CYTOTOXICITY OF HEXANE AND ETHANOL PIPER SARMENTOSUM EXTRACTS ON HUMAN HEMATOPOIETIC STEM CELLS

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Abstract: Piper sarmentosum or also known as 'kaduk' in Malaysia, is one of the well-known plants with its ethnopharmacological benefits which have various biological activity such as antimalarial, antituberculosis, antioxidant and fracture healing properties. The objective of this study is to determine the cytotoxicity effect of hexane and ethanol P. sarmentosum extracts on human hematopoietic stem cells (hHSC). The isolated peripheral blood mononucleated cells were cultured in the complete medium for 14 days to observe the in vitro proliferation ability of the cells. The expression of stemness marker of the cells was then determined using RT-qPCR. The cytotoxicity of P. sarmentosum was determined by treating the hHSC using hexane and ethanol extracts. Trypan blue exclusion dye assay was used to evaluate the viability of hHSC. Proliferation graph showed significant increase for the number of viable cells for 14 days with activation of SLAMF1 gene. After 48- and 72-hours exposures, IC_{50} of hexane extract treatment were 96.6 µg/mL and 95.83 µg/mL, respectively, while hHSC treated with the ethanol extract showed IC_{50} of 168.38 µg/mL and 83.72 µg/mL, respectively. This showed that ethanol extract of P. sarmentosum possessed slightly higher cytotoxicity towards hHSC when compared to hexane extract. The results suggested that P. sarmentosum's exhibit moderate cytotoxicity towards human's cells, which can be further used in the cellular therapeutic approach.

Keywords: Piper sarmentosum; human hematopoietic stem cells; cytotoxicity; RT-qPCR

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INTRODUCTION

Piperaceae family belongs to the Nymphaeifloraea superorder and comprised of 1,000 species in tropical and subtropical regions of the world with 300 can be found in Southeast Asia (Dyer and July 2004). Piper sarmentosum has been used extensively for its ethnopharmacological benefits. Almost all parts of the plant possess their own uses wherein the leaves and roots are used to treat toothaches, pleurisy and headache, while the fruits are used as an expectorant by locals in Thailand (Rukachaisirikul et al., 2004; Zakaria et al., 2010).

Despite all the ethnopharmacological properties exhibited, P. sarmentosum has also shown toxicity at certain concentrations where LD_{50} values in rats and mice are more than 10 and 5 g/kg respectively (Peungvicha et al., 1998; Ridtitid et al., 2007). However, there is less evidence reported on cytotoxicity level of the extract on human cells. Without knowing the effect and the optimum dose that can be consumed, the plant extract has been frequently used traditionally to treat diseases. Hence, a controlled cytotoxicity study was carried out to determine the toxicity of P. sarmentosum on human hematopoietic stem cells (hHSC). Stem cell is defined as unspecialized cell that able to proliferate for indefinite periods of time, differentiate into various cell lineages and repopulate a host in vivo (Shahrul Hisham et al., 2005; Weissman 2000). hHSC is a good in vitro model to study the cellular toxicity profiles because the cells are not only able to repopulate all cells from hematopoietic lineage such as platelets, erythrocytes and lymphocytes, but it also capable to undergo transdifferentiation into important nonhematopoietic cells such as myocytes and hepatocytes (Gussoni et al., 1999; Lagasse et al., 2000 Shahrul Hisham et al., 2005; Siti Norhaiza et al., 2014).

In this study, the cytotoxic activity of two types of P. sarmentosum extracts on hHSC (i.e., hexane and ethanol) has been observed. Trypan blue exclusion assay was used to observe the viability of hHSC after three incubation times. The generated result can be used for further studies on the safety of P. sarmentosum extracts on human's health.

MATERIALS AND METHODS

Plant Materials

Fresh leaves of P. sarmentosum were collected from Forest Research Institute of Malaysia (FRIM) and further identified by a botanist from Faculty of Applied Science, Universiti Teknologi MARA (UiTM). The leaves were cleaned and dried in an oven at 50°C, before being ground into fine powder. The extraction procedures were then conducted at the Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM) laboratory.

Preparation of Extracts: Sequential extraction by Soxhlet apparatus of 20g oven-heated dry-powdered leaves was carried out using hexane and ethanol with the ratio 1:10. Then, the extracts were concentrated under reduced pressure using rotary evaporator. The extracts were left in the fume hood for a few days to ensure the solvent had evaporated completely. The dried extracts were stored in -20°C until required. Then, 1% dimethyl sulfoxide (DMSO) was used to generate a range of concentrations (1-500 µg/mL) for further analysis.

