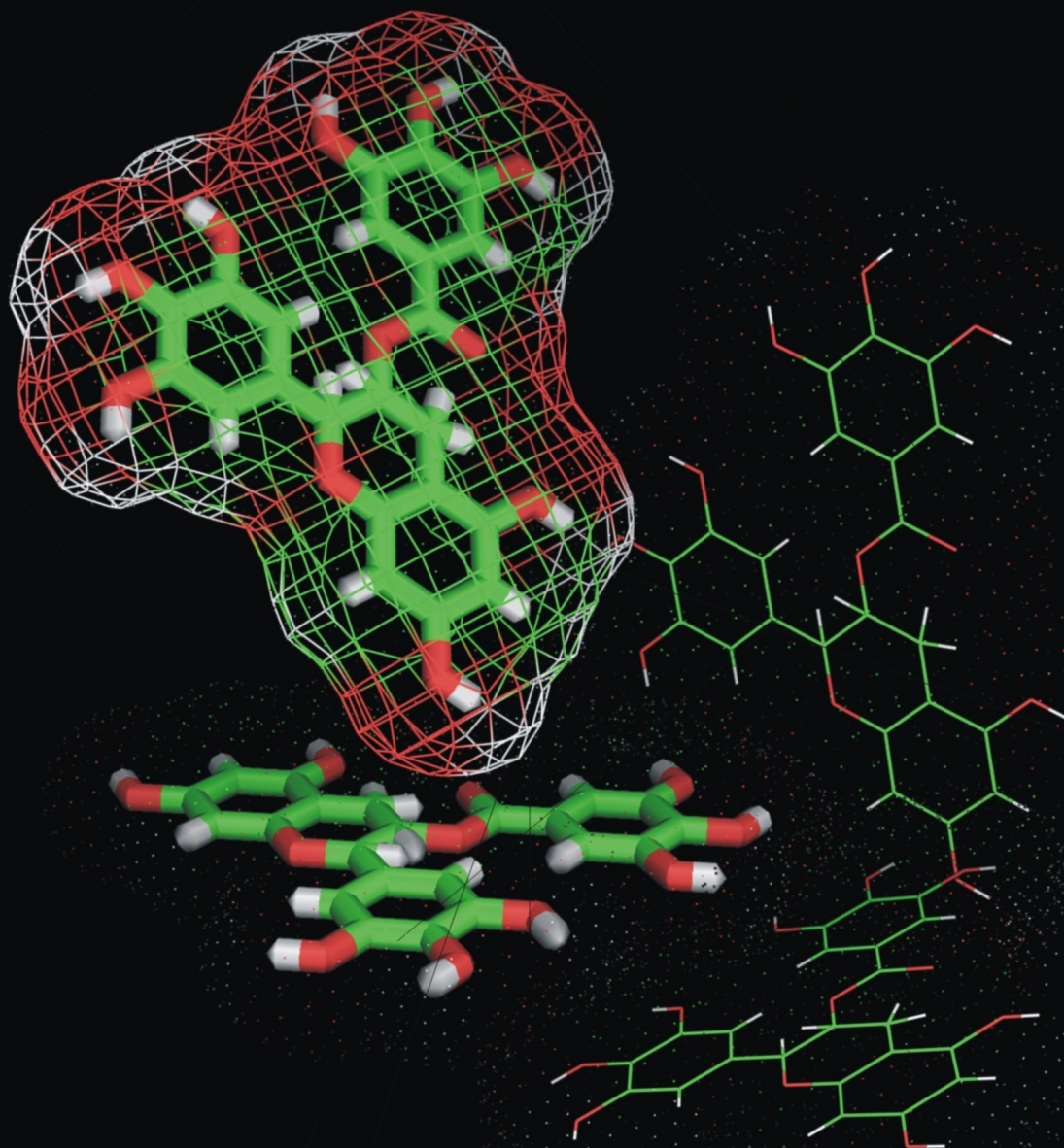


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Effects of Cadmium Exposure on Lipid Peroxidation and Chlorinative Stress of Rat Kidney

