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The Effect of Ciplukan (*Physalis minima*) Leaf Extract on Mesenchymal Stem Cell Proliferation and Population Doubling Time (PDT) In Vitro

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Abstract. *Physalis minima*has been used as herbal medicine because it is believed by the community to cure neurodegenerative and cardiovascular diseases. This study aims to determine the effectiveness of *P.minima* extract in increasing the proliferation of mesenchymal stem cells (MSC) from mouse (*Musmusculus*) bone marrow (BM). BM from the femur and tibia were isolated using a flushing method. BM-MSC primary culture was conducted in mesenPRO® medium at 37°C in a 5% CO_2 incubator until it reached a 70% confluence.BM-MSCs were sub-cultured overnight in Dulbecco's Modified Eagle's Media (mDMEM). The mDMEM was replaced with a treatment medium on the second day of subculture. The treatment medium was changed every three days and evaluated under an inverted microscope by counting the number of cells at the beginning and the end of the incubation period. The proliferation rate is expressed as PDT, which was statistically analyzed using ANOVA at a significance level of 0.05% and followed-up with Duncan's test. Statistically, *P. minima* leaf extract could significantly reduce the PDT value. The optimum dose of *P. minima* leaf extract that can increase the proliferation of BM-MSC was 0.8 mg/ml. It is concluded that *P. minima* leaf extract was effective as an inducer of BM-MSC proliferation. The data obtained is the preliminary data on the use of *P.minima*extracts in stem cell-based therapy. The results of this study provide important information in scientifically proving the potential of *P. minima* extract on stem cell proliferation.

Key words: Bone marrow, Culture, Mesenchymal-like cell, *Physalis minima*, Proliferation

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INTRODUCTION

Physalis minima is an important species of the physalis genus, a promising medicinal plant. This plant grows wild in tropical and subtropical regions (Ma et al., 2007); distributed in several countries, including Indonesia, Malaysia, India, Baluchistan, Afghanistan, tropical parts of Africa, Singapore, and Australia (Chotani and Vaghasiya, 2012). *P. minima* is commonly found in dry rice fields, forests, edges of irrigation embankments, and post-harvest plantations in Indonesia. The plant has a high tolerance to various environmental conditions (Hadiyanti et al., 2018).

P. minima are often used as raw material for herbal medicines (Kemenkes RI, 2015). From an ethnobotany perspective, the community believes this plant can cure neurodegenerative and cardiovascular diseases. *P. minima* alsodemonstrate antipyretic, analgesic, anti-inflammatory, cytotoxic, antiarthritis, antioxidant, immunomodulatory, and antidiabetic properties.

Furthermore, it affects the cardiovascular system and induces re-endothelialization in endothelial dysfunction (Singh and Prakash, 2014; Karpagasundari and Kulothungan, 2014; Daud et al., 2016; Nugrahenny et al., 2017; Wu et al., 2018). The pharmacological activity of *P. minima* is due to its flavonoid content, such as gallic acid, ellagic acid, quercetin, tannic acid, and many more (Karpagasundari and Kulothungan, 2014). However, *P. minima* have not been tested to initiate the proliferation of mesenchymal stem cells (MSCs).

The phytochemical properties of *P. minima* allow it to be used in stem cell-based therapy because stem cells can proliferate, self-renew, and differentiate. A mesenchymal stem cell is a type of stem cell sourced from adult tissue, one of which is bone marrow (Majka et al., 2017). The proliferative capacity of MSCs can be increased by modifying the growth medium by inducing *P. minima* extract; thereby, a suitable

niche will be formed, and an increase in BM-MSC proliferation can happen. A suitable niche will provide a paracrine effect, which will modulate the protection of damaged tissue and initiate endogenous repair mechanisms; therefore, degenerated cells can improve their viability (Ng Kin, 2014). Optimal viability can increase cell proliferation (Browne and Al-Rubei, 2011).

