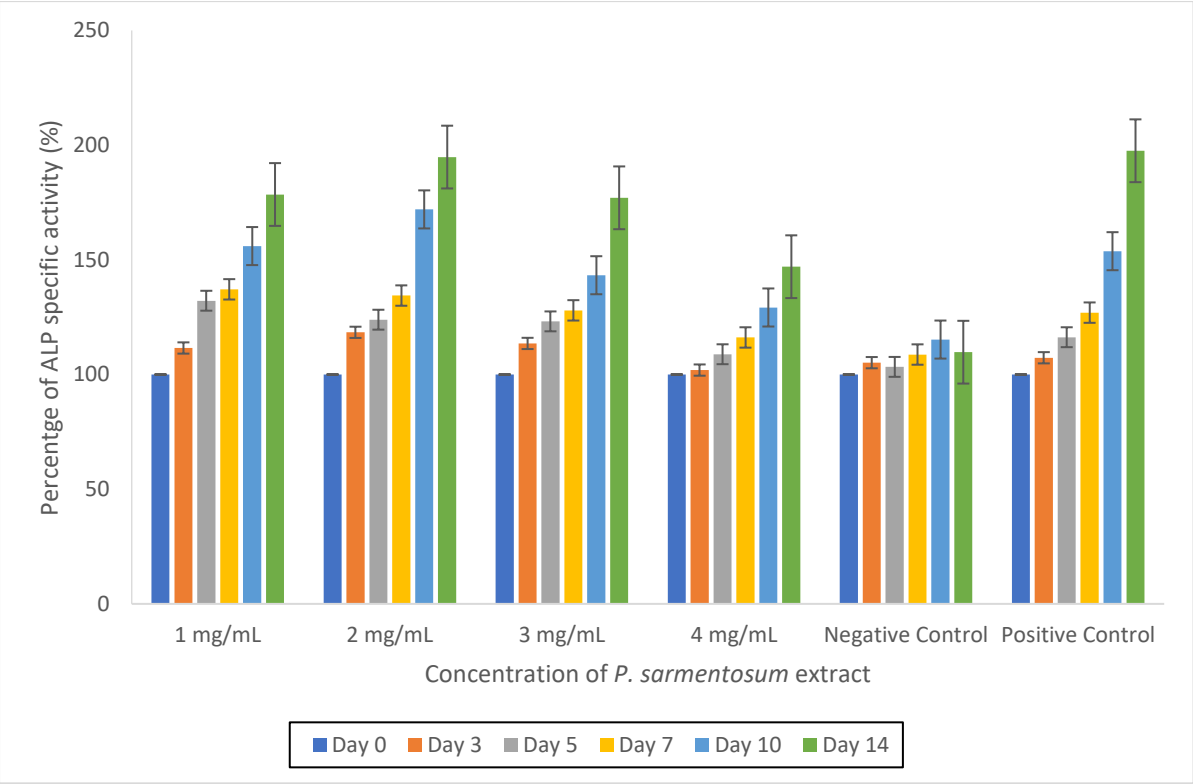


Figure 1. Effect of P. sarmentosum aqueous extractat various concentration on ALP specific activityduring osteoblast differentiation. ALP assays were performed on days 0, 3, 5, 7, 10 and 14 using 1 mg/mL, 2 mg/mL, 3 mg/mL and 4 mg/mL of P. sarmentosum aqueous extract. The data obtained are the average ± standard deviation from three different experiments.

