Biosaintifika 12 (3) (2020): 384-391 p-ISSN 2085-191X | e-ISSN 2338-7610
Journal of Biology & Biology Education https://journal.unnes.ac.id/nju/index.php/biosaintifika

Alkaline Phosphatase Expression From Mice Mesenchymal Stem Cells Induced By Flamboyant Flower (*Delonix regia*) Extract

Kartini Eriani[1*], Deby Anggraini[1], Yudha Bintoro[2], Ichsan Ichsan[3], Al Azhar[4] , Silmi Mariya[5]

[1]Department of Biology, Faculty of Mathematics and Natural Science, Universitas Syiah Kuala, Indonesia
[2]Department of Molecular Biology, Faculty of Medicine, Universitas Abulyatama, Indonesia
[3]Department of Microbiology, Faculty of Medicine, Univesitas Syiah Kuala, Indonesia
[4]Faculty of Veterinary Medicine, Universitas Syiah Kuala, Indonesia.
[5]Primate Research Center, IPB University, Indonesia.
*Email: kartini_eriani@unsyiah.ac.id

Submitted: 25 August 2020. Revised: 13 September 2020. Accepted: 13 October 2020

Abstract. Flamboyant flower (*Delonix regia*) extract can increase proliferation and differentiation rates of mesenchymal stem cells (MSCs) into specific cells such as bone, nerve, and fibroblast cells. The extract possess metabolic compounds that may act as antibiotics, anti-inflammatory, antimicrobial, diuretic, anthelmentic, astringent, leucorrhoea, and potentially increase the body's metabolism normally. This study aimed to investigate expression level of alkaline phosphatase (ALP) by mice MSCs treated with flamboyant flower extract *in vitro*. Here, mice bone marrow cell cultures were treated with flamboyant flower extracts of 0.6 mg/ml (P1), 0.7 mg/ml (P2), 0.8 mg/ml (P3), and 0.9 mg/ml (P4). Untreated cell culture was used as negative control (P0). Expression of ALP gene was measured by RT-qPCR method. The results showed that mice mesenchymal stem cell could differentiate into bone, nerve, and fibroblast cells. The addition of flamboyant flower extract ranged from 0.6-0.9 mg/ml significantly ($p<0.05$) influenced the expression of ALP by differentiating MSCs. The highest expression was found at the stem cells treated with flamboyant flower extract of 0.8 mg/ml, 0.13 times compared with control. In conclusion, flamboyant flower extracts treatment might increase the expression of ALP in differentiating MSCs. This information can be used as a basis for finding an appropriate biomarkers for tracking the differentiation and profileration of tissue originated MSCs induced by extracts of medicinal plants.

Key words: Alkaline Phosphatase; Actin Beta; Flamboyant Flower Extract; qPCR

How to Cite: Eriani, K., Anggraini, D., Bintoro, Y., Ichsan, I., Azhar, A., & Mariya, S. (2020). Alkaline Phosphatase Expression From Mice Mesenchymal Stem Cells Induced By Flamboyant Flower (*Delonix regia*) Extract. *Biosaintifika: Journal of Biology & Biology Education*, *12*(3), 385-392.

DOI: http://dx.doi.org/10.15294/biosaintifika.v12i3.25433

INTRODUCTION

Flamboyant (*Delonix regia*) is a medicinal plant with potential to be used as a traditional medicine. Previous phytochemical analysis showed that the plant contains terpenoids, tannins, cardiac glycosides, and anthroquinones that may act as antioxidants, anti-diarrheals, anti-inflammatories, hepatoprotectives, and antimicrobials (Singh & Kumar, 2014). Flower extract contains carotenoids that function to regulate enzyme activities and communication between cells, as well as to provide immune response, neoplastic transformation, and growth control (Milani et al., 2017).

Previous studies have identified the role of flamboyant flower extract in improving human health (Wang et al., 2016a), and in particular, extracts from the leaf may be beneficial in immunostimulatory (Eriani et al., 2018a). In general, degenerative diseases are difficult to cure, and the involvement of this plant can play a role in the development of new medical advances in the use of stem cells to cure disease. This flower extract has been predicted by Eriani et al. (2018b) to increase the rate of cell culture differentiation *in vitro*. The number of compounds contained in the flower extract is positively correlated with the rate of cell growth within the culture medium. Based on morphological observations, Eriani et al. (2018b) reported that the addition of flamboyant flower extract to culture media was able to promote differentiation of mice stem cell into fibroblasts and nerve-like cells. This previous research focused on determining optimal dosage of flamboyant flower extract for the induction of cell culture differentiation based on morphological observation, with a standard microscopic method using hematoxylin eosin (HE) staining. The results showed that an increase in flamboyant flower extracts was able to induce cell differentiation into osteoblasts. These cells had a dark, rounded nucleus, located either on the side or at the edge of the cell (Eriani et al., 2018b). The accuracy of determining stem cell differentiation based on morphological evaluation, however, might be limited by

morphological similarities of different cells (Haasters et al., 2009). Additional methods are, therefore, needed to provide more convincing results such as by examining expression of specific genes or molecules involved in and required for cell division. Measuring the changes in expression of these molecules by actively differentiated cells could provide biochemical markers of cell differentiation.