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Abstract

In addition to a wide range of adverse effects on human health, toxic metals such as cadmium (Cd) can promote kidney damage. In this study, we proposed the toxicological properties of these metals which are partly related to lipid peroxidation and chlorinative stress. Thus our study aimed to measure the lipid peroxidation and chlorinative stress in kidney of rats exposed to Cd. The study was performed on 32 male rats (*Rattus norvegicus*) weighing 300 ± 10 grams with normal activity. Rats were acclimatized for a week before the treatment. The animals were divided into two major groups with one group for control (K_0) and the other groups were exposed to 3 mg/L CdSO_4 through the drinking water. The animals in each groups were divided again into two small groups with different time of exposure. The time of exposure are subacute (six weeks) and subchronic (eight weeks). After the exposure the kidney from all treated animals showed the significant increase of MDA and AOPP levels. In conclusion, subacute and subchronic exposure of Cd caused the kidney damage through lipid peroxidation and chlorinative stress, as shown by increasing of MDA and AOPP levels.

Keywords: AOPP, Cadmium, Chlorinative Stress, Lipid Peroxidation, MDA

INTRODUCTION

Cadmium (Cd) is a heavy metal often used by humans for wide range of industrial materials, for example CdS and CdSeS compound widely used as dye, CdSO_4 used in the battery industry for Weston cell compartment, CdBr_2 and CdCl_2 used in photography, $(\text{C}_2\text{H}_5)_2\text{Cd}$ used in the tetraethyl-Pb producing, etc. In other side, industrial waste containing cadmium can enter into the water and at certain concentration it will become the source of toxin to aquatic life organism. Cadmium pollution can disrupt the stability and diversity of aquatic ecosystems. From the ecological aspect, degradation of aquatic ecosystems due to pollution of Cd can be determined through level and continuity of incoming contaminants into the water, toxicity characteristic, and bioaccumulation [1].

In South Kalimantan, Cd levels from construction and mining industry activity has polluted the aquatic environment. It was reported that Cd level in sediments sampled from Trisakti, Basirih, and Banjar Raya at South

Kalimantan already exceeded the threshold value ($<1 \text{ mg.L}^{-1}$) up to 1.019 mg.L^{-1} , 1.138 mg.L^{-1} , respectively [2]. Other study showed that the abandoned acid mine drainage of coal mine contained Cd level reach 23.7 mg.L^{-1} [3]. In addition, it was also reported that levels of Hg, Pb, and Cd contained in water bodies of Barito river basin exceeded the threshold value. This is presumably caused by the transport and unloading of coal activities passed through the river stream [3-4]. Increase levels of Cd in the waters cause some marine biota also has been polluted by Cd. A study mentioned that some species of shrimps and crabs in Takisung and Batakan South Kalimantan has been contaminated by Cd (mean of 0.213 mg.kg^{-1}) [5].

The pollution cause Cd enters into the body. Cadmium will lead to damage to various organs, especially liver and kidney which begun of cellular damage level. Cd is metabolized through biotransformation mechanism involving cytochrome P-450 in the mitochondria. Additionally, Cd that entered the body can lead to cytokines activation by releasing various inflammatory mediators. Induction of Cd caused increasing Tumor Necrosis Factor Alpha (TNF- α) as mediator of inflammation in rat [6]. This is caused by the activation of macrophages that produce cytokines. Furthermore, cytokine produce various inflammatory mediators,

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including TNF- α and NF κ B [7]. On the other hand, macrophages also phagocyte cadmium by releasing H₂O₂ and •O₂.

Resulted H₂O₂ molecules will be catalyzed by myeloperoxidase to form hypochlorite anion (HOCl). Then, HOCl will react with the amino groups of proteins to form chloramine. If antioxidant capacity is not able to reduce the reactivity of HOCl so chlorinative stress will be occurred. Advanced Oxidation Protein Products (AOPP) is a good marker for chlorinative stress of derivative phagocytes determination. The increase of AOPP reflects increasing H₂O₂ formation, myeloperoxidase activity enhancement, and increasing reactivity of hypochlorite anion to biomolecules containing the amino groups such as proteins in the cell structure [8].

