Antioxidant and Anti-inflammatory Activity of *Salacca zalacca* (Gaertn.) Voss Peel Ethanolic Extract on Lead Induced Fibroblast Cells

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Abstract: Lead toxicity is a serious environmental disease and its effects on the human body are overwhelming. Lead can increase reactive oxygen species (ROS) levels in the body which results in oxidative stress. Elevated ROS levels can stimulate inflammation and cellular aging. Plants extract have the abilities as antioxidant and anti-inflammatory agent to prevent aging and toxicity including *Salacca zalacca* peels extract (SPE). Cytotoxicity assay of SPE towards fibroblast cells (BJ) was handle using MTS (3-4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). Intracellular ROS levels were observed by flow cytometry using DCF-DA fluorescent probe. Fibroblast cells were incubated at 37^0C, 5% CO_2, treated by 25 and 100 µg/ml SPE for 4 hours and followed by 400 µM Pb for 72 hours. Anti-inflammatory capacity was conducted using ELISA to measure IL-10 and TNF-α. SPE at 3.13-100 µg/ml were nontoxic to the BJ cells. Accumulation of intracellular ROS levels in lead-induced BJ cells were decreased by treatment using SPE at 25 and 100 µg/ml. SPE at 25 and 100 µg/ml elevated IL-10 and decreased TNF-α related to positive control (lead-induced cells). This research shows that *S. zalacca* peels extract has the ability as protective effect related to Pb poisoning.

1 INTRODUCTION

Lead (Pb) toxicity is a serious environmental disease and its effects on the human body are overwhelming (Wani et al., 2015). Lead can cause an increase in reactive oxygen species (ROS) levels in the body which results in oxidative stress. Oxidative stress is a condition where there is an inequality between the antioxidant defences and level of ROS, and causes oxidative damage (Redza-Dutordoir and Averill-Bates, 2016). The generation of ROS in cells is in equilibrium with the defence system against free radicals. Excessive formation of ROS can cause stress response which then causes an increase in the aging process of cells (Kuilman et al., 2010). Conversely, low ROS levels affect the lifetime of an organism (Davalli et al., 2016). Several disease and aging process affected by accumulation of ROS which induces apoptosis and can drive even skin cancer (Widowati et al., 2016). Furthermore, ROS production have a crucial role to the elevate of many inflammatory disorder (Mittal et al., 2014).

Salacca zalacca known as snake fruit is a plant species of the palm tree family (Arecaceae) that native to Indonesia. Snake fruit peel are the major waste of the consumption because is hard and inedible, even so the previous study discover that snake fruit peel contains important phenolic compound such as rutin, chlorogenic acid, caffeic acid, and protocatechuic acid (Girsang et al., 2019). Moreover, phytochemical compounds discovered in snake fruit peel were known to be active as an antioxidant (Gulcin, 2006; Kikuzaki et al., 2002; Liang and Kitts, 2015; Yang et al., 2008). In this study, we investigated the antioxidant and anti-inflammatory effects of SPE on lead-induced fibroblast cells (BJ) as aging role model.

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2 MATERIALS AND METHODS

2.1 Cytotoxicity Assay

Human fibroblast cell line (BJ) (ATCC® CRL-2522™) was received from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia. We detected to determine the maximum tolerance concentration of SPE on BJ cells and to determine the optimal oxidative damage concentration of lead (Pb) for the following experiments. BJ Cells were cultured in MEM (Biowest, L0416-500) suplemented with 10% fetal bovine serum (FBS) (Biowest, S1810-500), 1% Antibiotic/antimycotic (ABAM, Biowest, L0010100), 1% Nanomycopulitine (Biowest, L-X16-100), 1% Amphotericin B (Gibco, 1%), 0.1% Gentamicin (Gibco, 15750045). Cells were incubated at 37^0C in a humidified atmosphere with 5% CO_2. After that, 80% of cells confluency, 5 x 10^3 cells were seeded in each well of 96-well plate. After 24 h incubation, the cells were treated with SPE at various concentrations (3.13, 6.25, 12.5, 25, 50, and 100 µg/ml) for 24 h. To elect cell viability, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI, USA) was used. MTS was added to each well at a ratio of 1:10 (Novilla et al., 2017; Widowati et al., 2016) The plate was incubated in 5% CO_2 at 37^0C for 4 h. Absorbance was measured at 490 nm on a microplate reader. The data are given as the percentage of viable cells (%) and data were analyzed using ANOVA and continued by Tukey post hoc test.

