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Research Article

Cytotoxic and Osteoblast Differentiation Induction Properties of Crude Polar Extract of *Piper sarmentosum* leaves

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ABSTRACT

Piper sarmentosum or ‘kaduk’ is a well-known herb plant in Malaysia. Its extracts were found to exhibit a bone-protective effect against osteoporotic rats. Our study aim is to morphologically observe the effect of *P. sarmentosum* ethyl acetate extract on the differentiation of human peripheral blood stem cells (PBSCs) into osteoblasts. *P. sarmentosum* extracts (1-900 µg/mL) prepared using 1% dimethyl sulfoxide (DMSO) were used in the cytotoxicity assay. Then, the differentiation assay was performed using concentrations of 1-50 µg/mL. The untreated cells acted as the negative control, while the cells cultured in 50 µg/mL ascorbic acid and 10 mM β-glycerophosphate was a positive control. The cytotoxicity effect and proliferation capacity of the cells were analyzed using Trypan Blue exclusion method, while the differentiation of PBSCs was observed using von Kossa staining and *ALP* gene expression analysis. The result showed a decrease in cells’ viability in a dose-dependent manner during cytotoxicity assay. After 14 days of the differentiation, a constant rate of proliferation could be observed in the treated cells and positive control, while the untreated cells showed an increase in proliferation. The mineralization of extract-treated cells showed significant differences ($p<0.05$) as compared to the negative control. The expression of *ALP* was also upregulated on day 14 of differentiation compared to day 0. In conclusion, the extract was capable to induce osteoblast differentiation of human peripheral blood stem cell and potential to be used in tissue regeneration.

Keywords: Cytotoxicity, Differentiation, Ethyl acetate, Osteoblast, Piper sarmentosum

Introduction

Piper is a plant genus categorized under the family Piperaceae that is consisting of 1000 species; 300 of them can be found in Southeast Asia. One of the well-known species for its abundant benefits is *Piper sarmenstosum*, also known as ‘kaduk’ in Malaysia [1]. The locals commonly consumed *P. sarmentosum*’s parts by including it in the cooking or eating it raw as it is believed to be able to maintain health and treat diseases such as diabetes, headache, hypertension, colds, toothaches and pleurisy [2–4]. Multiple scientific studies have reported the biological activities exhibited by *P. sarmentosum* extracts including antimicrobial, antituberculosis, anticancer and antiplasmodial activities [2, 5–7]. *P. sarmentosum* has also exhibited bone protective effect as the rats experiencing ovariectomized osteoporosis showed enhanced fracture healing when treated with the aqueous extract [8]. On top of these biological activities, the extract of *P. sarmentosum* has exhibited toxicity activity in rats and mice as the LD_{50} exhibited were 10 g/kg and 5 g/kg, respectively [9,

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10]. Unfortunately, the information on cytotoxicity effect of the extract on healthy human cells is very limited.

In this study, the potential of *P. sarmentosum* extract as a bone protective agent at the cellular level was tested on human peripheral blood stem cells (PBSC). Stem cell is an unspecialized cell, that can undergo the proliferation process for indefinite periods, differentiate into various cell lineages, and repopulate the *in vivo* system of host cells. [11, 12]. Instead of differentiating only into hematopoietic lineage cells like platelets, lymphocytes and erythrocytes, PBSC is also able to undergo a transdifferentiation process, forming cells under other non-hematopoietic cells such as hepatocytes and myocytes, making it a great *in vitro* model for differentiation study [12–15]. Another non-hematopoietic cell involved in this study is the osteoblast, which originates from mesenchymal lineage. The osteoblast is an important component involved in the bone remodelling process, wherein its population in bone tissue needs to be balanced with the other bone cell, i.e., osteoclast. The homeostasis in the skeletal system is regulated by this vital process to maintain bone health, repair skeletal damage and prevent brittle bone [16].

There are a few *in vivo* studies that exhibited the bone protective effect of *P. sarmentosum* extract by repairing bone damage and preventing further bone loss in osteoporotic rats [17–19]. Due to the various health benefits presented, *P. sarmentosum* has been widely consumed by the locals without knowing the possibility of its effect on human cells. Other than that, currently, the extract involvement in the bone remodelling process at the cellular level is still not well-studied with very limited results published. So, in our study, the cytotoxicity effect and the potential to induce the differentiation of osteoblasts on human peripheral blood stem cells (hPBSC) were examined.