Alkaline phosphatase (ALP) is among the molecules exhibiting increased expression at the time of cell cleavage and differentiation (Fakhry et al., 2013). This enzyme is able to accelerate the formation of cell framework, increase cell links, and induce metabolic processes (Jin et al., 2018). ALP is closely related to the regulation of cell cycle (Nam et al., 2019), body minerals, and cell growth (Millán, 2006; Štefková et al., 2015). It is possible that the change in ALP gene expression might provide a specific molecular marker for the differentiation of mesenchymal stem cell cultures induced by different dosages of flamboyant flower extract. Therefore, this study was conducted to investigate the potential of flamboyant flower extracts to induce bone cell differentiation by analyzing the expression rate of ALP gene by the actively differentiated mesenchymal stem cells (MSCs).

Knowing the potential ALP expression in depicting the differentiation stage of bone originated MSC induced by the addition of flamboyant flower extract should assist current efforts to find an appropriate differentiation biomarkers required for the treatment and diagnosis of a variety of bone diseases. These, in turn, might bring some benefits for overcoming bone related health problems exist in the community.

METHODS

Flamboyant flower extraction

Flower extracts were prepared by maceration using ethanol 96% as a solvent. Briefly, simplicia powders (500 grams) were soaked in 1.5 liters of ethanol 96% for three days. Filtrate was obtained by filtering the macerate using a filter paper and stored in a bottle container at room temperature. The entire filtrate was concentrated using a rotary vacuum evaporator at 60 °C and 80-90 rpm for three days. Subsequently, the extracts were weighed and stored in a sealed, dark container.

Animals

Animals used in this study were male Balb-C strain mice aged two months old, with an average body weight of 30 g. The mice were kept in cages sized 48 cm x 36 cm x 13 cm containing dry rice husk. Food and drink were given *ad libitum*.

Preparation of Mesenchymal Stem Cells (MSCs) cultures from bone marrow

Petri dishes (35 mm in diameters) were coated with gelatin 0.1% for one hour at room temperature. Petri dishes were cleaned from gelatin by rinsing them in a 1x modified phosphate buffer saline (mPBS) solution and dried for 5 minutes. Each petri dish was then filled with 2 ml of modified Dulbecco's modified eagle's medium (mDMEM) and incubated at 37 °C in a 5% CO_2 incubator for 1 hour.

Isolation of primary culture of bone marrow MSCs

The mice were anaesthetized using a combination of 0.01 ml of ketamine (Troy Laboratories PTY Limited) and 0.01 ml of xylazine (Troy Laboratories PTY Limited). All mice were dissected for femur and tibia bones collection. Under a sterile condition, the bones were cleaned from muscles, washed in mPBS, and cut on both ends. The bone marrows were flushed out from bone cavity with mPBS using the syringe. Bone marrow suspensions were collected in a petri dish by repetitive pipetting and spun at 3000 rpm for 10 minutes. Cell pellets were washed four times with mPBS and once with mDMEM. The cells were resuspended in mDMEM, at final concentration of $1x10^6$, and incubated for 24 hours. Cells that adhered to plastic were considered as mesenchymal stem cell (MSC) (Halim et al., 2010). In duplicate, MSC-like cells were cultured in mDMEM medium supplemented with *D. regia* extracts of 0% (m/v) (control), 0.4% (m/v) (P1), 0.6% (m/v) (P2), and 0.8% (m/v) (P3) and 0.9% (m/v) (P4). Cell mixtures were then incubated at 37 °C in a 5% CO_2 incubator for 7 days with mDMEM replacement every two days. On day 8, cell pellets were harvested and subjected for RNA analysis by using RT-PCR.

