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Original Article

# The effect of *Cissus quadrangularis* Salisb. extract on maturation of rat mesenchymal stem cells

## O efeito do extrato de *Cissus quadrangularis* Salisb. na proliferação e diferenciação de células-tronco mesenquimais de rato

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**Abstract**

Degenerative diseases, such as osteoporosis, could be treated by stem cells. The aim of this study was to identify the gene expression of bone marrow mesenchymal stem cells (BM-MSC) derived from Sprague Dawley rats and to assess the effect of *Cissus quadrangularis* Salisb. extract on their maturation into bone cells. The BM-MSC were divided into three groups: (a) BM-MSCs + osteoblast cell growth basal medium as the positive control; (b) BM-MSCs + Dulbecco's modified eagle's medium (DMEM) + 0.3 mg/mL methanol extract of *C. quadrangularis* as methanol group; and (c) BM-MSC + DMEM + 0.3 mg/mL ethyl acetate extract of *C. quadrangularis* as ethyl acetate group. A relative quantification approach using was used to analyze the expression of the *alp* (alkaline phosphatase) gene, with the *beta-actin* gene was used to normalize the expression of the *alp* gene. The intra-assay variation was calculated to validate the RT-qPCR data. Our study found that the intra-assay variation value was acceptable, with most of the coefficients of variability (CV) value <5. Ethyl acetate solvent outperformed methanol solvent in extracting the active compound *C. quadrangularis*. In the ethyl acetate extract group, the expression of the *alp* gene increased three times compared to the positive control. In methanol extract group, the expression of *alp* gene was lower six times compared to positive control. This study suggests that *C. quadrangularis* extracts using ethyl acetate could induce the maturation of BM-MSCs. However, further studies are warrant to confirm this effect using different indicators.

**Keywords:** Cissus quadrangularis, osteoporosis, mesenchymal stem cell, qPCR.

**Resumo**

Doenças degenerativas, como a osteoporose, podem ser tratadas por células-tronco. O objetivo deste estudo foi identificar a expressão gênica de células-tronco mesenquimais da medula óssea (BM-MSCs) derivadas de ratos Sprague Dawley, bem como sua proliferação e diferenciação em células ósseas afetadas pelo extrato de *Cissus quadrangularis* Salisb. As BM-MSCs foram divididas em três grupos: (1) BM-MSCs + meio basal de crescimento de osteoblastos como controle positivo; (2) BM-MSCs + meio de Eagle modificado por Dulbecco (DMEM) + extrato de metanol; e (3) BM-MSCs + DMEM + extrato de acetato de etila de *C. quadrangularis*. Uma abordagem de quantificação relativa foi usada para analisar a expressão do gene alp (fosfatase alcalina), com o gene da beta-actina sendo usado para normalizar a expressão do gene alp em cada tratamento. A variação intraensaio foi calculada para validar os dados de RT-qPCR. Os resultados mostraram que o valor da variação intraensaio é aceitável, com a maioria dos valores dos coeficientes de variabilidade (CV) < 5. Além disso, no tratamento T2, a expressão do gene alp aumentou três vezes em relação ao controle positivo. No tratamento T1, o gene alp apresentou nível de expressão seis vezes menor em comparação com o controle positivo. Além disso, o solvente acetato de etila superou o solvente metanol na extração do composto ativo *C. quadrangularis* Salisb. Este estudo demonstra que extratos de *C. quadrangularis* Salisb., particularmente com acetato de etila como solvente, podem induzir a proliferação e diferenciação de BM-MSCs. Com base na expressão do gene alp, *C. quadrangularis* tem o potencial de corrigir a perda óssea.

**Palavras-chave:** *Cissus quadrangularis*, osteoporose, células-tronco mesenquimais, qPCR

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## 1. Introduction

Osteoporosis is a degenerative disease that decreases bone density and leads to an increased risk of fractures (Halim et al., 2010; Lukman and Juniarti, 2008). Approximately 200 million people worldwide have osteoporosis, and 8.9 million of them have fracture each year and those aged older than 50-years-old, osteoporosis affects one in three women and one in five men (International Osteoporosis Foundation, 2015; Wardhana, 2012). Since the majority of osteoporosis drugs used today have side effects that include bone resorption and blood clotting, the stem cell could be a promising therapy due to its capability of cell renewal and differentiation in restoring bone mass (Sandhaanam et al., 2013).