Material and Methods

Preparation of Piper sarmentosum extract

P. sarmentosum leaves were collected and identified by a botanist from the Faculty of Applied Science, Universiti Teknologi MARA (UiTM). The leaves were cleaned and dried at 50°C before being ground into fine powder. Approximately 20g powdered leaves were extracted using Soxhlet apparatus with 200 mL ethyl acetate solvent. After the extraction process for four hours, the extraction product was transferred into a rotary evaporator to remove the excess solvent. To ensure a complete solvent removal, the extract was left to evaporate in a fume hood for a few days. The dried extract that was present in a sticky form was stored at -20°C until further use. Prior to conducting further tests, the ethyl extract was solubilized and diluted using 1% dimethyl sulfoxide (DMSO) to produce a range of stock concentrations ranging from 10 µg/mL to 9000 µg/mL.

Cell isolation and in vitro culture

Ethical approval (Reference number: UKM PPI/111/8/JEP-2019-612) from Research Ethical Committee Universiti Kebangsaan Malaysia was obtained before conducting sample collection. Peripheral blood samples alongside with the consent form were collected from healthy donors, aged 18 to 25 years old. The collected samples were diluted with Hanks' balanced salt solution (HBSS) in the ratio of 1:3. The diluted blood was then layered onto Ficoll-Paque™ PLUS (1:1.5) and centrifuged at 400xg, 37°C for 20 min. The centrifugation step would produce four layers of supernatants with the buffy coat containing the mononucleated cells observed as the second layer. The buffy coat layer was taken out and washed three times using phosphate-buffered saline (PBS). Finally, the cells were cultured in a complete medium containing alpha medium essential medium (AMEM), 2% (v/v) penicillin-streptomycin and 10% (v/v) heat-inactivated newborn calf serum (NBCS) at 37°C for seven days before further experiments.

Cytotoxicity assay

After 7 days, approximately 1×10^5 cells/mL were seeded into each well of the culture plate and treated with various concentrations of *P. sarmentosum* extract, i.e., 1-900 µg/mL. The untreated cells served as the negative control for the cytotoxicity assay. The viability of the cells was observed after 24-, 48- and 72-hours using Trypan Blue Exclusion Dye.

Differentiation assay

A few concentrations were chosen based on the cytotoxicity assay for the differentiation assay. The cells were seeded at 1×10^5 cells/mL and treated with extract with multiple concentrations, i.e., 1, 15, 35 and 50 µg/mL. The untreated cells served as the negative control, while the cells

treated with a combination of 50 µg/mL ascorbic acid and 10 mM β-glycerophosphate acted as a positive control. The differentiation assay was carried out for 14 days.

von Kossa staining of differentiated cells

Approximately 1×10^5 cells/mL of PBSC were collected and washed using PBS and fixed onto a glass slide using 10% (v/v) formalin in PBS for 30 min. Then, the slide was rinsed using deionized water before the cells were stained with 5% (v/v) silver nitrate solution for 30 minutes. After that, the staining was developed with 5% (v/v) sodium carbonate in 25% (v/v) formalin followed by final fixation using 5% (v/v) sodium thiosulfate for 2 min. The slide was rinsed using deionized water after each step. The slide was left for air-dried before observation under the microscope.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

RNAs were extracted from the treated cells on day 0 and day 14 of the differentiation assay using a Trizol reagent. The extraction process was carried out according to the manufacturer's instructions. The extracted RNAs were subjected to a reverse transcription process that was carried out for 10 min at 25°C, 15 min at 42°C and followed by 5 min at 85°C using Sensifast cDNA Synthesis Kit. The resulting strands were then used as templates for amplification using Thunderbird Next SYBR qPCR Mix according to the manufacturer's instructions. PCR was carried out for 30 sec at 95°C, followed by another 5 sec at 95°C and 60°C (*GAPDH*)/54°C (*ALP*) for 10 sec for a total of 40 cycles. The reactions were analysed and normalized to the housekeeping gene, *GAPDH*. The following primer sequences were used: *GAPDH* (forward: 5'-GACCACTTTGTCAAGCTCATTTC-3'; reverse: 5'-CTCTCTTCCTCTTGTGCTCTTG-3') and *ALP* (forward: 5'- GGAGTATGAGAGTGACGAGAAAG -3'; reverse: 5'-GAAGTGGGAGTGCTTGTATCT -3').