2.2 Intracellular Reactive Oxygen Species Analysis

The intracellular ROS levels were detected by flow cytometry using a DCF-DA fluorescent probe (invitrogen) in accordance with the method of Jie et al (Jie et al., 2006; Prahastuti et al., 2019; Widowati et al., 2014) with slight modification. After 7 days of culture, BJ cells were digested with trypsin-EDTA and 10^5 cells were incubated with 10 µM DCF-DA at 37^0C for 30 min, after that incubated with SPE (25 and 100 µg/ml) for 4 h and followed by 400 µM Pb for 3 days. The intracellular ROS levels were analyzed using Milteny Flow Cytometer (MAQS quant). BJ Cells treated with Pb without SPE treatment showed as controls. The analyzed fluorescence values were expressed as a percentage of control.

2.3 IL-10 and TNF-α Evaluation

Evaluation of Interleukin 10 (IL-10) and Tumour necrosis factor-α (TNF-α) were conducted using ELISA Kit, IL-10 (Elabsci, E-EL-H0103) and TNF-*α* (Elabsci, E-EL-H0109), conditioned medium (CM) was used as a sample. Conditioned medium was collected after culture of fibroblast cells and treatment using SPE (25 and 100 µg/ml for 4 hours) following by inducted using Pb (400 µM for 72 hours). Cells were incubated at 37^0C in a humidified atmosphere with 5% CO_2. The method was in accordance with manufacturer protocol. Sample absorbances were read at 450 nm using spectrophotometer (Multiskan GO, Thermo Scientific). Colour changes of samples are evaluated then read immediately at 450 nm wavelength and the IL-10, TNF-*α* concentration can be determined based on a protein standard curve (Noverina et al., 2019; Widowati et al., 2018, 2017).

3 RESULTS AND DISCUSSION

Skin aging is a perplexing natural phenomenon defined by continuous loss of structural stability and function of the skin, beside skin aging can be induced by environmental factors such as lead exposure. Continuous lead exposure can lead to increased physical changes in the skin and connective tissue over intracellular ROS and cell contents (Widowati et al., 2016).

3.1 Cytotoxicity Assay

Many cell biological studies using fibroblast for standard cell line. Fibroblasts are liable for the metabolism and synthesis of most connective-tissue components and also play an effective part in the body's natural immune responses. Fibroblasts are the major cells in granulation tissue and scar forming over inflammation. In this research we define the cytotoxicity of SPE toward fibroblast cells, depend on the results SPE decreased cell growth only at the highest concentration (100 µg/ml).

To employ this natural compound to health concern, toxicity assay was conducted to assure safety. Tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) has been used for this assay. The MTS compound is bio-reduced by cells into a colored formazan product due to conversion by dehydrogenase enzymes in metabolically active cells (Novilla et al., 2017).

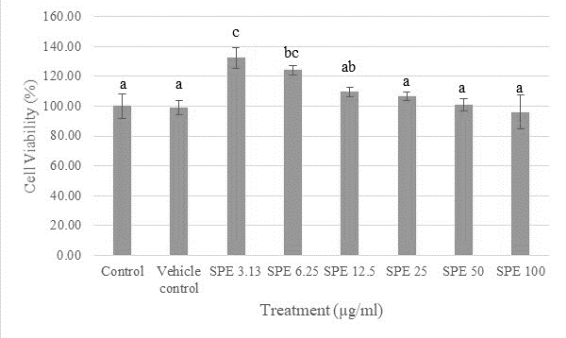


Figure 1: Effect of *S. zalacca* peels ethanolic extract (SPE) on cytotoxicity of fibroblast cells. *The histograms are presented as mean ± standard deviation. The data were analyzed with ANOVA and continued with Tukey post hoc test. Different letters (a, ab, bc, c) indicate significant differences among treatment. Control: cells without any treatments; Vehicle Control: cells with DMSO 10% treatment.

The total of each concentration of SPE were found non-cytotoxic and safe for fibroblast cell (3.13, 6.25, 12.5, 25, 50, and 100 µg/ml) (Figure 1), as the viability of the cells was more than 75% (Kanlayavattanakul et al., 2012). The lower number of cell viability was obtained from the highest concentration of SPE but not cytotoxic. The data of viable cell number were presented in percentage of viable cell. Depend on the post hoc test results, the safe concentration that can be used for further experiments and not harmful for cells are found in SPE concentrations 25 and 100 µg/ml.