One of the factors affecting the differentiation of stem cells is growth factors that could be obtained from phytochemicals (Halim et al., 2010; Kartini Eriani, 2022; Vandebroek et al., 2004). *Cissus quadrangularis* Salisb., a widely-known medicinal plant for rheumatism and fractures in Indonesia, contains a high level of phytoestrogens which are plant-based compound that mimic human estrogen. The compounds of phytoestrogens such as flavonoids, resveratrol and piceatannol, have been proven to have antiosteoporosis activities (Mustafa et al., 2011; Rao et al., 2007). Additionally, the plant also consists of a high level of calcium and phosphorus which are essential for bone growth (Shah, 2011).

Osteoporosis develops as a result of low estrogen levels, particularly in postmenopausal women. Phytoestrogens could help substituting the estrogens and subsequently stimulate osteoblasts and osteocytes through estrogen receptors in osteogenic cells for the osteogenesis process (Ceriana et al., 2015; Djuwita et al., 2012; Ohashi et al., 1991; Štefková et al., 2015).

The present work aimed to assess the effect of *C. quadrangularis* extract with two different solvents on maturation of bone marrow mesenchymal stem cells (BM-MSC) into bone cells by targeting *alp* gene as the marker of MSC maturation.

## 2. Methods

### *2.1. Study design*

To determine the effect of *C. quadrangularis* extract on maturation of BM-MSCs that were isolated from Sprague Dawley rats, an in vitro experiment was conducted. The extraction of *C. quadrangularis* was conducted using two types of solvents: polar (methanol) and semi-polar (ethyl acetate). Three groups were used: T1 (Dulbecco's modified eagle's medium (DMEM) + BM-MSCs + *C. quadrangularis* methanol extract, 0.3 mg/mL), T2 (DMEM + BM-MSCs + *C. quadrangularis* ethyl acetate extract, 0.3 mg/mL), and a positive control group (osteoblast cell growth basal medium + BM-MSCs). After ten days of incubation and exposure, the effects of *C. quadrangularis* extracts on maturation of MSCs were assessed.

The use of 0.3 mg/mL of *C. quadrangularis* extract was based on the results of previous study (Ceriana et al., 2015). The study used *C. quadrangularis* ethanol extract at various concentrations ranged from 0.1 mg/mL, 0.3 mg/mL, 0.6 mg/mL, and 0.9 mg/mL and the best result demonstrated at a concentration of 0.3 mg/mL (Ceriana et al., 2015). The present study used two types of solvents, methanol which is polar and ethyl acetate which is semi polar, to determine which solvent is better for extracting the active compounds from the *C. quadrangularis*.

### *2.2. Extraction of C. quadrangularis Salisb.*

Briefly, *C. quadrangularis* was washed by water and sun-dried for three days. Dried plants were powdered using a DF-15 grinder (CGOLDENWALL, Hammer Mill Grinder Commercial Electric, USA). A total of 500 g of simplicial powder was soaked in 1 L of technical grade ethyl acetate for three days until the surface was submerged by the solvent. The maserat was then collected and filtered. At a temperature of 40°C, the filtrate was evaporated using a BUCHI R-300 Rotary Evaporator (BÜCHI Labortechnik AG, Postfach, Germany). The residue was passaged for three times every three days with 1 L technical grade methanol. The concentrated extract was then weighed and refrigerated.

### *2.3. Dulbecco's modified eagle's medium (DMEM) preparation*

Briefly, 1 L DMEM stock culture medium was prepared by homogenizing 1 g of DMEM powder with aquadest. The solution was mixed with 0.37 g of $NaHCO_3$, 100 µL of non-essential amino acids (Sigma-Aldrich Pte Ltd, Singapore), 100 µL of insulin transferrin selenium (Thermo Fisher Scientific, Carlsbad, CA, US), 100 L of gentamicin (Sigma-Aldrich Pte Ltd, Singapore) and 10 mL of 10% fetal calf serum (Sigma-Aldrich Pte Ltd, Singapore). The mixture was then sterilized using microfiltration with a 0.22 µm diameter (Eriani et al., 2018).

### *2.4. The animals*

Eight-week-old male Sprague Dawley rats were acclimated and fed *ad libitum* with a standardized diet for one week in laboratory settings which included a 12-hour light-dark cycle, 60% humidity and temperature approximately 23°C. Three rats were used, with the assumption that one rat's MSCs were sufficient for one set of treatments for a group. Before being sacrificed, the animals were kept in a facility that was cleaned and disinfected regularly. The facility was kept quiet during one week of the acclimation period, with controlled environmental conditions. The animals were sacrificed using a cervical dislocation by a certificated veterinarian. To reduce the pain, the rats were injected with 0.01 ml of ketamine and 0.01 ml of xylazine (both from Troy Laboratories PTY Limited, NSW, Australia) before the euthanasia.