Isolation of Human Mononucleated Cells: Mononucleated cells were isolated from peripheral blood of healthy donors aged 18 to 25 years old after the donors' consents were obtained. The blood samples diluted with Hanks' balanced salt solution (HBSS) (1:3), were then layered onto Ficoll-Paque™ PLUS with the ratio 1:1.5. The application of density-gradient separation method using this reagent results in the formation of four layers where the mononucleated cells would be at the second layer. The samples were centrifuged at 400 ×g for 20 min at 27°C. The mononucleated cells layer was taken out and washed three times using phosphate buffer saline (PBS) and cultured in 24-well plates in complete medium containing alpha medium essential medium (AMEM), 2% (v/v) penicillin-streptomycin and 10% (v/v) new-born calf serum (NBCS) at 37°C in a humidified atmosphere containing 5% CO_2 for 4 days before further experiments.

Proliferation of Human Mononucleated Cells: Suspension cell was transferred into new plates with the density of 1×10^5 cells/mL and incubated for 14 days. The proliferation of the cells was observed using Trypan blue exclusion dye method for 14 days and the medium was replaced every three days. The live cells could be distinguished by the clear cytoplasm due to the presence of intact cell membranes that exclude the trypan blue dye. Meanwhile, dead cells appeared dark because of the absence of intact membrane. The viability of the mononucleated cells was counted daily using haemocytometer (Hirschmann, Germany). The viable cells were measured using the following formula:
Viable cells = Average viable cell count per square × Dilution factor × 10^4

RT-qPCR Analysis
Total RNAs were isolated from the cultured cells on day 0 and 14 using Trizol reagent according to the manufacturer's instructions. Reverse transcription of total RNA was carried out for 10 min at 25°C, 15 min at 42°C and followed by 5 min at 85°C using SensiFAST™ Real-Time PCR Kit for RT-PCR. The resulting single-strand cDNA molecules were then used to perform qPCR using the following primers: GAPDH (forward: 5'-GACCACTTTGTCAAGCTCATTTC-3'; reverse: 5'-CTCTCTTCCTCTTGTGCTCTTG-3') and SLAMF1 (forward: 5'-GGAAAGCAGGAAGGAGGA-3'; reverse: 5'-GCAGCCCAGTATCAAGGT-3'). Reactions were performed using Thunderbird Next SYBR qPCR Mix which consisted of 10 µL Thunderbird Next SYBR qPCR Mix, 6 pmol each forward and reverse primers, 2 µL template strand and sterile RNAse free water. The PCR conditions used were 95°C for 30 sec followed by 95°C for 5 sec and 60°C for 10 sec for a total of 40 cycles. All reactions were run in triplicate and GAPDH was used for the normalization.

In vitro Cytotoxicity Assay: Cells were plated at a density of 1×10^5 cells/mL and treated with various concentrations of P. sarmentosum extracts, i.e., 1-500 µg/mL. The untreated cells (0 µg/mL) were used as negative control in this study. The cells were incubated at 37°C and the viability was observed after 24, 48 and 72 hours. The percentage of viability was calculated and IC_{50} values were analysed.

Statistical Analysis: Data analysis was carried out using paired t-test and significant differences between treatments were indicated by the value of $p<0.05$.

Ethical Approval: his research was done according to the guideline set in Helsinki Declaration of 1975. The approval from REC UKM with approval reference number UKM PPI/111/8/JEP-2019-612 was obtained before starting the research.

RESULTS

Proliferation of Human Mononucleated Cells in Complete Medium
Self-renewal is the main characteristics of stem cells. Therefore, the ability of the isolated cells to survive and proliferate in the complete medium was observed every day for 14 days. Figure 1 shows that the number of viable cells was increased significantly ($p<0.05$) throughout 14 days of culture, i.e., Day 1 ($p=0.0010$), Day 2 ($p=0.0005$), Day 3 ($p=0.0001$). Day 4 ($p=0.0004$). Day 5 ($p=0.0007$), Day 6 ($p=0.0004$), Day 7 ($p=0.0003$), Day 8 ($p=0.0003$), Day 9 ($p=0.00007$), Day 10 ($p=0.0002$), Day 11 ($p=0.00004$), Day 12 ($p=0.0003$), Day 13 ($p=0.0001$) and Day 14 ($p=0.0003$).