Beside to induce inflammation, Cadmium also able to bind covalently with various ligands, such as -OH, -COO-, -OPO₃H-, -C=O, -SH, -SS-, -NH₂ and -NH, which abundant contained in amino acids as protein or enzyme constituent. Bond between Cd and ligands will inhibit the activity of antioxydative enzymes resulting in oxydative balance disorders [9,10]. This imbalance will lead to increase free radicals and oxidants so it also damage biomolecules of membrane constituent through formation of lipid peroxidation.

Since lipid peroxidation and chlorinative stress might be involved in kidney damage during Cd exposure. Thus our study was aimed to investigate effect of Cd exposure in kidney of rats through lipid peroxidation via MDA and chlorinative stress via AOPP formations.

MATERIALS AND METHODS

This study design was a true experimental study design, and examine the impact of Cd exposure in kidney of rats for different time of exposure. The rats were divided into two major experimental groups, for each groups consists of 16 rats and housed in standard rat cages. One set of rats in a group served as the control (K₀) while the other served as the test (K₁). The control groups were provided with distilled water as drinking-water while the test groups were provided with aqueous solution of CdSO₄ containing the equivalent of 3 mg.L⁻¹ (K₁) for six and eight weeks. Animals had free access to standard laboratory rat pellet and water ad libitum. At the end of treatment period the kidney was removed surgically under the anesthesia. Then the kidney was cut into small pieces and ground to form a liquid. Subsequently,

5 ml of the solution was taken and centrifuged at 3500 rpm for 10 minutes. The top layer of 200 mL were taken to be examined.

Preparing kidney homogenate

After dissection, the kidney removed and immediately fixed in phosphate buffer solution pH 7. Then the kidney was cut into small pieces and crushed to formed liquid. Subsequently, 5 mL of the solution was taken and centrifugated at 3500 rpm for 10 minutes. Supernatant was taken as much 200 μ L to be checked levels of malondialdehyde (MDA) and Advanced Oxidation Protein Products (AOPP).

MDA level determination

A total of 1 mL of kidney homogenate was added with 1 mL of distilled water, then put in the Eppendorf tube. After that, 100 μ L TCA 100%, 100 μ L Na-Tiobarbiturat 1%, and 250 μ L HCl 1 M were successively added. The solution was heated at 100°C for 20 minutes then centrifugated at 3500 rpm for 10 minutes. Thereafter, the supernatant was taken and added with distilled water until 3500 μ L. Level of MDA (absorbance) was measured using spectrophotometer with maximum wavelength of 540 nm [11].

AOPP level determination

Sample solution was prepared by mixing 200 μ L of homogenate, 600 μ L of 20 mM phosphate buffer pH 7,4 and 100 μ L of KI 1.16 M. While, blanko solution was prepared by mixing 800 μ L of phosphate buffer with 100 μ L of KI 1.16 M. Sample and blanko solutions were left for two minutes then added with 200 μ L of 10% acetic acid, and measured the absorbance at maximum wavelength of 340 nm. AOPP concentration was calculated by $A = \epsilon b C$ where $\epsilon = 26 \text{ mM}^{-1} \text{ cm}^{-1}$ dan $b = 1 \text{ cm}$ [12].

Data analysis

The obtained data was shown as mean and the standard deviation ($\pm$ SD). The data was prior to analyzed using Kolmogorov-Smirnov normality test and Levene homogeneity. If the data was normally distributed and homogeneous then tested by one-way analysis of variance test (One Way ANOVA) and continued using Tuckey HSD test. Data analysis was performed using SPSS version 17.0 with 95% confidence level ($\alpha = 0.05$).