Statistical analysis

Data distribution was tested using the Shapiro-Wilk test in Graphpad Prism 8. The statistical analysis was carried out using one-way analysis of variance (ANOVA) with the Dunnet post hoc test and the difference between extract treatments and positive control was considered to be significant at $p < 0.05$.

Results and Discussion

Cytotoxicity effect of Piper sarmentosum on PBSC

The cytotoxicity of *P. sarmentosum* extract at various concentrations (1-900 µg/mL) was observed on the cells after 24, 48 and 72 hours. Figure 1 shows the reduction of the cell viability percentages in a concentration-dependent manner, wherein the higher the concentration of the extract, the lower the viability of the cells observed. The viability of cells treated with all concentrations (1-900 µg/mL) exhibited a significant decrease ($p<0.05$) compared to the untreated cell (0 µg/mL) that served as negative control after 24, 48 and 72 hours. The lowest percentages of cell viability were recorded after 72 hours treatment with 7% (900 µg/mL), 9% (800 µg/mL) and 13% (700 µg/mL) of cell viability. The extract concentrations that reduced the cell viability by 50% (IC_{50}) were determined from the cytotoxicity assay graph. The cells treated for 24 hours exhibited cell viability higher than 50% with the lowest recorded were 52% (900 µg/mL), 54% (800 µg/mL) and 55% (700 µg/mL). Hence, the IC_{50} value was not achieved after 24 hours of treatment. However, the IC_{50} were achieved after 48 and 72 hours of treatments, with values of 153 µg/mL and 84 µg/mL for the respective durations.

P. sarmentosum is one of the herbal plants that has been found to have numerous phytochemicals contributing to its multiple biological activities. These phytochemicals interact with certain solvents based on their polarities. In this study, ethyl acetate was used as a solvent to extract the phytochemicals from *P. sarmentosum* leaves. Ethyl acetate is a polar solvent that is capable to extract sterol, aglycon, glycoside compounds, terpenoids, flavonoids and alkaloids [20, 21]. Other than that, the ethyl acetate has been used to extract compounds that are involved in scavenging free radicals and reactive oxygen species (ROS) such as phenolic and nitrogenous compounds [22, 23]. In addition, ethyl acetate extract of *Piper betle* exhibited the highest antioxidant and anti-proliferative effect on the MCF-7 cell-line as compared to hexane, methanol and aqueous extracts [24]. The high content of flavonoids was also observed in plants that possess antioxidant activity such as *Boswellia dalzielii*, *Daniellia oliveri* and *Cayratia trifolia* [25, 26]. However, the phytochemicals in ethyl acetate extract that might be involved in the reduction of PBSCs viability are yet to be reported. This

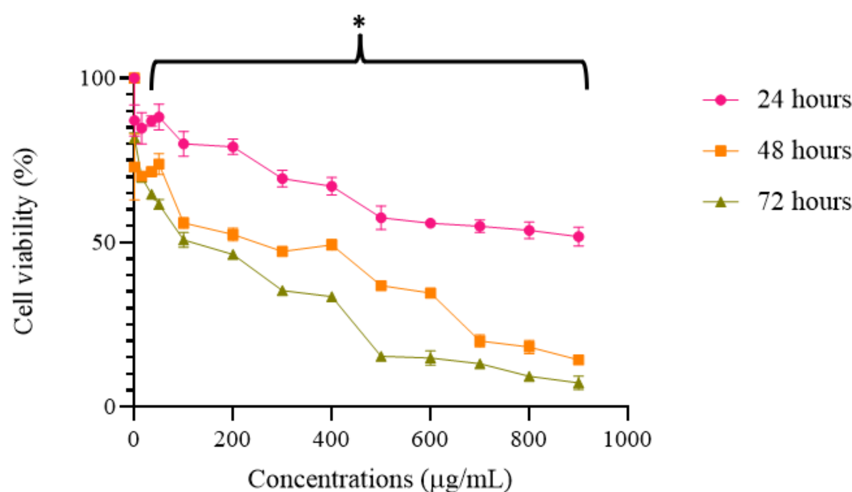

Figure 1. The percentage of PBSCs viability during the treatment with *P. sarmentosum* ethyl acetate extract in a range of concentrations (1-900 µg/mL). The data are presented as mean values ± standard deviation, n=3. *denotes a significant difference p<0.05 when compared to the untreated cells.