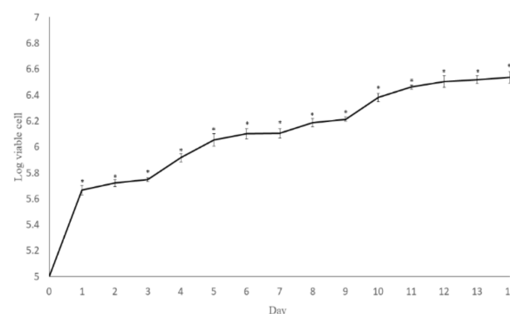

Figure 1 Proliferation of isolated human mononucleated cells cultured in complete medium for 14 days. Mean ± standard of the mean is given for 3 separate experiments (n=3).

Expression of Stem Cell Marker
Self-renewal capacity alone is not sufficient to prove the cultured cells are stem cell. The existence of hematopoietic

stem cells in suspension mononucleated cells was done using molecular approach (RT-qPCR analysis). The total RNAs were extracted on day 0 and 14 to observe the regulation of hematopoietic stem cells marker (SLAMF1). The expression level was analysed using qPCR. The expression of SLAMF1 was found significantly increased (p=0.03) as the duration of culture increased with 2.57 folds of increment on day 14 (Figure 2).

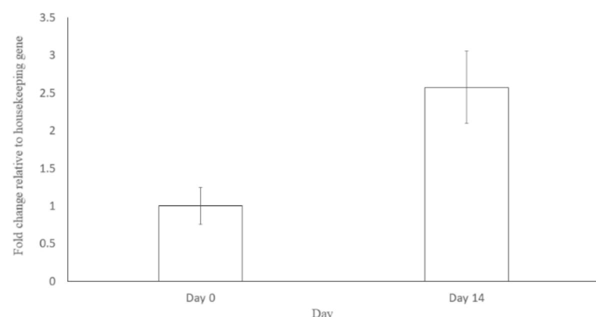

Figure 2. RT-qPCR analysis of human mononucleated cells in 14 days culture (n=3).

Cytotoxicity Activity of P. sarmentosum Extracts

The effect of cytotoxicity of P. sarmentosum extracts on hHSC using two solvents, i.e., hexane and ethanol (various concentrations) at three treatment durations, i.e., 24, 48 and 72 hours was observed. There was no significant decreased (p>0.05) of cell viability by both extracts after 24 hours treatment showed that there was no cytotoxicity effect within 24 hours treatment (Figure 3 and 4). The percentage of cell viability showed that both extracts exhibited cytotoxicity in a concentration-dependent manner after being exposed for 48 and 72 hours (Figure 3 and 4). Hexane extract showed the lowest viability percentage at 22.70% after 72 hours treatment using 500 µg/mL (Figure 3). Meanwhile, the lowest percentage of viability for ethanol extract was recorded at 15.22% after 72 hours treatment using 500 µg/mL extract (Figure 4).

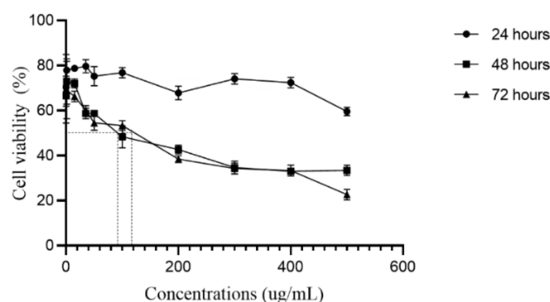

Figure 3 Percentage of cell viability treated with P. sarmentosum hexane extract (n=3).

The concentrations of extracts that reduced the cell viability by 50% (IC_{50}) were also determined. The 50% inhibitions of cell survivability (IC_{50}) were only exhibited after 48 hours treatment using both extracts with IC_{50} values of 96.6 µg/mL (hexane) and 168.38 µg/mL (ethanol). Meanwhile, P. sarmentosum ethanol extract showed the lowest IC_{50} value of 83.72 after 72 hours treatment, as compared to hexane (95.83).

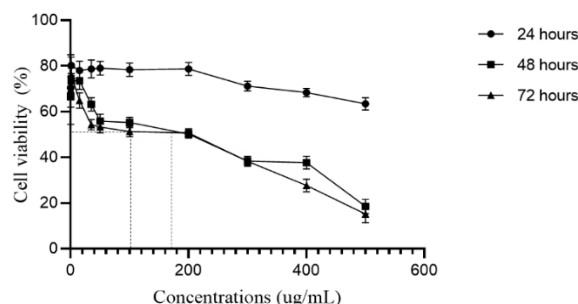

Figure 4 Percentage of cell viability treated with P. sarmentosum ethanol extract (n=3).