RNA Analysis

RNA isolation was performed using ReliaPrep™ RNA Cell Miniprep System (Promega, Z6011). In brief, cell cultures were transferred to a 1.5 ml micro-tube and spun at 300 rpm for 5 minutes. Cell pellets were washed in a sterile cold PBS and spun at 300 rpm for 5 mins. Supernatants were added with BL and TG buffers (250 µl each) and mixed by short vortex. Isopropanol, 85 µl, was added to the mixture and homogenized by short vortex. All solution was transferred to a mini-column (in a collection tube) and spun at 14,000 pm for 10 minutes. After discarding filtrate, mini column was put back to the collection tube, added with 500 µl of RNA Wash Solution, and spun at 12,000-14,000 rpm for 30 seconds. DNase I incubation (30 µl) was added to mini column and incubated at 20-25 °C for 15 mins. The column was added with 200 µl of Wash Solution and spun at

12,000-14,000 rpm for 15 seconds. RNA Wash Solution (500 µl) was added dan the column was spun at 12,000-14,000 rpm for 30 seconds. The mini column was transferred to a new collection tube, added with 300 µl of RNA Wash Solution, and spun at 14,000 for 2 mins. Mini column was transferred to a new elution tube, added with 30 µl of nuclease-free water, and spun at 14,000 rpm for 1 min. Purified RNA obtained was stored at -70 °C.

cDNA Synthesis

The cDNA formation process was performed using the GoScript™ Reverse Transcription System (Promega, A5000). Here, a RNA reaction mixture was prepared by mixing 1 µl of RNA sample, 0.1 µl of random primer, and 3.9 µl nuclease free water, and heated at 70 °C for 5 minutes. Reverse transcription reaction mixture was prepared by mixing 4 µl of GoScript™ reaction buffer, 1.2 µl of $MgCl_2$, 1 µl of PCR nucleotide mix, 0.5 µl of Ribonuclease inhibitor recombinant, 1 µl of GoScript™ reverse transcriptase, and 7.3 µl of nuclease free-water. Reverse transcription master mix, 15 µl, was added to each reaction sample. Reverse transcription reaction was run using a BioRad thermal-cycler at the following condition: annealing at 25°C for 5 mins, extension at 37°C for 1 hour, and reverse transcriptase inactivation at 70°C for 15 mins. The cDNA solutions obtained were stored at -20°C.

qPCR master mix preparation

The RT-qPCR process was performed using the GoTaq® qPCR Master Mix (Promega, A6001). Briefly, a 25 µl reaction mixture was prepared by mixing 12.5 µl of GoTaq® qPCR master mix, 7.5 µl of nuclease-free water, 2 µl of each primer, and 3 µl of cDNA samples. The qPCR reaction was run at a Biorad RT-PCR machine. ALP primers used were 5′-CAGACCCTCCCCACGAGT-3′ (forward), and 5'-GTCTTGGAGAGGGCCACAAA-3' (reverse). Beta actin gene amplified using forward primer 5′-ATGAAGATCCTGACCGAGCG-3′ and reverse primer 5'-TACTTGCGCTCAGGAGGAGGC-3' was use as a reference.

Ethics

All procedures involving animals were performed at Syiah Kuala University after ethics approval from the IACUC of this facility. This research used the protocols approved by the Research Ethics Committee of the Faculty of Veterinary Medicine Syiah Kuala University**.**

Statistical Analysis

Data of gene expression was statistically analyzed using unpaired single tailed t-test with level of significance of 0.05.

Bioinformatics Analysis

Primary predictions of ALP were analyzed in silico to see RT-qPCR amplification optimization using online resources namely http://genatlas.medecine.univ-paris5.fr and http://.primer3plus.com/cgibin/dev/primer3plus.cgi

RESULTS AND DISCUSSION

Stem cell differentiation

Microscopic analysis showed morphological characteristics of cells differentiated from MSCs (Figure 1). Three cells might differentiate from the stem cells originated from mice bone marrow were fibroblasts, nerve cells, and bone cells. As shown in Figure 1A-B, fibroblast-like cells had morphology characteristics of branching in cytoplasm, oval-shaped, and large-pore and pale cytoplasm with oval cores accumulated by rough endoplasmic reticulums. Most fibroblasts have components of collagen fibers that function to maintain the integrity of cell membranes.

Morphologically, cells resemble to nerve cells had cytoplasmic elongations in some parts and a centrally located nucleus. These protein-rich cells normally play important roles in electrical polarization of cells by forming a synapse bridge along neurons for signal transduction (Koleske, 2013). Young nerve cells had more cytoplasmic components, but relatively short streaks. The development of young nerve cells into mature nerve cells required many organic components, especially proteins that are important for maintaining integrity of cell membrane (Alberts et al., 2002). This is contrast to adult nerve cells that already have a relatively stable cytoplasmic volume and many axons and dendrites accompanied by long stretches (Koleske, 2013)**.**

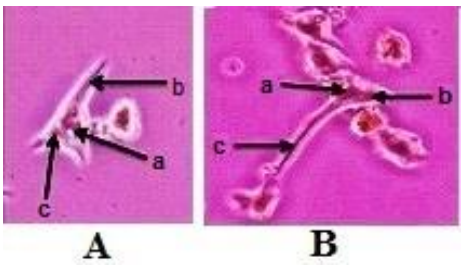

Figure 1. Morphology of fibroblast and nerve-like cells in culture media. The images show morphology of young (A) and adult (B) nerve cells. a. Cell nucleus, b. Cytoplasma, c. Axon.