### *2.5. Isolation and culture of BM-MSCs*

After the euthanasia, the femur and tibia were extracted. Phosphate buffered saline (PBS) solution was used to clean the bones of muscle and fat tissue. After placing the bone in a Petri dish with a sterile PBS solution, both ends of the femur and tibia were cut with scissors.

The bone was then placed in a Falcon tube with 10 ml of sterile PBS containing 1% antibiotic and centrifuged at 3000 rpm for 15 minutes until the bone marrow emerged from the femur. The supernatant was discarded and 10 mL of PBS was added and centrifuged again 2 times. The final centrifugation included the addition of a growth medium. The cell precipitate was added to the growth medium before being grown in a T25 culture flask in a 5%$CO_2$ incubator at 37°C. Subcultures were performed when the cell population reached 70–80% confluence and the growth medium was changed every 3–4 days.

### *2.6. BM-MSCs characterization using the PCR technique*

The BM-MSCs were characterized by targeting genes encoding MSC surface proteins (CD73 and CD105) as well as markers of hemopoietic stem cells (CD34 and CD45). To ensure that isolated cells were not contaminated with hematopoietic cells, hematopoietic stem cell markers were also screened (CD34 and CD45). PCR was carried out using the protocol on GoTaq G2 Master Mixes (Promega, Wisconsin, USA, catalogue number #M2782A), and 2 µL forward and reverse primers. The detailed primer sequences are presented in Table 1. The PCR product was then analysed on 1% agarose gel electrophoresis with TAE buffer run at 80 V for 60 min and then visualized using UV light at 312 nm.

### *2.7. Osteocyte differentiation*

The MSC suspension was plated at 1x$10^6$ cells/$cm^2$ in DMEM for the treatment groups while the positive control was in the osteoblast cell growth basal medium. After one day of incubation at 37°C, each plate was washed with PBS to exclude nonadherent cells followed by media exchange. The treatment groups were respectively added 0.3 mg/mL of methanol and ethyl acetate extract of *C. quadrangularis,* while positive control received only standard media for osteocyte differentiation (OBM Osteoblast Growth and Differentiation Basal Medium, Lonza, Tuas, Singapore). The media was exchanged every 3-4 days until the tenth day.

### *2.8. mRNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)*

The Rneasy kit (Qiagen, Maryland, USA) was used to extract mRNA from BM-MSCs of each experimental group. The concentration of mRNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, California, USA) at wavelengths of 260 and 280 nm. Using the SuperScript III Reverse Transcriptase kit (Invitrogen, Massachusetts, USA), the mRNA samples were reverse transcribed into cDNA strands. The mRNA extraction and reverse transcriptase PCR procedures followed to the manufacturers' protocols. The reversed transcriptional cDNAs were used in PCR and quantitative polymerase chain reaction (qPCR).

### *2.9. Quantitative polymerase chain reaction (qPCR) analysis of alp gene expression*

To assess the maturation osteoblast, the expression of *alp* gene was determined. The *alp* gene encodes alkaline phosphatase and is a key marker of MSC differentiation into bone cells. The mRNA extraction was carried out according to the method described above. The qPCR process was carried out using the GoTaq® qPCR Master Mix kit (Promega, Wisconsin, USA). The qPCR Master Mix reagent was prepared by mixing 12.5 µl of GoTaq® qPCR Master Mix, 7.5 µL of free-water nuclease, and 2 µl of primer each. The primer sequence used for alp forward 5' CAGACCCCCCACGAGT 3', and reverse 5'GTCTTGGAGGGCCACAAA3'. Primer for beta-actin forward 5'ATGAAGATCCTGACCGAGCG3' reverse 5'TACTTGCGCTCAGGAGGAGC3'. The qPCR Master Mix was then mixed with 3 µL of cDNA samples in each test tube. All samples were evaluated using RT-PCR (Bio-Rad, California, USA).

### *2.10. Statical analysis*

To compare the expression of alp gene among groups, analysis of variance (Anova) was used followed with Tukey honestly significant difference (HSD) analyses. The p-value <0.05 was considered significant. The analyses were conducted using SPPS for Windows version 20.