The effectiveness of the phytochemical content of *P. minima* in stem cell-based therapy has not been reported before. Therefore, it is critical to conduct this research to obtain scientific information related to the effect of *P. minima* extract in increasing the proliferation of BM-MSC in vitro. The result of this study is expected to be the first step in the treatment of diseases caused by cell damage due to degenerative diseases. Damaged cells will be replaced by proliferating BM-MSC cells, accelerating the curative process and restoring the cell functions.

METHODS

Time and Location of Study

The study was conducted at the Research Laboratory, Faculty of Mathematics and Natural Science, Syiah Kuala University, from March to August 2021.

Plant Material

The *P. minima* leaves were obtained from the Panton Labu area, Tanah Jambo Aye District, North Aceh Regency. The leaves were cleaned, cut into small pieces, and air-dried at room temperature. The dried leaves were ground with a grinder into coarse simplicia. The coarse simplicia were macerated using ethanol and filtered. The filtrate was evaporated using a vacuum rotary evaporator at a temperature of 60°C and a speed of 80-90 rpm.

Isolation and Primary Culture of Bone Marrow MSC

The cervical dislocation was carried out in four three-month-old male mice of the Balb-C strain. The method of isolation and primary culture of MSC bone marrow in this study were based on the protocol of Eriani, *et al.*, (2018).

Induction of *P. minima* Ethanol Extract

The primary culture of the mouse bone marrow ended when the confluence reached 70%. The trypsinization in mesenchymal-like-cell colonies by adding trypsin at a concentration of 0.2%. The number of cells was counted using a hemocytometer using the formula, i.e., total cells (cells/ml) = average number of cells in 5 boxes x diluent factor x 10^4 (Djuwita et al., 2012).The cells were then cultured in a Corning 35 mm tissue culture dish (TCD) with a surface diameter of 9 cm^2. A total of twenty TCDs were used for five treatments (positive control, negative control, and three doses of *P. minima* extract) with four replications.

All cells were cultured in mDMEM and incubated in a 5% CO_2 incubator at 37°C for 24 hours. The positive control was treated using the STEMdiffTM Forebrain Neuron Differentiation Kit medium, while the negative control was only treated with mDMEM without adding *P.minima* leaf extract. The three test cultures were added with 0.6 mg/ml, 0.8 mg/ml, and 1.0 mg/ml of *P. minima* extract in mDEM, respectively. The treatment dose was determined based on Eriani et al. (2018). The medium was changed every three days until the 28th day of subculture. The cells were then evaluated and counted using a hemocytometer.

Evaluation of the Culture Product

Morphological Characteristics of Mesenchymal Like-Cell

Evaluation was carried out after the 7th day of incubation. The shape and extension of the cells, as well as the shape, position, and size of the nucleus, were evaluated.

Cell Proliferation

Cell proliferation was evaluated by comparing the number of cells at the beginning and the end of the incubation period. The difference in the number of cells describes the cell proliferation data.

Population Doubling Time (PDT)

PDT is the time required for the cell population to double from its original number. PDT was calculated using the following formula (Davis, 2011):

$$PDT = \frac{\frac{1}{(log)finalcellcount - loginitialcellcount x\ 3.32}}{time}$$

Statistical Analysis

The morphological characteristics of the MSCs were analyzed descriptively. Meanwhile, cell proliferation data, which is in the form of PDT values, were analyzed using the statistical analysis of variance (ANOVA) test at a 95% confidence level. Further tests were carried out using Duncan's test if the treatment caused an effect.