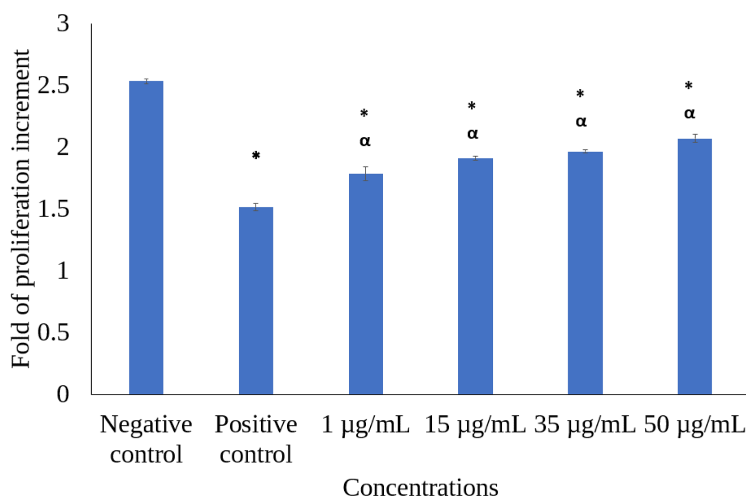

Figure 2. The increment fold of cell number on day 14 compared to day 0. The data are presented as mean values ± standard deviation, n=3. α denotes a significant difference p<0.05 when the extracts were compared to the positive control. *denotes a significant difference p<0.05 when compared to the negative control.

study suggests that ethyl acetate extract contains phytochemicals that are safe for the cells at certain concentrations but could reduce the viability of the cells at high concentrations.

Proliferation ability of PBSC treated with Piper sarmentosum extract

Following the cytotoxicity assay, extract concentrations of 1, 15, 35 and 50 µg/mL were chosen to be used in the differentiation assay. During the assay, the effect of the extract on the proliferation ability of the cells was observed using Trypan Blue exclusion dye on days 0 and 14. Figure 2 shows that the treated cells, either using the differentiation medium (positive control) or *P. sarmentosum* extracts exhibited decreased proliferation rate when compared to the untreated cells (negative control). The untreated cells were able to proliferate well until day 14 as compared to the treated cells; both a positive control and the extract-treated cells. All treated cells exhibited a significant decrease (p<0.05) when compared to the negative control, while the cells treated with 1, 15, 35 and 50 µg/mL showed a significant increase compared to the positive control.

As the cells were induced to differentiate by the -addition of differentiation factor, the cell division rate in the culture was reduced. This is be-

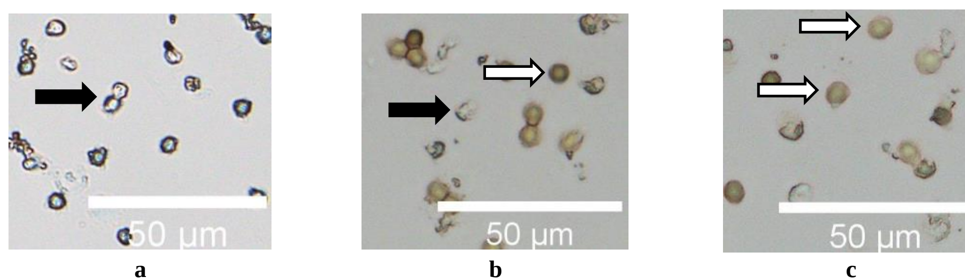

Figure 3. The morphology of PBSCs following *von Kossa* staining on day 14 of differentiation assay. (a) The untreated cells (negative control). (b) The cells were treated with a differentiation medium (positive control). (c) The cells were treated with *P. sarmentosum* ethyl acetate extract. The black arrow shows the morphology of undifferentiated cells. The white arrow shows the morphology of differentiated cells. (Magnification 400×).