### *2.11. Ethical clearance*

The Animal Ethics Commission of PT. Bimana Indomedical approved the procedure involving animals in this study (No R.07-20-IR). Efforts were made to reduce the pain, suffering, and distress felt by the rats. The animals were given appropriate housing and ad libitum feeding, anesthesia was used to minimize pain before the animals were sacrificed, and all procedures were carried out by a certified veterinarian with animal care training.

**Table 1.** Primers used for detection of markers of mesenchymal stem cells

| Primer | Forward and reverse sequence | References |
|---|---|---|
| CD73 | F 5′CCCGGGGGCCACTAGCACCTCA3′<br>R 5′GCCTGGACCACGGGAACCTT3′ | (Tamajusuku et al., 2006) |
| CD105 | F 5'CTGGAGCAGGGACGTTGT3'<br>R 5'GCTCCACGCCTTTGACC3' | (Sympson et al., 1994) |
| CD34 | F 5'CGGCTATTTCCTGATGAACC3'<br>R 5'CCGTGTAATAAGGGTCTTCACC3' | (Nagasaki et al., 2011) |
| CD45 | F 5'GGGAGGGTGCAACGAAATG3'<br>R 5'TGCCTGGACGAGCTGCTT3' | (Nagasaki et al., 2011) |

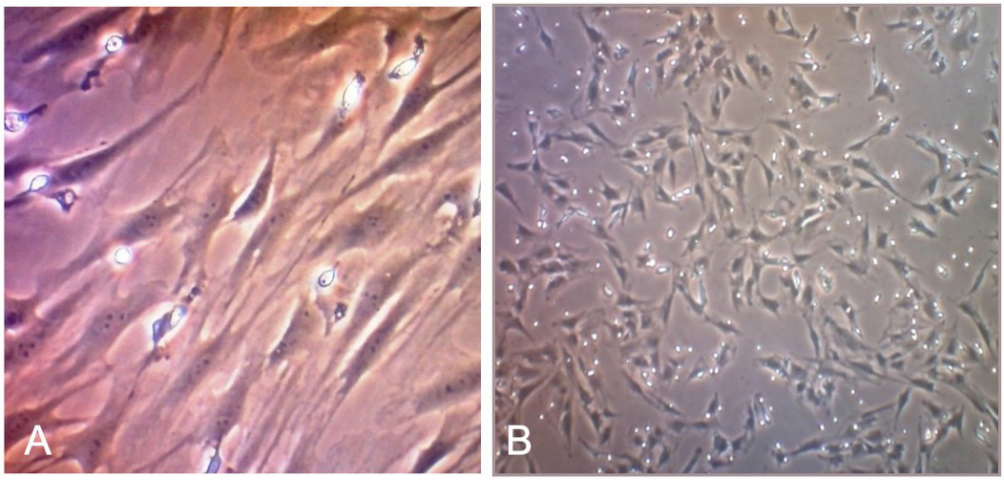


**Figure 1.** The culture of mesenchymal stem cells (MSCs) (A) The MSCs population was attached to the substrate and the culture. (B) The MSCs with fibroblast-like morphology in 60-70% confluence with 32x magnification.

## 3. Results

### *3.1. Mesenchymal stem cell morphology*

Primary cell culture observations revealed the presence of mesenchymal-like cells with cytoplasmic projections in the form of polyhedral and more than one visible nucleolus in each cell. These were because the cells in the culture medium have not been fully identified, but the isolated cells demonstrated the unique characteristics found in MSC. The morphology of the mesenchymal like-cell obtained from observations in the culture medium is presented in Figure 1.

### *3.2. Morphological analysis of bone cells*

On the 10$^{th}$ day of morphological observation, we discovered two types of osteoblasts: undifferentiated and differentiated osteoblasts (Figure 2). Undifferentiated osteoblast cells have an intact shape and no cytoplasmic extensions. While differentiated osteoblast cells have multiple cytoplasmic projections in preparation for differentiation into osteocytes.

### *3.3. Surface marker analysis of mesenchymal stem cells*

The genes of MSCs were identified by targeting genes encoding surface marker proteins in the form of a cluster of differentiation (CD) and the PCR results for each marker are presented in Figure 3.

### *3.4. RT-qPCR analysis of gene expression*

Using the *alp* gene-encoded alkaline phosphatase as a key marker and beta-actin as a calibrator, an intra assay variation study (CV) was performed in each gene, both beta-actin and alp genes, to validate and test the repeatability of the RT-qPCR data obtained. The results of the calculation of intra assay variation for each gene are shown in Table 2 and 3. The expression of the alp gene increased 3 times compared to the positive control, in the treatment with ethyl acetate extract *C. quadrangularis* 0.3 mg/ml (Figure 4).