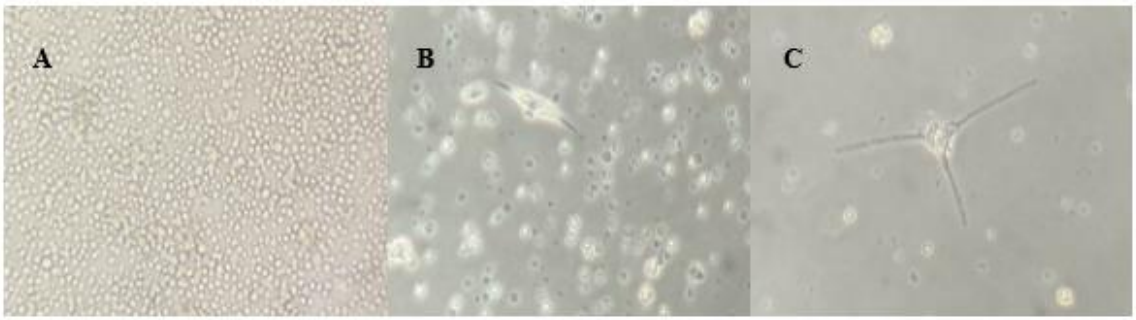

Figure 1. The morphology of BM-MSC primary culture in mesenPRO medium.A: Day 1 of culture, the cells appeared round. B: Day 2 of culture, the cells appeared as fibroblast-like cells.C: Day 5 of culture, the cells appeared as mesenchymal stem-like cells.

RESULTS AND DISCUSSION

The Morphology of Mesenchymal Stem-Like Cell

On the first day of the Bone Marrow-Mesenchymal Stem Cell (BM-MSC) primary culture, cells appeared to be round (Figure 1. A), and BM-MSCs were still mixed with other types of cells. On the second culture day, the cells were seen floating on the surface of the medium. It happened because MSCs began to expand at the bottom of the substrate, while other types of cells died and floated on the surface. Dead cells were discarded when changing the culture medium.

On the second day, the number of cells at the bottom of the petri dish had decreased substantially. The cell morphology was observed microscopically and showed cell extensions with a central and oval nucleus resembling fibroblast-like cells (Figure. 1. B). Fibroblast-like cells indicate that the growing cells are MSC populations (Gnecchi and Mello, 2009; Asumda and Chase, 2011).

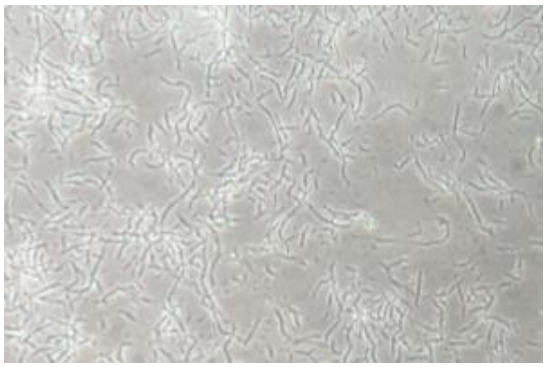

Figure 2. The morphology of mesenchymal stem-like cells on the eighth day

Microscopic observation on the fifth day discoveredtriangular-shaped cells, forming three extensions, with a large and central nucleus (Figure 1. C). According to Rinendyaputri and Noviantari (2015), these are characteristics of mesenchymal stem-like cells. Round cells with a larger diameter than the first day of culture were also observed. The round cells had completed their mitotic division (Rinendyaputri and Noviantari, 2015). On the eighth day, the cells had changed into a fusiform shape, i.e., the cells looked elongated, formed many extensions, and were attached to the base of the petri dish (Figure 2). Mesenchymal like-stem cell morphology was observed in all cells inside the petri dish.

In general, MSCs have specific characteristics. It expresses MSC-specific surface markers, i.e., CD90, CD105, and CD73, as well as negative expression of hematopoietic markers, i.e., CD14, CD34, and CD45 (Teixiera et al., 2013). The rodent BM-MSCs are specifically characterized by the expression of CD105 and CD51 surface markers. CD105 is a marker of primitive progenitor population lineage with self-renewal capacity (Chan et al., 2009; Chan et al., 2015). MSCs can also attach to a substrate surface and differentiate into mesenchymal lineages, such as osteoblasts, adipocytes, chondroblasts, endothelial cells, and cardiomyocytes. In addition, MSCs can also differentiate into non-mesenchymal lineages such as hepatocytes (Crapnell et al., 2013) and nerve cells (Eriani et al., 2018).