DISCUSSION

Extensive literatures on ethnopharmacological potential of P. sarmentosum are available, however, cytotoxicity study on human cell is currently limited. Therefore, in vitro cytotoxicity screening can become an efficient approach as an early assessment of natural herbal products.

The heterogenous morphology of cells appeared on the day of isolation was caused by the presence of several types of cells. After 4 days, only mononucleated cell existed in the medium. Majority of differentiated and precursor cells that were isolated together died due to their short lifespans, i,e., monocytes (5-7 days) and platelets (3-5 days) (Intan Zarina et al., 2010; Muhammad Dain et al. 2011; Shahrul Hisham et al., 2010; Sørensen et al., 2009; Whitelaw and Bell 1966). The increase of mononucleated cells observed in Figure 1 showed that the cells could proliferate in vitro in the complete medium. Proliferation ability alone is not sufficient to prove that the mononucleated cells are stem cells. The existence of hematopoietic stem cell (HSC) marker in the suspension mononucleated cells was further characterized by a molecular approach. The expression of signaling lymphotic activation molecule family I (SLAMF1) gene was observed using RT-qPCR. SLAMF1 or also known as CD150 belongs to SLAM subfamily of CD2-like family of proteins that involves in the regulation of the proliferation and activation of lymphocytes that is expressed on T- and B-lymphocytes, dendritic cells and macrophages (Cocks et al., 1995; Wang et al., 2004). Hematopoietic stem cells have also been found to exhibit SLAMF1 as a specific positive marker that is important in the self-renewal of HSCs (Kiel et al., 2005; Kent et al., 2009). The significant role of SLAMF1 in the maintenance of the undifferentiated hHSCs in the complete medium by stimulating proliferation was shown in the upregulation of SLAMF1 expression along the increase of hHSCs proliferation for 14 days.

The cell viability showed that P. sarmentosum extracts potentially contain active compounds that contributed to its cytotoxicity. These two solvents were chosen for their different polarities with polarity index values were 0.1 (hexane) and 5.2 (ethanol). The solvent's polarity was an important factor in bioactive compound extraction to determine the ability to engage in strong interactions with other polar molecules in the plant (Barwick 1997). Most bioactive compounds in plants are highly polarizable because of the presence of aromatic delocalized µ-electron that enables the compounds to interact with polar solvents (Kolář et al., 2002). Since the type of

compounds that were likely to be extracted depended on the nature of the solvent, each P. sarmentosum extracts used in this study potentially possessed different compounds that were liable to the cytotoxicity.

Ethanol is considered as one of the common solvents for compound extraction because of its intermediate polarity that enables it to interact with both polar and non-polar compounds (Karadeniz et al., 2005). On the other hand, hexane soxhlet extraction had successfully extracted an aromatic alkaloid called 1-nitrosoimino-2,4,5-trimethoxybenzene from aerial parts of P. sarmentosum that was found to contribute to the cytotoxicity towards HeLa and MCF-7 cell lines with IC_{50} values were 11.6 µg/mL and 14.4 µg/mL respectively (Ee et al., 2009). Previous studies also reported the cytotoxicity of P. sarmentosum extracts on other cell lines such as human hepatic carcinoma cell line (HepG2) and HUVEC (Hussain et al., 2009; Shahrul Hisham et al., 2009). Even though the interest on the cytotoxic activity of P. sarmentosum extract on human cells are increasing, most of them were only focusing on the fate of cancerous cells, not on the primary normal cells. It is also important to ensure that the active compounds in the plant extract could inhibit the survivability of the cancerous cell lines but not the healthy or normal primary cells. Therefore, both extracts of P. sarmentosum possessed cytotoxic effect towards hHSC as the IC_{50} values after 48- and 72-hours treatment. Concentrations 1-50 µg/mL for both extracts can be considered safe and may be further used in in vitro and in vivo studies to observe the cytotoxicity and other potential biological activities of this extract on normal human cells.

These findings showed that P. sarmentosum extracts exhibited cytotoxicity on hHSC after 48- and 72-hours treatment. The generated result in this study could be used as a guideline for more in vitro studies of P. sarmentosum's extracts on normal cells.

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ABBREVIATIONS

1. hHSC = human hematopoietic stem cells
2. DMSO = dimethyl sulfoxide
3. HBSS = Hank's balanced salt solution
4. PBS = phosphate-buffered saline
5. AMEM = alpha medium essential medium
6. NBCS = newborn calf serum
7. RT-qPCR = reverse transcription quantitative polymerase chain reaction
8. REC UKM = Research ethics committee Universiti Kebangsaan Malaysia

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