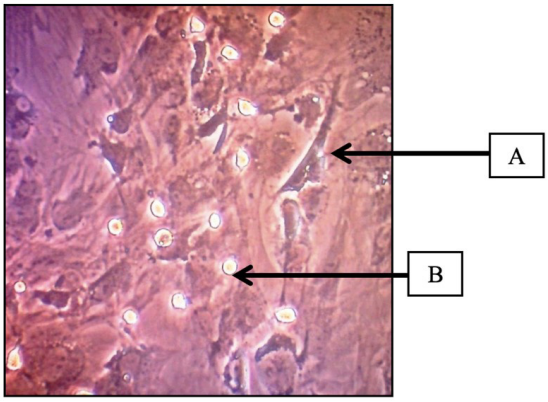


**Figure 2.** The morphology of osteoblast-like cells. (A) Differentiating osteoblasts, (B) Undifferentiated osteoblasts.

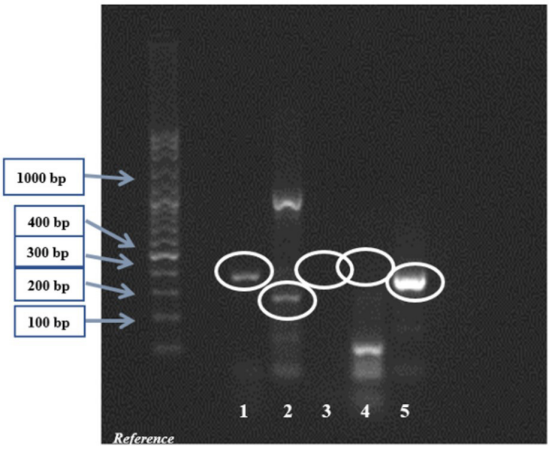


**Figure 3.** MSC confirmation PCR results targeting genes encoding surface marker CD (1. CD73 403bp (positive); 2.CD105 225 bp (positive); 3.CD34 450 bp (negative); 4.CD45 450 bp (negative); 5. GAPDH 352 bp (positive)),

**Table 2.** Intra-assay coefficients of variability (CV) for beta-actin gene in each sample.

| Sample | Mean cycle threshold value | Standard deviation | % CV |
|---|---|---|---|
| Positive control | 25.80 | 0.75 | 2.94 |
| P1 (*C. quadrangularis* methanol extract 0.3mg/ml) | 26.09 | 0.83 | 3.21 |
| P2 (*C. quadrangularis* ethyl acetate extract 0.3mg/ml) | 25.18 | 0.81 | 3.22 |

**Table 3.** Intra-assay coefficients of variability (CV) for the *alp* gene in each sample.

| Sample | Mean cycle threshold value | Standard deviation | % CV |
|---|---|---|---|
| Positive control | 29.34 | 1.22 | 4.17 |
| P1 (*C. quadrangularis* methanol extract 0.3mg/ml) | 31.41 | 1.82 | 5.81 |
| P2 (*C. quadrangularis* ethyl acetate extract 0.3mg/ml | 27.42 | 0.99 | 3.64 |

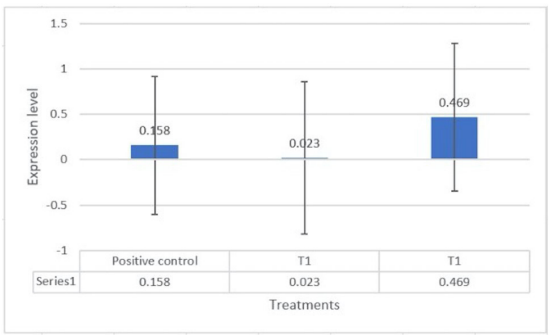


**Figure 4.** A graph comparing the levels of *alp* gene expression in each treatment to positive controls.

In contrast, the alp gene experienced a 6 times lower pattern of expression level decline than the positive control in the treatment with 0.3mg/ml *C. quadrangularis* methanol extract. However, our Anova analysis found that there was no significant different of alp gene expression among groups (p=0.880). Post-hoc analyses using Tukey HSD also found no different between control and methanol extract groups (p=0.944); between control and acetate extract groups (p=0.872); and between methanol and acetate extract groups (p=0.983).