The MSCs in this study were harvestedfrom the femur and tibia marrow of three-month-old mice (*Musmusculus*). It is vital to select young mice because it is a significant factor in BM-MSC primary culture's success. According to Asumda

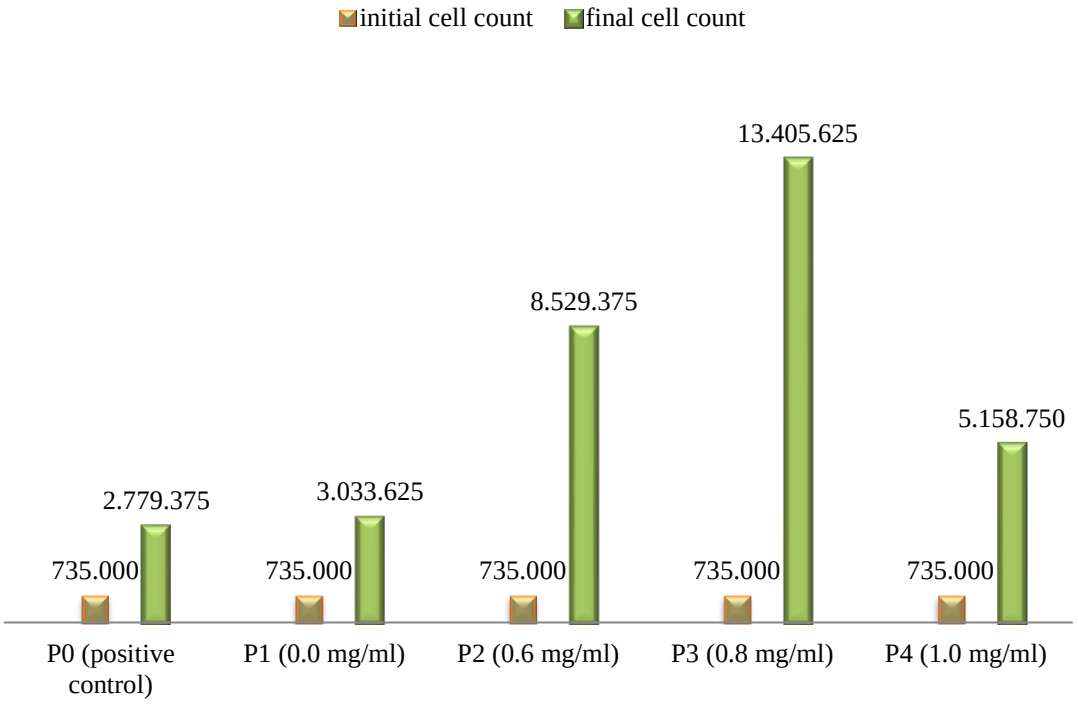

Figure 3. Histogram of the comparison of mean number BM-MSC Proliferation

and Chase (2011), the BM-MSC of four-month-old rats had a higher level of plasticity than fifteen-month-old rats. The primary culture of BM-MSC from young mice expanded faster and reached 80% confluence in just four days. In addition, the BM-MSC cultures of young mice were fibroblast-like cells, which formed long extensions (Figure. 1. B). Thus, it can be concluded that the test animal's age affects the effectiveness of MSCs in mediating cell regeneration.

The primary culture of BM-MSC in this study used a specific medium, namely mesenPRO, that can grow pure MSCs without mixtures of hematopoietic stem cells (Mendez-Ferrer et al., 2010) and fibroblast cells (Baddoo et al., 2003). According to Xu et al. (2010), purification of BM-MSC cultures in rodents, such as *M. Musculus*, is more complex than in humans and other animal species because it requires periodic medium change (Nadri et al., 2007).

Cell Proliferation

The cell proliferation rate was determined based on the difference between the initial cell count (first day) and the final cell count (28th day) during the incubation period.