RESULT AND DISCUSSION

The concentrations of MDA and AOPP in the kidney homogenate exposed to Cd in different time of exposure are shown in Table 1, with corresponding levels in controls. After the administration of 3 mg.L⁻¹ cadmium, MDA levels in the kidney homogenate increased significantly in both time of exposure compared to controls ($p < 0.05$). There was a time dependent increase in MDA levels in the kidney homogenate and they were higher in the subchronic exposure.

Table 1. Mean of MDA (μ M) and AOPP (μ M) levels in rat kidney before and after Cd treatment

Group (μ M)	Exposure time		
	Control	Subacute	Subchronic
MDA	46.14 $\pm$ 2.49	105.57 $\pm$ 4.62*	136.02 $\pm$ 4.58*
AOPP	0.97 $\pm$ 0.36	10.28 $\pm$ 0.25*	11.68 $\pm$ 0.43*

Malondialdehyde (MDA) was a parameter of lipid oxidative damage caused by free radicals and oxidants. The formation of MDA was started from polyunsaturated fatty acids containing two or more double bonds which highly susceptible to oxidation by hydroxyl radicals and peroxide compounds. Hydroxyl radical or peroxide compounds would attract hydrogen atoms of the double bond of unsaturated fatty acids and formed lipid peroxy radicals. This radical compounds then reacted with other unsaturated fatty acids to formed lipid hydroperoxide and new lipid peroxy radicals. This oxidation reaction continued to other lipid, or well-known as lipid autoxidation or lipid peroxidation process. The process would also form cyclic endoperoxide which broke down into malondialdehyde. MDA was one of molecules formed during lipid peroxidation as product of endoperoxide breaking down. MDA became into measuring tool widely used as an indicator of lipid peroxidation [13].

In this study, MDA levels increased along with the increasing concentration and duration of Cd exposure. This was caused by several mechanisms:

- Cd involved in Fenton or Haber-Weiss reaction that transformed peroxide compounds into reactive hydroxyl radicals. The hydroxyl radicals subsequently oxidized unsaturated fatty acids and formed stable MDA [13].
- The mechanism of respiratory burst due to cadmium caused increasing formation of peroxide compounds. Increase of peroxide would oxidize unsaturated fatty acids and formed MDA.

Thus, along with increase of concentration and duration of Cd exposure, the MDA levels would also increase. This was consistent with studied by Kara, the rats were injected subcutaneously with CdCl₂ each dose of 0.5 mg.kg⁻¹, 1 mg.kg⁻¹, 2 mg.kg⁻¹, 4 mg.kg⁻¹. After 24 hours, rats were dissected and measured levels of MDA serum. The results of that study could be concluded that the increase of CdCl₂ injected dose caused enhancement of MDA serum levels formation [14].

Another study also reported that the treatments of CdCl₂ with 1 μ M, 50 μ M, and 150 μ M concentration to human erythrocytes significantly increased the levels of MDA [15]. Along with enhancement of Cd concentration, production of MDA was also higher.

This results were supported by Aflanie *et al.* [16] who treated liver homogenate using Cd²⁺ with concentration of 0.003 and 0.006 mg.L⁻¹. The observation was carried out on days 2, 4, and 6. The results showed that there was positive correlation between reaction time with the formation of MDA ($R^2 = 0.81$; $p < 0.05$), which indicated the longer exposure time of Cd²⁺, MDA formed would also increase. In addition, the enhancement of Cd²⁺ concentration also increased the levels of MDA.

The result from table 1 also showed that Cd exposure could increased the AOPP level significantly, compared to controls. Also, there was a time dependent increase in MDA levels in the kidney homogenate and they were higher in the subchronic exposure.

Advanced Oxidation Protein Products (AOPP) was good marker for chlorinative stress of derivatives phagocytes determination. Increase of AOPP reflected increase in the formation of H₂O₂, enhancement myeloperoxidase activity; and increasing reactivity of free radical to biomolecules containing the amino groups, such as protein in the cell structure [8,17].