3.2 Intracellular ROS Analysis

The accretion of ROS which generate apoptosis is then an main contributor to several disorders and aging (Orr and Sohal, 1994). The accretion of intracellular ROS in aging process can drives to loss of skin elasticity and cause formation of wrinkle, brown spots, uneven pigmentation, and even skin cancer (Widowati et al., 2016). Our outcome in accordance with previous research which express that lead exposure can elevate the ROS levels (Lopes et al., 2016). *Salacca zalacca* peel extract has natural compound such as chlorogenic acid (Liang and Kitts, 2015). Addition of chlorogenic acid can decrease ROS levels, in accordance with Hoelzl et al (Hoelzl et al., 2010), chlorogenic acid can decrease levels of ROS approximately 20.3% after hydrogen peroxide exposure.

Fluorescence intensity used as an indicator of ROS production levels. Figure 2 shows significant increase of ROS levels (relatively 25%) in cells induced lead (400 µM) for 72 hours compared to negative control (Cells stained using DCF-DA). The outcome of treatment using SPE with concentration of 25 and 100 µg/ml can significantly decrease ROS levels (relatively 15% and 23%). The better optimal SPE concentration in decreasing ROS levels was 100 µg/ml, but the concentration did not contrast significantly from SPE concentration of 100 µg/ml based on Tukey post hoc test.

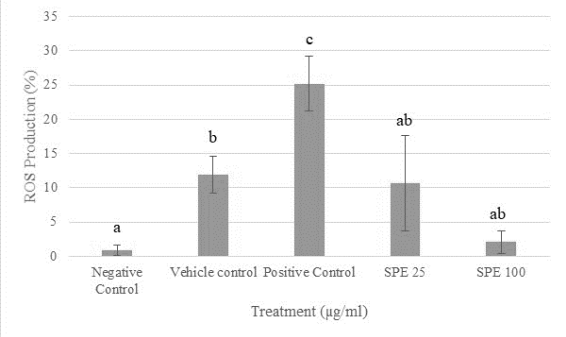
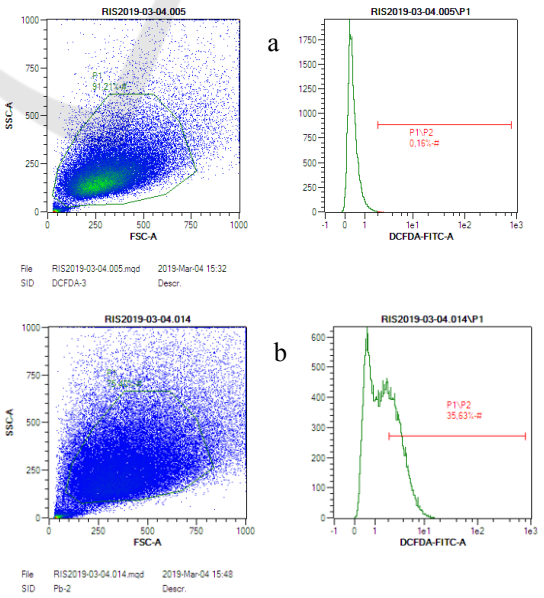


Figure 2: Effect SPE toward ROS production on lead-poisoned fibroblast cell. The histograms are presented as mean ± standard deviation. The data were analyzed with ANOVA and continued with Tukey post hoc test ($p<0.05$). Different letters (a, ab, b, c) indicate significant differences among treatment. Negative control: cells without any treatments; Vehicle control: cells with DMSO 10% treatment; Positive control: cells with Pb induced; SPE 25: cells treated Pb + SPE 25 µg/ml treatment; SPE 100: cells treated Pb + SPE 100 µg/ml treatment.