Morphological characteristics of bone cells are similar with osteoblasts with round-shaped cells that have a large, centrally-located nucleus (Figure 2). The transformation phase of osteoblast cells into osteocytes was accompanied by decreased cell diameter, cytoplasm elongation, and had less density of apparatus and reticular endoplasmic reticulum, but had more lysosomes compared to osteoblasts.

The expression of alkaline phosphatase gene

To provide more accurate cell differentiation validation, one osteoblast differentiated from MSCs was subjected to RT-qPCR method for quantification of ALPgene expression. Previous studies on osteoblast formation indicated that ALP is progenitor of osteoblast cell development (Fakhry et al., 2013). Here, beta actin mRNA was used as a positive control for gene expression. The results showed that ALP gene amplification was seen through the PCR amplification curve of osteoblast differentiated from mice MSCs treated with all four doses (0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml and 0.9 mg/ml) of flamboyant flower extracts (Figure 3). The highest ALP expression occurred at cells treated with flamboyant flower extract of 0.8 mg/ml (Figure 4).

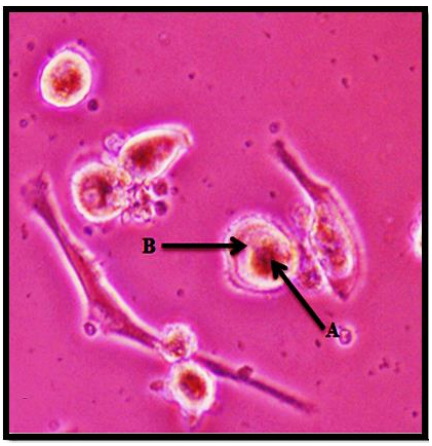

Figure 2. Morphology of osteoblast cells in culture medium added with ethanol extract of flamboyant flower (*D. regia*). (A) Cell nucleus, (B) cytoplasm