Figure 3 shows the difference in the average number of cells at the beginning and the end of the incubation period. The final average cell count is higher than the initial average cell count. The samples added with *P. minima* leaf extract produced a higher cell count than those not added with the extract. Sample P3 produced the highest cell count, while the least cell count was produced by sample P0. The number of cells in P3 increased eighteen times from the initial count and more than four times the number of cells of P0. Out of the three extract treatments, the lowest cell count was observed in P4 or the sample with the highest extract dose. The data shows that the addition of *P. minima* extract into the culture medium maintained and increased the cell's proliferation capacity throughout the incubation period.

The P0 treatment produced more cells than P1 because mDMEM was more effective than STEMdiff™ Forebrain Neuron Differentiation Kit medium in mice BM-MSC culture. STEMdiff™ Forebrain Neuron Differentiation Kit medium is more effective when combined with STEMdiff™SMADi Neuron Induction medium in neural, pluripotent stem cell-derived, neural stem, and human-based progenitor cell cultures (Technologies, 2022). The study shows that the negative control (P1) generated better results than the positive control (P0).

The cell proliferation rate is determined based on the Population Doubling Time (PDT), i.e., a value that indicates the time required by a population of cells to double its number

Table1. The Population Doubling Time (PDT) of Mesenchymal Stem Cells (MSCs) from Mice (*M. musculus*) Bone Marrow that is Treated with *P. minima* extract

Treatment	Mean PDT Value (Day) ± SD
P0 (positive control)	15.61 ± 3.94^{b}
P1 (0.0 mg/ml)	15.12 ± 5.34^{b}
P2 (0.6 mg/ml)	8.08 ± 1.15^{a}
P3 (0.8 mg/ml)	6.68 ± 0.5^{a}
P4 (1.0 mg/ml)	10.66 ± 2.91^{ab}

compared to the cell count at the start of the incubation. The PDT value is inversely proportional to the cell proliferation rate. A high PDT is related to a low cell proliferation rate, while a low PDT is related to a high cell proliferation rate.

The data is considered homogenous because the homogeneity test results in sig. 0.06 or sig > 0.05. Meanwhile, the normality test (Kolmogorov-Smirnov) resulted in sig. 0.17 or > 0.05 (Appendix), indicating that the data is normally distributed. The two tests indicate that the average PDT value met the criteria for an ANOVA test. PDT value analysis using ANOVA yielded a sig value of 0.004 (P<0.05). The result indicates that adding *P. minima* leaf extract affected the PDT; therefore, Duncan's follow-up test was carried out.

Duncan's follow-up test shows that samples P3 and P2 differed significantly from the positive control (P0) and negative control (P1).The positive control (P0) was not significantly different from the negative control (P1). Meanwhile, sample P4 did not significantly differ from the other samples. Statistically, samples P3 and P2 could maintain and increase cell proliferation compared to the control with an optimum dose at 0.8 mg/ml, based on the PDT mean value. The result demonstrates that the induction of *P. minima* leaf extract could positively increase cell proliferation.

Table 1 shows that the lowest PDT value was produced from the P3 treatment. For the P3 treatment, the cells required 6.68 or 7 days to double their number. Meanwhile, P2 required 8 days, P4 required 10.66 or 11 days, P1 required 15.12 or 15 days, and P0 required 15.61 or 16 days. However, P4 showed a higher PDT value than previous treatments, i.e., the proliferation rate decreased at a 1.0 mg/ml dose of extract but still higher than the positive and negative controls. All samples with added *P. minima* leaf extract resulted in lower PDT values than the positive and negative controls, which indicate a high proliferation rate. It is concluded that adding *P. minima* leaf extract increased the cell proliferation rate. The optimum dose to double the number of the initial cells was 0.8 mg/ml.