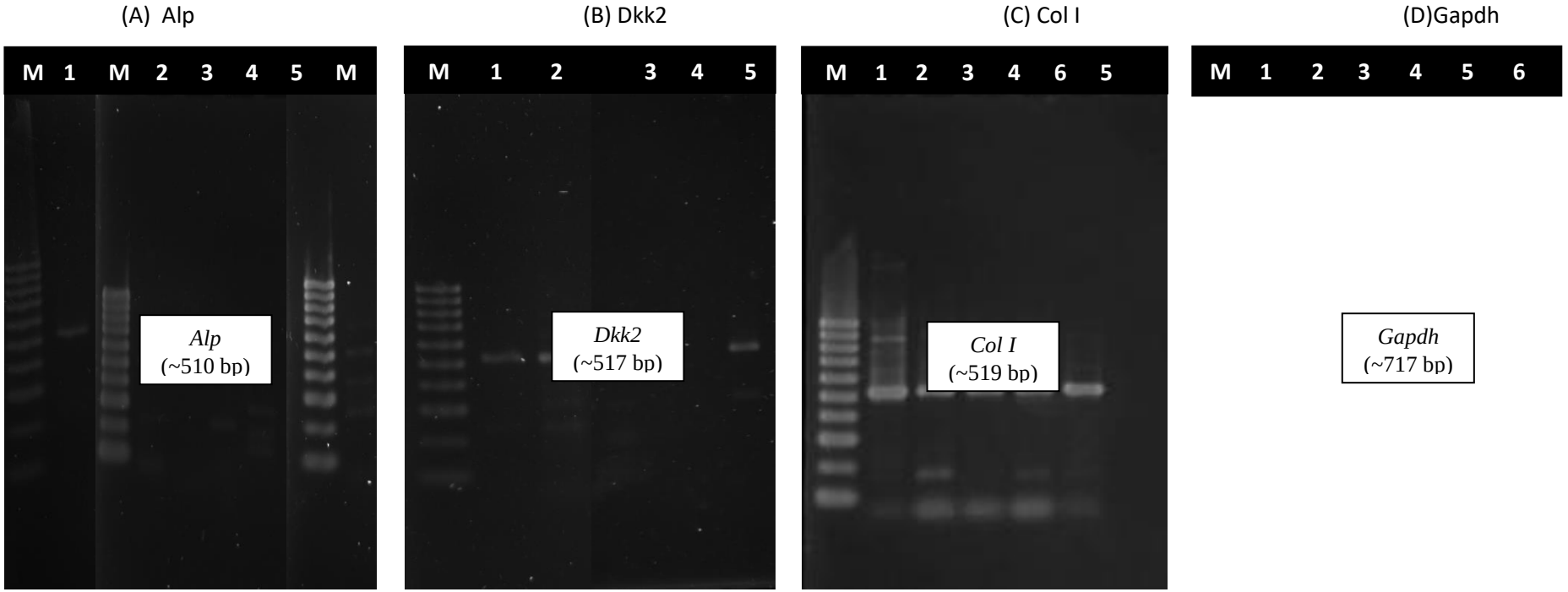
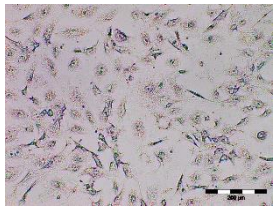
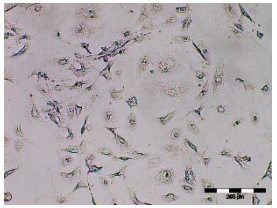
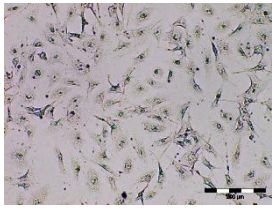
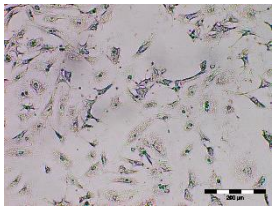
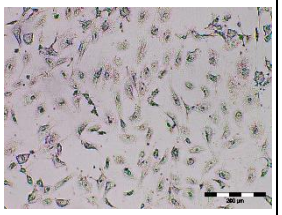
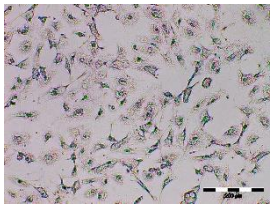
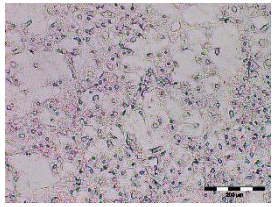
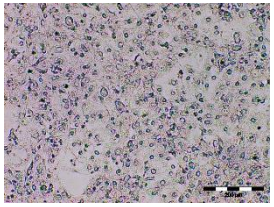
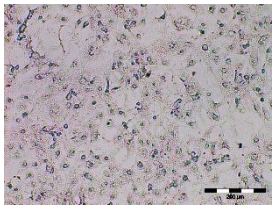
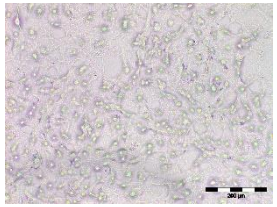
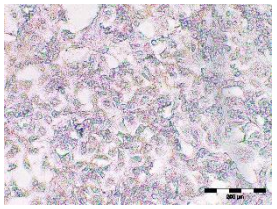
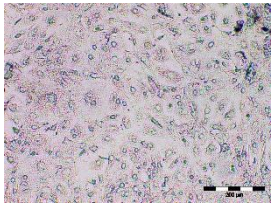
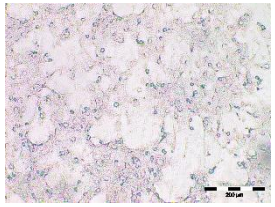
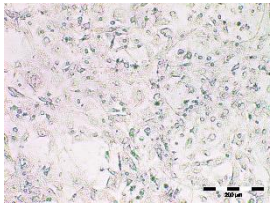
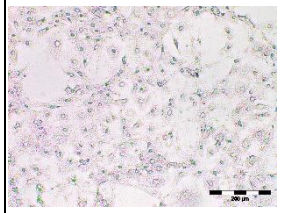
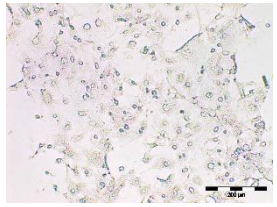
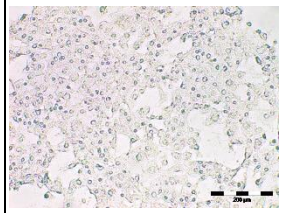
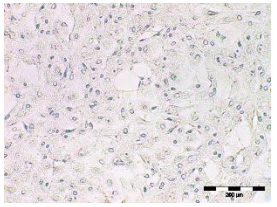