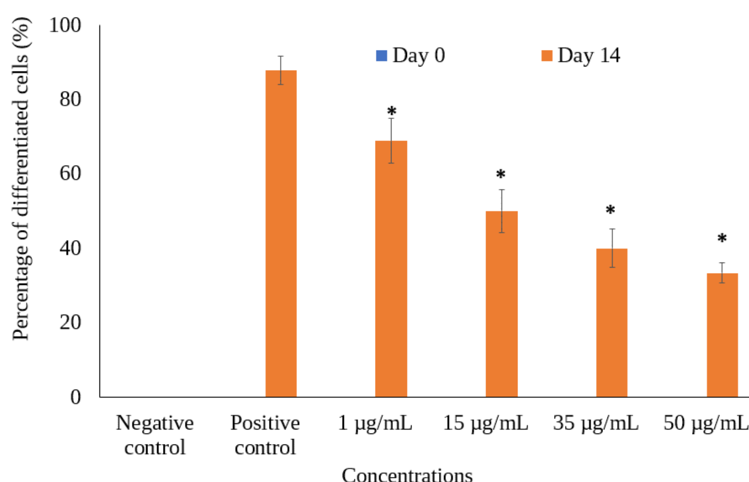

Figure 4. The percentage of differentiated cells treated with *P. sarmentosum* ethyl acetate extract with concentrations of 1, 15, 35 and 50 µg/mL. The data are presented as mean values ± standard deviation, n=3. *denotes a significant difference p<0.05 when compared to the positive control.

cause both of these processes, i.e., the differentiation and cell division process cannot be carried out or co-occur in a cell. Hence, as observed in Figure 2, the cells treated with the differentiation medium (positive control) and the extract exhibited reduced cell proliferation caused by the decreased rate of cell division. The reduction of cell proliferation in differentiation-induced cells could also be observed in other studies involving the differentiation of dental pulp stem cells into neuron-like cells and ascorbic acid-induced differentiation of PBSCs into osteoblasts [15, 27]. On the other hand, the untreated cells (negative control) were able to proliferate well until day 14 because of the absence of a differentiation inducer in the culture, allowing the cells to maintain the cell division cycle.

Piper sarmentosum induced cell mineralization of PBSC

The cells were treated with multiple concentrations of extract for 14 days to determine the potential of the extract to induce osteoblast differentiation which can be observed via cell mineralization. To observe the mineralization process, the cells were fixed onto glass slides and stained using the *von Kossa* technique. Following the staining step, the mineralization process occurred could be seen as the differentiated cells appeared brownish (stained), while the undifferentiated cells appeared translucent (unstained). Figure 3 shows the morphologies of differentiated (white arrow) and undifferentiated (black arrow) cells. There were no stained cells observed in the untreated culture (Figure 3a) after 14 days of treatment. Meanwhile, Figures 3b and 3c show the morphology of cells treated with the differentiation medium (positive control) and *P. sarmentosum* extract on day 14 of the differentiation assay.

Following the morphological observation, the mineralization process was quantified by determining the percentage of differentiated cells. Fi-

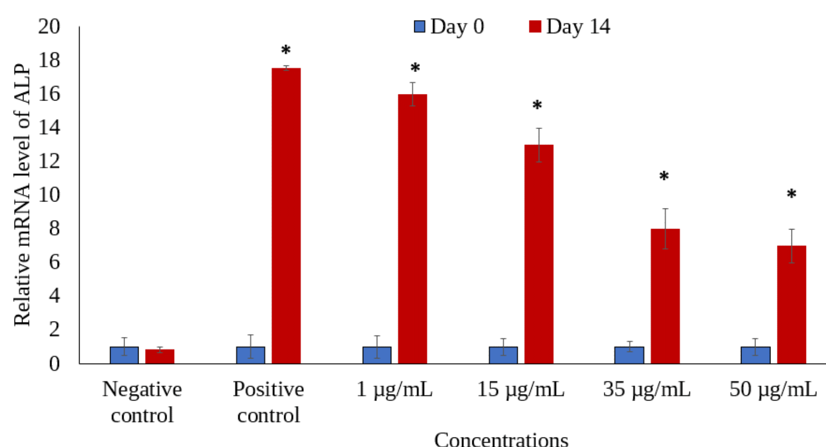

Figure 5. The expression of *ALP* gene on days 0 and 14 of the differentiation assay. The data are presented as mean values ± standard deviation, n=3. *denotes a significant increase ($p<0.05$) when compared to the expression on day 0.