Cell proliferation is an increase in the number of cells caused by cell activity in dividing or multiplying. The rate of cell proliferation is strongly influenced by various factors (Yadav *et al.,* 2014*)*. Extrinsic and intrinsic factors strongly influence the MSC's ability to maintain its proliferation capacity. The extrinsic factor is the MSC's microcellular environment (niche), while the intrinsic factor is the initiation of the transduction signal into the nucleus. The niche determines the direction of the MSC proliferation, either towards self-renewal or differentiation (Putra, 2019). *P. minima* leaf extract is an extrinsic factor that acts as a membrane or receptor-level proliferation signal inducer.

P. minima leaf extract is thought to initiate MSC niche towards self-renewal by increasing cell division and differentiation. This condition is demonstrated based on the data in Table 4.1., which shows a cell count increase after the treatment. *Physalis minima* leaf extract contains essential compounds such as catechol, gallic acid, ellagic acid (Karpagasundari and Kulothungan, 2014,) and quercetin from the flavonoid group (Banothu et al., 2017), which affects the proliferation and differentiation of cultured MSCs.

Quercetin is an inducer of the Neural Growth Factor (NGF), which plays a vital role in stimulating and maintaining neurite growth (Nakajima et al., 2011). NGF initiates the proliferative regulatory activity of MSCs via the phosphatidylinositol-3-kinase (PI3K)/Akt pathway (Amara et al., 2015), which is one of the transduction pathways vital for cell proliferation and differentiation (Brown, 2015). Specifically, activating the PI3K/Akt signaling pathway involves releasing pluripotent transcription factors

produced by MSCs, such as Oct4, Sox2, and Nanog proteins. These transcription factors play a role in controlling the genetic transcription rate of DNA to mRNA by binding to specific DNA sequences that have stemness potentials towards self-renewal pathways (Putra, 2019).

The key molecules in the PI3K/Akt signaling pathway are Receptor Tyrosine Kinase (RTK), phosphatidylinositol 3-kinase (PI3K), phosphatidylinositol-4,5-bisphosphate (PIP2), phosphatidylinositol-3,4,5-bisphosphate (PIP3), and AKT/protein kinase B. PI3K is a protein kinase consisting of two domains, namely the catalytic domain (P110) and the regulatory domain (P85 and P55) (Akinleye et al., 2013). The PI3K/Akt transduction pathway is initiated by quercetin from the *P. minima* extract, which induces the NGF release as a Growth Factor. NGF acts as a ligand for the RTK receptor. NGF then binds to RTK to form the NGF-RTK complex. After forming the ligand-receptor complex, the PI3K catalytic domain will be removed, and the regulatory domain will be activated. Activation of the regulatory domain causes the phosphorylation of PIP2 to PIP3. Then PIP3 is phosphorylated and binds to the PH domain to form PDK-1. ADK-1 is phosphorylated, binds to the pleckstrin homology domain, and activates the Akt protein. The Akt protein is phosphorylated and activates intracellular signals that encourage proliferative activity towards self-renewal (Akinleye et al., 2013; Putra, 2019;).

Various studies related to the pharmacological activity of P.minima extract have been widely carried out both ethnobotanically (Kemenkes RI, 2015) and in vivo(Singh dan Prakash, 2014; Karpagasundari and Kulothungan, 2014; Daud *et al.*, 2016; Nugrahenny *et al.*, 2017; Wu *et al.*, 2018). However, the potential of *P.minina* extract *in vitro* on MSC was only reported from the results of this study. *P.minima* extract has been shown to have effectiveness against increased proliferation of BM-MSC. The results of this study are very important preliminary data in the application of degenerative disease healing applications based on *stem cell* therapy using medicinal plant extracts.

CONCLUSION

P. minima leaf extract was proven to increase the proliferative capacity of BM-MSC cells at an optimum dose of 0.8 mg/ml. Induction of *P. minima* leaf extract was able to maintain the proliferative capacity of BM-MSC cells. In addition. *P. minima* leaf extract could reduce the PDT value, increasing the cell proliferation capacity. Therefore, it is necessary to carry out further molecular research to obtain scientific data regarding the potential of P. minima extract in expressing nerve cell genes as reported by Eriani *et al.,* (2022)

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