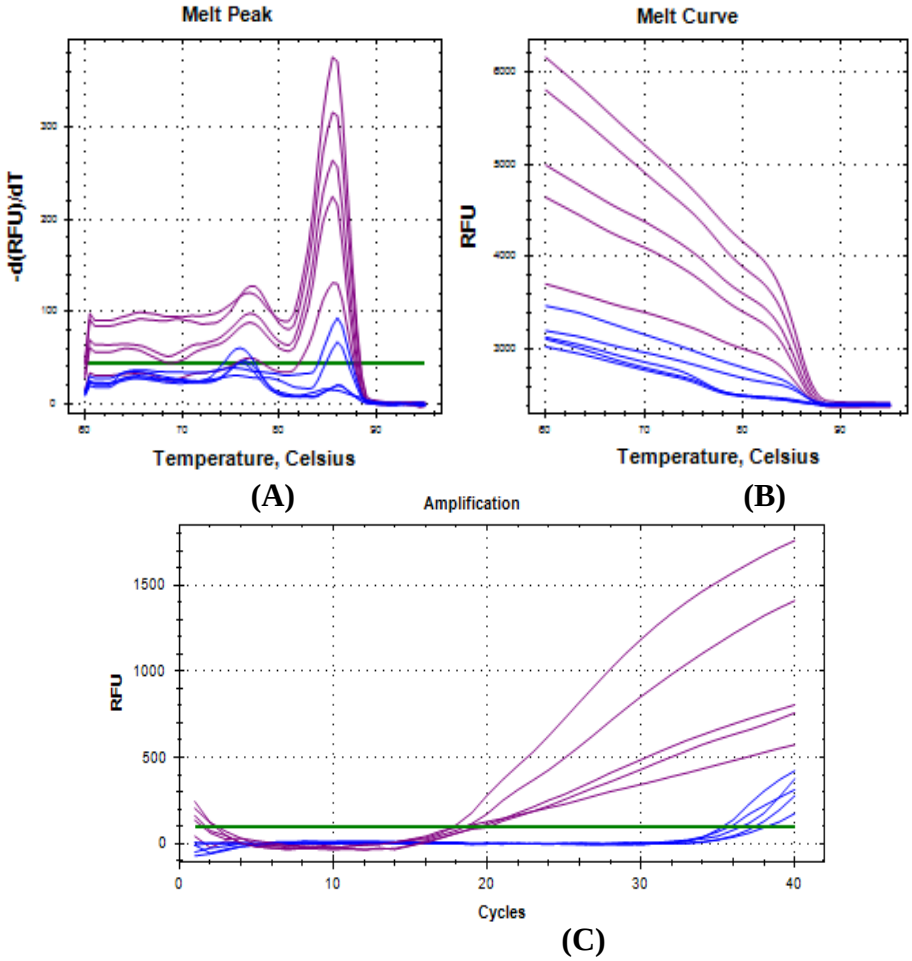

Figure 3. The qPCR amplification of ALP gene expressed by mice MSCs treated with *different concentratins of D. regia* flower extract. The blue and purple curves show the expression of alkalin phosphatase (ALP) and beta actin genes, respectively. A. Melting peak of DNA template. B. Melting curve of DNA template, and C. Amplification cycle starts from cycle 20 to 36.

As illustrated in Figure 3-C, the ALP amplicons were formed in cycle 36 and that of beta-actin were formed in cycle 20. Melting peak of amplification curves (Figure 3-A) indicated variation in temperature requirements for elongation (annealing) of the two protein DNAs. Melting peak of beta actin amplification was about 85 °C and those of ALP ranged from 75-85 °C.

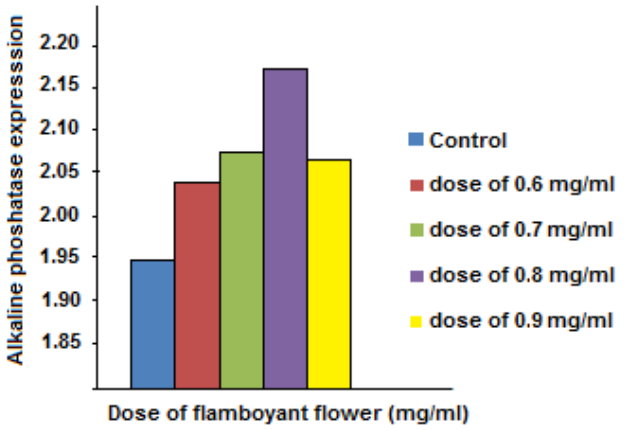

Figure 4. Level of ALP expression among stem cells treated with different doses of flamboyant flowers extract.

This study is an early stage performed to find out potential use of ALP as a specific marker for successful MSCs differentiation induced by flamboyant flower extract. Systematically, preliminary studies were done to investigate the effects of flamboyant flower extract on MSC differentiation based on morphological observation. Standard microscopic examination using hematoxylin eosin (HE) staining protocol showed that the administration of flamboyant flower extract in the concentrations ranged from 0.6-0.9 mg/ml successfully induced the differentiation of MSCs into fibroblast, nerve-like cells, and bone-like cells (Figure 1-2). The results obtained, therefore, support previous findings reported by Eriani et al. (2018a) that flamboyant flower extract was able to induce the differentiation of MSCs originated from mice bone marrow into fibroblast and nerve cells. The study presented additional data about potential of the extract to stimulate differentiation of bone marrow MSCs into bone cells.

Next, moleculer analysis was done to validate cell differentiation and to identify the potency of ALP as a molecular marker for bone stem cells differentiation. This was done by qPCR using forward and reverse primers specific for ALP gene. Samples used were mRNA extracted from fibroblast differentiated from MSCs. Beta actin DNA amplified using appropriate primers was used as positive control. The approach successfully observed ALP expression in all treatment groups (Figure 3). MSCs treated with flamboyant flower extracts of 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, and 0.9 mg/ml expressed 0.09, 0.13, 0.23, and 0.12 times ALP more than that of untreated controls respectively. These were significant ($p<0.05$) compared to that of negative control. The best treatment for the highest ALP expression was the administration of 0.8 mg/ml flamboyant flower extracts (Figure 4). Altogether, results obtained showed potential of ALP as the specific marker for MSCs differentiation into osteoblast cells.

Most celullar biological processes in the body require the involvement of chemical metabolites, encoded gene, and related pathways. During differentiation process, several molecular messengers are synthesized to initiate the cascade of cell formation. The OCT4, SSEA3, SSEA4, SSEA1, TRA-1-60, and TRA-1-81 are among molecules play important roles in cell differentiation (Calloni et al., 2013). Previous study using human embryonic stem cell found increased synthesis of OCT4, NANOG, ALP, and stage-specific embryonic antigen-1 (SSEA1) during cell differentiation *in vitro* (Bram-brink et al., 2008; Li et al., 2017). Therefore, differentiation of mesenchymal, embryonic, and hematopoeietic stem cells might be traced based on the presence of these signaling molecules in cells.