In vivo condition, AOPP was produced from macrophages activity which would phagocytized cadmium with released H₂O₂ and superoxide anion. H₂O₂ compound would be catalyzed by myeloperoxidase and formed hypochlorite anion (HOCl⁻). Furthermore, hypochlorite anion reacted with the amino groups of protein to formed chloramine. If the endogenous antioxidant capacity was not able to reduced reactivity of hypochlorite anion, chlorinative stress would be occurred [8]. Thus, if the chlorinative stress occurred in the kidney, the AOPP levels also increased.

In addition, the AOPP was also one of biochemical parameter indicated protein oxidative damage, primarily albumin. The damage was caused by free radicals and oxidant compound, and characterized by the structural modifications of proteins that showed with formation of dityrosine cross-link, disulfide bond, and carbonyl groups [18-19]. AOPP could be formed during the glycation process by forming H_2O_2 as residue molecule produced through oxidation of 2,3-enediol with presence of Cd and oxygen. On the other hand, due to the Fenton and Harber-Weiss reaction, H_2O_2 was converted into highly reactive hydroxyl radical. This hydroxyl radical would oxidize tyrosine aromatic amino acid residue into dityrosine, tryptophan into hydroxytryptophan, and phenylalanine into 2,3-hydroxyphenylalanine. In addition, hydroxyl radical also oxidized cysteine, methionine, and histidine, so that the protein structure to be modified [20-21].

Increase of AOPP levels caused kidney disorder characterized by tubular fibrosis and glomerulosclerosis, proteinuria which led to kidney dysfunction [22]. Kidney disorder was started from the amino acids oxidation from protein that made up the cell membrane, resulted in deformation of biomolecule structure of cell membrane constituent. This condition affected the osmotic pressure of the kidney and activity of Na^+K^+ -ATP ase enzyme [23].

The results of this study was consistent with our previous study, which concluded that four weeks of Cd exposure through the drinking water could increased the AOPP level. Cd exposure would result in respiratory burst involving NADPH oxidase activation to formed superoxide anion and other free radicals. Reaction of free radical with protein caused modifications of protein, measured as AOPP. In addition, formed superoxide anion modulated the production of $TNF-\alpha$, which was one of the proinflammatory factor. Between AOPP and $TNF-\alpha$ showed strong positive correlation, so AOPP could be used as inflammation parameter of the kidney [17,23]. Besides as inflammation and oxidative stress parameters, AOPP also played important role as cellular homeostasis. AOPP could provide information about the molecular mechanisms underlying kidney disorder related to oxidative stress. AOPP was modified form of cellular protein that could lead to kidney dysfunction, [23]. The modification included formation of disulfide bond from amino acid containing

cysteine, which could be used as cellular redox sensor.

CONCLUSION

Subacute and subchronic of cadmium exposure significantly triggered lipid peroxidation and chlorinative stress, indicated by increasing levels of MDA and AOPP.

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Effects of Low Temperature on Somatic Embryos Growth, Maturation and Plantlet Regeneration of Citrus Mandarin var Batu 55 (*Citrus reticulata* Blanco.)

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Abstract

This study was aimed to determine the effect of incubation at 4°C temperature during multiplication stage of somatic embryos on growth, maturation and plantlets regeneration of citrus Mandarin var Batu 55. Globular somatic embryos were cultured on MT (Murashige & Tucker) medium and incubated at 4°C temperature for 0, 2, 4, 6, 8 weeks. Maturation of somatic embryos was induced by transferring globular somatic embryos on maturation medium (MT + 50 mg l⁻¹ malt extract + 73 mM sorbitol + 73 mM galactose). Cotyledonary embryo was regenerated on MT medium + 50 mg l⁻¹ malt extract + 30 g l⁻¹ sucrose + 2 ppm GA₃. The research showed that maturation of somatic embryos at 4°C temperature inhibited growth and maturation of somatic embryos. Fresh weight of somatic embryo incubated at 4°C for 2 weeks lower than fresh weight of embryo cultured without incubation at 4°C, and continuous decline in longer incubation period. Maturation percentage of embryos without incubation at 4°C temperature was 15%, but embryos incubated at 4°C temperature were lower than 9%. There was no effect of incubation at 4°C temperature during somatic embryos multiplication stage on plantlet regeneration percentage.