gure 4 shows the percentage of mineralized cells on days 0 and 14. As observed from the graph, the extract has exhibited that lower concentration has higher potential to induce osteoblast differentiation. The highest percentage of mineralized cells on day 14 was recorded in the treatment using 1 µg/mL (68%), followed by 15 µg/mL (50%), 35 µg/mL (40%) and 50 µg/mL (34%). On the other hand, the cells treated with the differentiation medium (positive control) showed the highest differentiation percentage with 87% mineralization recorded on day 14. There was no mineralized cell observed in the untreated cell (negative control) until day 14 of the differentiation assay. All extract-treated cells exhibited significant differences ($p<0.05$) when compared to the positive control.

The differentiation and formation of osteoblasts can be observed using multiple approaches such as the expression of gene markers, alkaline phosphatase (ALP) specific activity and the morphology of the mineralized cells. In this study, *von Kossa* staining and *ALP gene* expression were carried out to observe the differentiated cells. This happened through the reaction of silver ions from the stain with phosphate and calcium that are present in osteoblasts, producing brownish precipitates [28, 29]. The stained brownish cells observed in Figure 3 indicate that the cells were induced to undergo differentiation and mineralization, proving the potential of *P. sarmentosum* extract to be an inducer for osteoblast differentiation. The percentage of mineralized cells showed that *P. sarmentosum* ethyl acetate extract exhibited the highest potential as an osteoblast inducer when being used at the concentration of 1 µg/mL. This is determined by the highest percentage of the differentiated cell when treated using this concentration. At the same time, treatment using 1 µg/mL exhibited the lowest cell proliferation observed on day 14 when compared to other concentrations. These results indicate that 1 µg/mL of *P. sarmentosum* extract possessed the highest potential to induce the cells to initiate differentiation process, thus reducing the cell division cycle. A previous study has reported the ability of *Piper sarmentosum* aqueous extract on the osteoblast differentiation of the MC3T3-E1 cell-line; a murine cell-line of immature osteoblasts [30]. The study showed that the aqueous extract of *P. sarmentosum* was able to increase the rate of MC3T3-E1 cell-line differentiation into osteoblasts by observing the formation of black or dark brown nodules via *von Kossa* staining.

The morphological observation of cell mineralization was followed by the analysis of *ALP* gene expression. As shown in Figure 5, the gene expression was downregulated in a dose-dependent manner, wherein the highest expression was recorded in the cells treated with 1 µg/mL (16 folds), followed by 15 µg/mL (13 folds), 35 µg/mL (8 folds) and 50 µg/mL (7 folds). The cells treated with the differentiation medium (positive control) showed the highest *ALP* expression on day 14 with 18 folds. All treated cells exhibited a significant increase in *ALP* expression on day 14 compared to day 0. *ALP* is a well-known marker for osteoblast and its expression was analysed in numerous osteoblast differentiation studies [31, 32]. *ALP* is an early-stage differentiation marker that is involved in the maturation of osteoblasts,

wherein its expression would induce the calcification process [33, 34]. The upregulation of *ALP* expression on day 14 compared to day 0 indicates that *P. sarmentosum* extract can induce the mineralization process of osteoblasts.

Conclusion

These findings showed that *P. sarmentosum* only caused the decrease in hPBSC viability at higher concentrations. On the other hand, the morphological analysis and the expression of *ALP gene* exhibited the ability of the extract to induce the osteoblasts differentiation with the best differentiation potential shown by the extract with a concentration of 1 µg/mL. These results showed that the ethyl acetate extract of *P. sarmentosum* is safe for consumption and could be used as an alternative inducer for osteoblast differentiation.

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