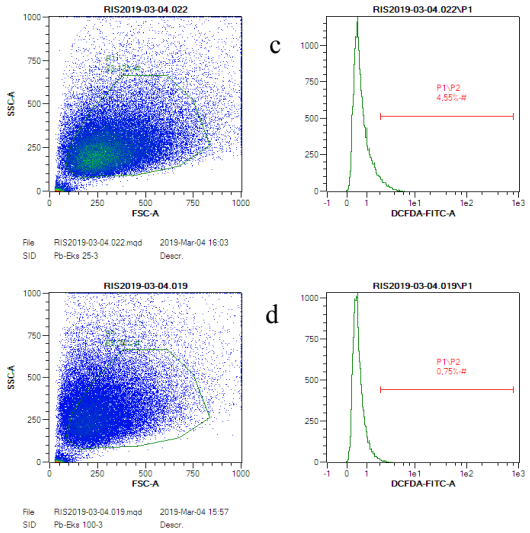


Figure 3: The representative of dot blot of various concentrations of SPE treatment on lead-poisoned fibroblast cell toward ROS level. TBHP = tert-butyl hydroperoxide, DCFDA = 2',7'-dichlorofluorescin diacetate. a: Negative control (Cell only + DCFDA) (0.16%); b: Positive control (Cell only + Pb) (35.63%); c: *S. zalacca Peels Extract* induced 25 µg/ml (4.55%); d: *S. zalacca Peels Extract* induced 100 µg/ml (0.75%).

3.3 IL-10 and TNF-α Evaluation

Accumulation of intracellular ROS production can improve an increase the expression of NF-kB, leading to the upregulation of factors elaborated in inflammation. Tumour Necrosis Factor-α (TNF-α) effective releasers of IL-6 which is one of the basic cytokines to be related to the aging process (Morley and Baumgartner, 2011). The effect of TNF-α can turn triggers effects that elevate inflammation that can indicate with elevated ROS levels (B. Marcu et al., 2010). Contrary, IL-10 is a cytokine which has a role as an anti-inflammatory and manage by stimulating antagonist proteins against TNF-α as pro-inflammatory cytokine (Wojdasiewicz et al., 2014).

During inflammation, IL-10 and TNF-α are cytokines that responsible. TNF-α and IL-10 has contrary role, TNF-α has a role as pro-inflammatory cytokine (Laksmitawati et al., 2016), while IL-10 has a role of anti-inflammatory cytokine (Morley and Baumgartner, 2011). Evaluation of IL-10 and TNF-α have been conducted using ELISA method. Fibroblast cells induced with lead has the highest levels of TNF-α among others, although SPE inclusion shows significant decrease in TNF-α. Besides, fibroblast cells lead-induced has the lowest levels of IL-10 and inclusion of SPE can elevate the IL-10 levels. SPE at 25 and 100 µg/ml can reduce the TNF-α levels but not significant, as well as IL-10 levels, both SPE concentration can elevate IL-10 levels but the results are not significant based on post hoc test.

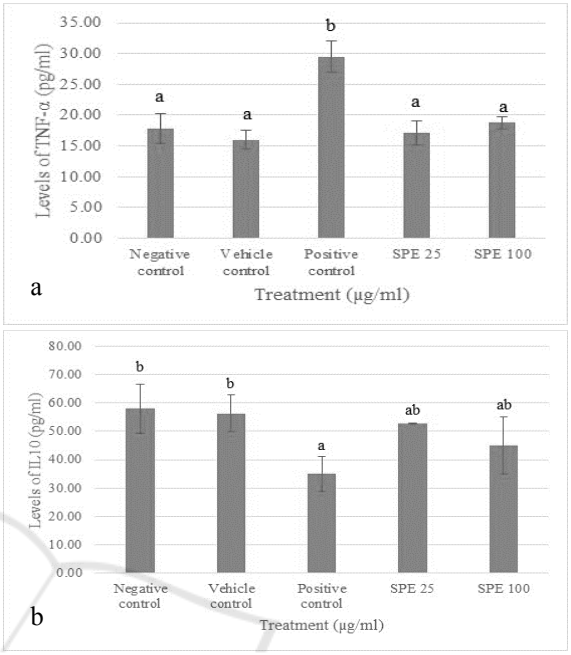


Figure 4: Effect of SPE toward TNF-α level (a) and IL-10 level (b) on lead-poisoned cells model. The histograms are presented as mean ± standard deviation. The data were analyzed with ANOVA and continued with Tukey post hoc test ($P<0.05$). Different letters (a, b) on figure A and (a, ab, b) on figure B indicate significant differences among treatment. Negative control: cells without any treatments; Vehicle Control: cells with DMSO 10% treatment; Positive control: cells with Pb induced; SPE 25: cells treated Pb + SPE 25 µg/ml treatment; SPE 100: cells treated Pb + SPE 100 µg/ml treatment.

4 CONCLUSIONS

This current study exhibit the compatibility of *S. zalacca* peels ethanolic extract as a promising antioxidant and anti-inflammatory agent through decrease of intracellular ROS levels and anti-inflammatory abilities to decrease pro-inflammatory cytokine (TNF-α) and increase anti-inflammatory cytokine (IL-10) on lead-poisoned human fibroblast cells.

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