Keywords: *Citrus reticulata*, maturation, plantlet regeneration, somatic embryo

INTRODUCTION

Citrus Mandarin var Batu 55 is one of the most economically important citrus variety in Indonesia caused of sweet taste a bit sour, soft textured fruit, easy to peel and almost seedless. This superior characteristic caused high market demand. But this productivity could not been able to fulfill the national citrus demand. One of the efforts to increase productivity was through the provision of citrus seedling. Micropropagation through somatic embryogenesis is an effective method of plant propagation in large scale for mass seedling production such as citrus [1], apple [2], mango [3] and other fruit plant.

Regeneration of citrus Mandarin var Batu 55 through culture techniques have been successfully carried through somatic embryogenesis techniques using nucellar as an explant [4]. However plant regenerated from in vitro technique has long juvenile period ranging from 3-5 years [5]. Thus it was capable for producing citrus rootstock. Therefore it was necessary to find a method to produce citrus scion with short juvenile period.

Modification of culture conditions such as photoperiod and low temperature treatment has been able to reduce the juvenile period of plants seedling regenerated from in vitro culture.

Low temperature exposure successfully stimulated flowering in some plants such as in *Cinchorium intybus* [6] and *Oenothera fruticosa* [7]. Low temperature treatment given during somatic embryos multiplication stage could produce plant with short juvenile period, but it was also affected the growth and development of somatic embryos. This study aimed to evaluate the effect of low temperatures treatment on somatic embryos growth, maturation and plantlets regeneration of citrus Mandarin var Batu 55.

MATERIALS AND METHODS

Induction and multiplication of somatic embryos

Somatic embryos were obtained from nucellus explant cultured on MT medium +50 g l⁻¹ sucrose + 3 ppm BAP + 50 mg l⁻¹ malt extract, cultured incubated at room temperature as long as two months [8]. Embryogenic calli subcultured on MT medium + 30 g l⁻¹ sucrose every 6 weeks, and cultured at 25°C ± 2°C. Somatic embryos that have undergone three times subculture used as explants for low temperature treatment.

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Low temperature treatment

Globular stage somatic embryos were incubated at 4°C for 2, 4, 6, 8 weeks, whereas the control was embryos incubated in temperature room. Each treatment was repeated 5 times (5 bottles), each bottle was cultured with one clump embryos (0.1 g). After each incubation period, embryos were incubated in room temperature as long as two months. Somatic embryo growth was examined by measuring somatic embryo fresh weight.

Somatic embryo maturation and plantlet regeneration

Embryo without incubation at 4°C and embryos in temperature 4°C treatment were transferred to MT + 50 mgL⁻¹ malt extract + 73 mM sorbitol + 73 mM galactose. Culture incubated in room temperature for three months, then number of cotyledonary embryo and maturation percentage was counted.

Cotyledonary somatic embryos were germinated on MT medium + 30 gL⁻¹ sucrose + 50 mgL⁻¹ malt extract + 2 ppm GA₃. Each bottle was cultured with 10 embryos and incubated at room temperature for three months, then number of plantlet and plantlet regeneration percentage were counted.

Data Analysis

The data of somatic embryo growth, percentage of maturation and percentage of plantlet regeneration were analyzed by the ANOVA test ($p < 0.05$). Significant difference was tested by Duncan's multiple range test ($p < 0.05$).

RESULT AND DISCUSSION

Effect of low temperature treatment on somatic embryo growth

Low temperature did not affect the morphology of embryos, but affected somatic embryo growth reflected by different size of embryo clump in each incubation period. Increasing incubation period at 4°C increasingly inhibited the growth of somatic embryos, it is shown in smaller clump size. Incubation at 4°C temperature during embryo multiplication stage decreased somatic embryo fresh weight. Fresh weight of somatic embryo incubated at 4°C for 2 weeks was lower than embryos incubated at room temperature and continuous decline in longer incubation period. Fresh weight of untreated embryo reaches 0.61 g, treated embryos are lower than 0.5 g. The lowest fresh weight was shown by somatic embryo cultured

in 4 °C treatment for 8 weeks in the amount of 0.17 g (Fig. 1).