Activation of these so called transcriptional factors might accelerate differentiation process, initiate cell division, and speed up cellular metabolisms. Mediator 1 (Med1), for example, was able to induce Noct1 signal stimulates the transcription of ALP gene, probably by facilitating the interaction between transcription factors and RNA polymerase (Yoshizaki et al., 2017). The importance of Med1 in the formation and differentiation control of most cells including hematopoietic cells, lumen cells and epidermal tissue keratine has been reported (Birmingham et al., 2012**;** Rutkovskiy et al., 2016; Wang et al., 2016b)

ALP is one of molecules plays an important role in the growth and differentiation of bone cells, digestive system organs, and tissue formation. Under normal conditions, concentration of ALP appears to incease in the formation of ATP, osteoblast, immune response, and food digestive organs as well as in cell division (Behera et al., 2017; Nam et al., 2019). Increased ALP expression proportional to exponential increases of bacterial populations indicated its function as one of important factors for cell growth (Behera et al., 2017). The enzyme belongs to orthophosporic monoester phosphorhydroxylase family that was almost detectable in all parts of the body's developing cells (Sharma et al., 2014).

Molecular examination detects ALP presence in the newborn serum, placenta, renal plasma, pregnant women, and gastrointestinal organs (Behera et al., 2017).

The involvement of ALP in phosphate regulation and other chemicals metabolism as well as in regulating activity of cells, tissues and organs has been reported. At the beginning of cell development, ALP concentration increases to meet cellular requirement for active bone and teeth mineralizations (Coulibaly et al., 2012). The formation of cell frameworks becomes more robust and important to enhance cell integrity essential for the continuity of metabolic processes of living cells (Bateman et al., 2015; Coulibaly et al., 2012). At the end of differentiation, cellular ALP decreases because the enzyme is degraded into simple compounds that then excreted into body circulation (Coulibaly et al., 2012).. One study reported that ALP concentration decreases to near normal when healing process of fractures was near perfect, indicating a complete calcification (Coulibaly et al., 2012).

The ability of flamboyant flower extract given to induce and activate growth factors might be related to chemicals content of the plant. Flamboyant flower extract contains auroxantine, mutatochrome, and pyruvic acid that serve as diurectic, anthelmintic, astrigent and leucorrhoea medications . According to Jahan et al. (2010). lupeol, epilupeol, β-sitosterol, stigmasterol, and p-methoxybenzaldehyde from flamboyant flower extracts can suppress microbial populations and degrade toxic properties of chemical compounds. High concentration of the extract, however, might be toxic (Penha-Silva et al., 2007; de Freitas et al., 2008). Administration of 2.8 mg/ml of flamboyant flower extract affects the density of *Plasmodeum breghei in vivo* (Fatmawaty et al., 2017).

Results of analysis on the formation of melting peak curves showed the presence of osteoblast cells in samples test of P1, P2, P3, and P4 because the sigmoidal graph of melting peak curve existed above the threshold base line whereas osteoblast cells were negative in control sample (U0). In addition, it can be said that cells that morphologically resemble to osteoblast cells in MSCs treated to *D. regia* extract of 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, and 0.9 mg/ml were osteoblast cells that expressed ALP whereas in control sample, which also had morphologically osteoblast cells, was categorized into an ALP negative osteoblast cells. These explained that methanol extract of *D. regia* flower was able to induce proliferation and differentiation of MSCs into osteoblast as shown by a detectable ALP gene.

Among chemicals contained in the flower of *D. regia* that were able to trigger osteoblast differentiation is flavonoid, as reported by Torre (2017), that flavonoid compounds could trigger osteoblast differentiation so that the application of the extract in appropriate doses might be able to make MSCs differentiate into osteoblast. In addition, flamboyant plant belongs to legumes from the *Fabacea* family that was predicted to have phytoestrogen, a compound resembles to estrogen (Mazur et al., 1998). Important phytoestrogens contained in these plant family are isoflavonoids named genistein and daidzein (Mazur et al., 1998). Genistein could stimulate differentiation of bone marrow stem cells into osteoblast (Bateman et al., 2015; Torre (2017)). The differentiation occurs due to increased Cbfa-1 (Saud et al., 2017) and transforming growth factor beta-1 (TGFβ-1) (Cheng & Scadden, 2014). Cbfa-1 is a transcriptional factor in the differentiation of progenitor cells into osteoblast and plays a role in controlling developmental, differentiation, and maturation processes of osteoblast (Saud et al., 2017). The TGFβ-1 regulates gene transcription in cells via receptor signaling (Tan et al., 2017). Torre (2017) reported that genistein and daidzein could also enhance osteoblast proliferation via protein synthesis by controlling the synthesis of Cbfa-1 and bone morphogenetic protein-*2* (BMP-2).