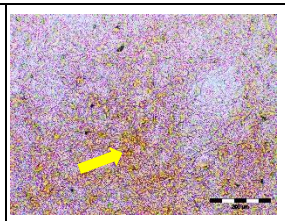
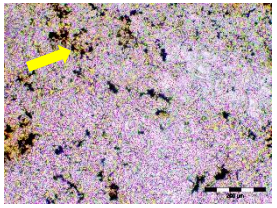
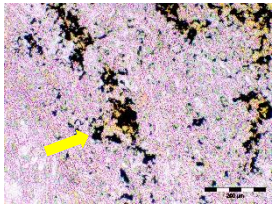
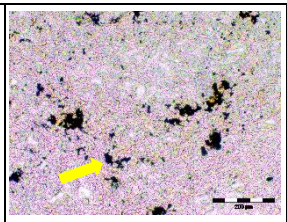
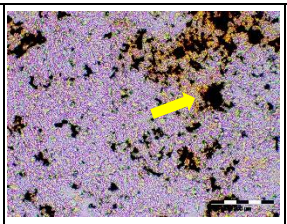
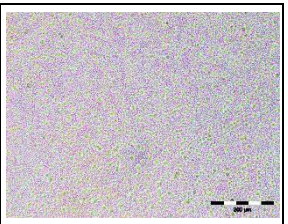
Figure 2. Expression of osteoblast specific genes in MC3T3-E1.

The RT-PCR analysis was performed using RNA isolated from MC3T3-E1 cells after induction into osteoblast cells. The expression of (A) Alp (~510 bp), (B) Dkk2 (~517 bp), (C) ColI (~519 bp)indicates differentiation into osteoblasts. (D) housekeeping gene; Gapdh (~717 bp) was used as positive control. Lane M:

100 bp marker. Lane 1: cell cultured in 1 mg/mL leaves extract; Lane 2: cell cultured in 2 mg/mL leaves extract; Lane 3: cell cultured in 3 mg/mL leaves extract; Lane 4: cell cultured in 4 mg/mL leaves extract; Lane 5: negative control; and Lane 6: positive control (50 µg/mL ascorbic acid plus 10 mM β-glycerophosphate).

(A)

	1 mg/mL P. sarmentosum	2 mg/mL P. sarmentosum	3 mg/mL P. sarmentosum	4 mg/mL P. sarmentosum	Positive Control	Negative Control
Day 0						
Day 5						
Day 10						

Day 14						

(B)

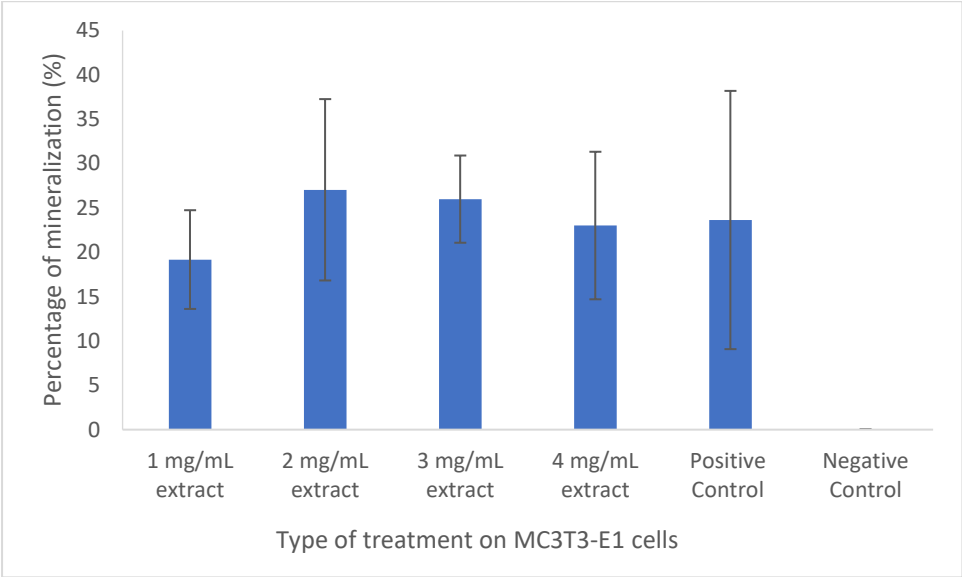


Figure 3. Mineralization of differentiated cells

MC3T3-E1 cells were cultured in the presence of P. sarmentosum extract (1, 2, 3 and 4 mg/mL) for 14 days prior to morphological analysis by von Kossa staining.(A) Brown and dark brown calcium nodules were observed on day 14 indicates MC3T3-E1 differentiation into osteoblasts. The yellow arrows show the formation of light-brown, dark-brown or black nodules. (B) Percentage of mineralization in MC3T3-E1 cells induced by various concentration of P. sarmentosum extracts. There was no significant difference ($P > 0.05$) of mineralization percentage when compared to the positive control (50 µg/mL ascorbic acid plus 10 mM β-glycerophosphate).