## 4. Discussion

MSCs have morphological characteristics such as high regeneration capacity, and spindle-shaped and flattened cells, with one or more nucleoli (Haasters et al., 2009; Kuehnel, 2003). They have a morphology similar to fibroblast cells (Figure 1A), with MSCs characteristics, aside from their ability to adhere to culture dishes (Prockop, 1997). Fibroblasts are extracellular connective tissues that are commonly found in bone marrow cell cultures. Our study showed the sufficient confluence of MSCs indicating that the cultured cells can be subcultured. Furthermore, the differentiated cells in large quantities denote that the cells can be harvested easily for prospective use (Gimble et al., 2007).

Morphological observations on 10th day revealed several cells, including osteoblasts (young osteocytes) with polygonal cell morphology, large size, and large nuclei (Figure 2). The absence of osteocytes (adult osteoblasts) might be attributed to their normal appearance period of days 14-28 (Hoemann et al., 2009). However, molecular identification is required to determine whether the cell type is MSCs.

In our study, the MSCs population in this study was not contaminated with hematopoietic stem cells and the expression of CD73 and CD 105 was presented in the differentiated MSCs. Previous findings explained that CD73 exhibits osteogenic differentiation capacity, helps bone grow bone upon fracture repair and aids in fracture healing. On the other hand, CD105 is often associated to chondrogenic potential. Through extracellular matrix secretion, the development of a cartilage callus or endochondral ossification, and the control of bone regeneration during the healing process, chondrocytes are involved in the healing of fractures. A study explained that MSCs have following characteristics: 1. adhered to plastic; 2. expressed surface MSCs specific markers (CD105, CD73, and CD90), but none of hematopoietic markers (CD19, CD14, and CD90). CD45) as well as HLA-DR; and 3. are able to differentiate into osteoblasts, adipocytes and chondroblasts (International Osteoporosis Foundation, 2015). The phenotype analysis using a multicolour flow cytometric (FACS) revealed that the BM-MSCs in rats expressed CD29, CD44, CD105, and Sca-1, but otherwise for CD34, TER-119, CD45, and CD11b (Sung et al., 2008).

We validate the RT-qPCR data by calculating the intra-assay coefficients of variability (CV) (Bustin et al., 2009; Kralik and Ricchi, 2017). Most of intra-assay CV for each gene in this study is less than 5%, except the alp gene in methanol extract group (5.8%) (Table 2 and 3). However, the intra-assay CV less than 10 is still acceptable.

The CV of ≤5% indicates the obtained data is qualified which reflects that the study has a high value of repeatability (Zheng et al., 2019).

The proliferation and differentiation of MSCs were confirmed molecularly to identify the presence of osteoblasts and osteocytes. Beta-Actin, the housekeeping gene (HKG), was used as a calibrator to normalize the data of alp gene expression derived from RT-qPCR to obtain robust and consistent results. This calibrator has been widely used and has stable gene expression (Chen et al., 2019; Rismaya et al., 2017). Meanwhile, The alp gene, which encodes alkaline phosphatase, has been identified as a key marker of MSC differentiation into the bone cells (Štefková et al., 2015).

Our study found that ethyl acetate was more effective than methanol in dissolving the active compounds of *C. quadrangularis*, which are required for the proliferation and differentiation of MSCs into osteoblasts and osteocytes (Figure 4). Ethyl acetate is a semi-polar solvent with a broad polarity range that can attract both polar and non-polar compounds and has low toxicity. On the other hand, methanol is a polar solvent that only dissolves polar compounds. Phytochemical studies on *C. quadrangularis* revealed high phytoestrogen compounds like flavonoids, resveratrol and piceatannol (Rao et al., 2007). Since lack of estrogens attributed to the bone loss and bone turnover, phytoestrogens may replace the estrogen deficits and help the calcium absorption which subsequently act as antiosteoporosis (Mustafa et al., 2011). The plants also contain a high calcium and phosphorus, which are important for bone growth (Shah, 2011). This study highlights *C. quadrangularis* is potentially applied to human bone cells to treat bone degenerative diseases.

## 5. Conclusion

The expression of *alp* gene increased three times in ethyl acetate extract of *C. quadrangularis* at 0.3 mg/ml compared to the positive control. On the contrary, the alp gene decreased six times in methanol extract than in the positive control. The active compounds required for the proliferation and differentiation of BM-MSCs from *C. quadrangularis* were extracted more efficiently in ethyl acetate than methanol.

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