Study performed by Eriani et al. (2018b), that also used ethanol extract of *D. regia* flowers, found morphologically osteoblast-like cells differentiated from MSCs treated by 0.8 mg/ml ethanol extract of *D. regia.* Based on the results of this study, therefore, it can be assumed that ethanol extracts of *D. regia* flowers 0.8 mg/ml and 0.9 mg/ml were proven to be able to induce proliferation and differentiation of MSCs into osteoblast. Based on aforementioned information, ethanol extract of *D. regia* flower in the doses of 0.8 mg/ml and 0.9 mg/ml can be used as induction factors for MSCs proliferation and differentiation into osteoblast in the culture media. The osteoblast cells resulted might be applied in stem cell therapy for osteoporotic patients in the future.

Results of this study proved that plant extract is able to cause differentiation of mesenchymal stem cells into bone cells. This approach, when passes comprehensive clinical tests, can be used as one of therapeutic choices for individuals suffered from bone fracture, osteoporosis, and other bone diseases.

CONCLUSIONS

The study showed that administration of flamboyant flower (*D. regia*) extracts ranged from 0.6-0.9 mg/ml (m/v) successfully induced differentiation of mice bone marrow mesenchymal stem cells into fibroblast, nerve, and bone cells. The potential use of alkaline phosphatase gene as a specific marker for mesenchymal stem cell

differentiation *in vitro* was showed by significant increased ($p<0.05$) of gene expression by the treated cells compared to that of by untreated, control cells.

ACKNOWLEDGEMENTS

This research was supported by the Research Institute of Universitas Syiah Kuala through Research Grant (Contract Number: 522/UN11/SPK/PNBP/2019).

REFERENCES

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). *Molecular Biology of the Cell*, 4th Ed. Garland Science, New York.

Bateman, M., Strong, A. L., Burow, M. E., Wang, G., Boue, S. M., Bunnell, B. A. (2015). *Bone Formation: Roles of Genistein and Daidzein in* Genistein and Daidzein: Food Sources, Biological Activity and Health Benefits. Nova Science Publishers, Inc., 111-167.

Behera, B. C., Yadav, H., Singh, S. K., Mishra, R. R., Sethi, B. K., Dutta, S. K., & Thatoi, H. N. (2017). Phosphate solubilization and acid phosphatase activity of Serratia sp. isolated from mangrove soil of Mahanadi river delta, Odisha, India. *J Gen Engin Biotech.* 15, 169-178.

Birmingham, E., Niebur, G. L., McHugh, P. E., Shaw, G., Barry, F. P., & McNamara, L. M. (2012). Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. *European Cells and Materials*. 23, 13-27.

Brambrink, T., Foreman, R., Welstead, G. G., Lengner, C. J., Wernig, M., Suh, H., & Jaenisch, R. (2008). Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. *Cell Stem Cell*. 7, 2(2): 151-159.

Calloni, R, Cordero, E. A. A/, Henriques, J. A. P., & Bonatto, D. (2013). Reviewing and Updating the major molecular markers for stem cells. *Stem Cells Develop.* 22(9), 1455-1476.

Cheng, T. & Scadden, D. T. (2014). Cell cycle regulators in stem cells. *In*: Lanza, R. & Atala, R. *Essentials of Stem Cell Biology*. Chapter 8. Academic Press, New York.

Coulibaly, M. O., Sietsema, D. L., Burgers, T. A., Mason, J., Williams, B. O., Clifford, B., & Jones, C. B. (2010). Recent Advances in the use of serological bone formation markers to monitor callus development and fracture healing. *Crit Rev Eukaryot Gene Expr*. 20(2), 105-127

de Freitas, M. V., Netto, R. D. C. M., da Costa Huss, J. C. et al. (2008). Influence of aqueous crude extracts of medicinal plants on the osmotic stability of human erythrocytes, *Toxicol in Vitro*. 22(1), 219–224.

Eriani, K, Ainsyah, Rosnizar, Yunita, Ichsan, & Azhar, A. (2018a). Immunostimulatory effect, of methanol extract of flamboyant leaf [*Delonix regia* (Boj. ex Hook.) Raf.] in mice. *Natural*. 18(1), 44-48.

Eriani, K, Suryani, I, Azhar, A, Nursanty, R, Ichsan, I, & Boediono, A. (2018b). Neurogenic differentiation of bone marrow mesenchymal-like stem cell induced by *Delonix regia* flowers extract. *Biosaintifika*. 10(2), 417-423

Fakhry, M., Hamade, E., Badran, B., Buchet, R., & Magne, D. (2013). Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts. *World J Stem Cells*. 5(4), 136-148.

Fatmawaty, Rosmalena, Amalia, A., Syafitri, I., Prasasty, V. D. (2017). Antimalarial effect of flamboyant (*Delonix regia*) bark and papaya (*Carica papaya* L) leaf ethanolic extracts against *Plasmodium berghei* in mice. *Biomed Pharmacol J*. 10(3), 1081-1089.

Jahan, I., Rahman, S. M., Rahman, S. M., Kaisar, A. M., Islam, S. M., Wahab, A., & Rashid, A. M. (2010). Chemical and biological investigations of *Delonix regia* (Bojer ex Hook.) Raf. *Acta Pharm*. 60, 207-215.

Jin, K., Ban, T. H., Jung, J. Y., Kim, A. J., Kim, Y., Lee, S. Y., et al. 2018. Stabilization of serum alkaline phosphatase in hemodialysis patients by implementation of local chronic kidney disease-mineral bone disorder management strategy: a quality improvement study. *Kidney Res Clin Pract*. 37, 157e66.

Štefková, K., Procházková, J., & Pacherník, J. (2015). Alkaline phosphatase in stem cells., *Stem Cells Int.* 2015, 1-11.

Koleske, A. J. (2013). Molecular mechanisms of dendrite stability. *Nat Rev Neurosci*. 14(8), 536-550. doi: 10.1038/nrn3486

Kurniawati, Y., Adi, S., Achadiyani, Suwarsa, O., Erlangga, D., & Putri, T. (2015). Culture of primary fibroblast: A preliminary research. *Artikel Penelitian*. 38(1), 34.

Li, D., Secher, J. O., Juhl, M., Mashayekhi, K., Nielsen, T. T., Holst, B., Hyttel, P., Freude, K. K., & Hall, V. J. (2017). Identification of SSEA-1 expressing enhanced reprogramming (SEER) cells in porcine embryonic fibroblasts. *Cell Cycle*. 16(11), 1070–1084

Milani, A., Basirnejad, M., Shahbazi, S., & Bolhassani, A. (2017). Carotenoids: Biochemistry, pharmacology and treatment. *British J Pharm*. 174, 1290-1324.

Nam, K. H., Vesela, I., Siismets, E., & Hatch, E. N. (2019). Tissue nonspecific alkaline phosphatase

promotes calvarial progenitor cell cycle progression and cytokinesis via Erk1,2. *Bone*, 120, 125-136.

Penha-Silva, N., Firmino, C. B., de Freitas Reis F. G. et al. (2007), Influence of age on the stability of human erythrocyte membranes. *Mechanisms of Ageing and Development*. 128(7-8), 444-449.

Rutkovskiy, E. A., Stensløkken, E. K. O., & Vaage, I. J. (2016). Osteoblast differentiation at a glance. *Med Sci Monit Basic Res*. 22, 95-106

Sharma, U. Pal, D. & Prasad, R. (2014). A novel role of alkaline phosphatase in the ERK1/2 dephosphorylation in renal cell carcinoma cell lines: A new plausible therapeutic target. Biochimie. 107, 406-409

Singh, S., & Kumar, N. S. (2014). A Review: Introduction to genus Delonix. *WorldJ Pharmacy Pharmaceut Sci*. 3(6): 2042-2055.

Saud, B. Malla, R. & Shrestha, K. (2017). A review on the effect of plant extract on mesenchymal stem cell proliferation and differentiation. *Stem Cells Int*. 2019, 1-13

Tan, W., Tan, Q., Wang, T., Lian, M., Zhang, L., & Cheng, Z. (2017). Calpain 1 regulates TGF-β1-induced epithelial-mesenchymal transition in human lung epithelial cells via PI3K/Akt signaling pathway. *Am J Transl Res*. 9(3):1402-1409

Torre, E. 2017. Molecular signaling mechanisms behind polyphenol-induced bone anabolism. *Phytochem Rev*. 16: 1183-1226

Wang, L. S., Lee, C. T., Su, W. L., Huang, S. C., & Wang, S. C. (2016). Delonix regia leaf extract (DRLE): a potential therapeutic agent for cardioprotection. *PloS one*, 11(12), e0167768.

Wang, C., Meng, H., Wang, X., Zhao, C., Peng, J., & Wang, Y. (2016b). Differentiation of bone marrow mesenchymal stem cells in osteoblasts and adipocytes and its role in treatment of osteoporosis. *Med Sci Monit*. 22, 226-233.

Yoshizaki, K., Hu, L., Nguyen, T., Sakai, K., Ishikawa, M., Takahasi, I., Fukumoto, S., DenBesten, P. L., Bikle, D. D., Oda, Y., & Yamada, Y. (2017). Mediator 1 contributes to enamel mineralization as a coactivator for Notch1 signaling and stimulates transcription of the alkaline phosphatase gene. *J Biol Chem*. 292(33), 13531-13540.