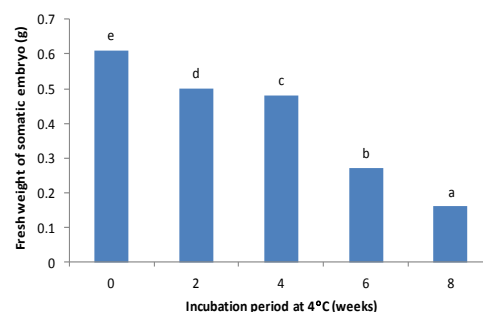


Figure 1. The effect of low temperature treatment on fresh weight of somatic embryo. The same letter for each incubation period showed no significant difference between means by Duncan test 5%.

Effect of low temperature treatment on somatic embryo maturation and plantlet regeneration

After transferred on maturation medium as long as two months embryos were mature, shown by changing in color of embryos, which initially appears milky white to yellow with green spots. After three months cultured on maturation medium, there were found globular phase, heart-shaped, torpedo and cotyledonary embryo (Fig. 2 A-E). Incubation at 4°C during multiplication stage inhibited somatic embryo growth and maturation. Somatic embryos incubated at 4°C produce fewer cotyledonary embryos than embryos incubated at room temperature (Fig. 2F-J). Somatic embryos incubated at room temperature and embryos incubated at 4°C able to regenerate into plantlets (Figure 3A-B). Plantlets which regenerated from embryos incubated at 4°C more than 4 weeks showed stunted growth (Figure 3C).

Incubation of somatic embryos at 4°C decreased maturation percentage. Somatic embryos incubated at 4°C show a higher percentage of maturation and number of cotyledonary embryos. The number of cotyledonary embryo and maturation percentage decreased with increasing incubation period at 4°C treatment. Maturation percentage of embryos incubated at room temperature was 15% with number of cotyledonary embryos was 25 embryos, whereas embryos incubated at 4°C for 4 weeks was 9% with number of cotyledonary embryo was 15. Maturation percentage and number of cotyledonary embryo in longer incubation period were less than 6% with number of cotyledonary embryo was about 5-11 embryos (Fig. 4A).

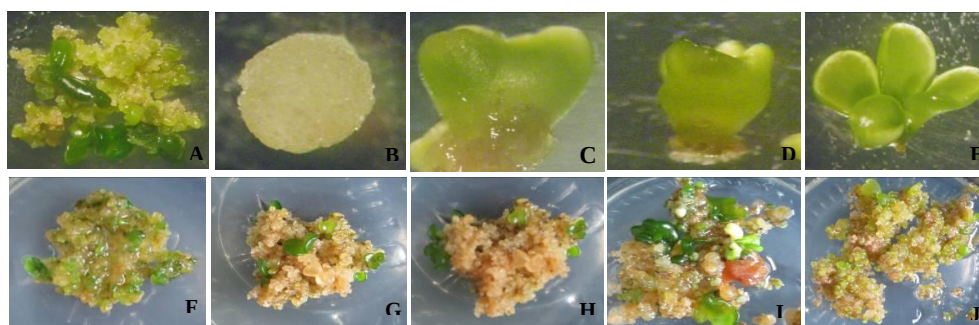


Figure 2. Developmental stage of *Citrus Mandarin var Batu 55* somatic embryos after incubated at 4 °C temperature. A. Embryo started mature; B. Globular phase; C. Heart-shaped; D. Torpedo; E. Cotyledonary; F. Embryos incubated at room temperature; G. Embryos incubated at 4 °C for 2 weeks; H. 4 weeks; I. 6 weeks; J. 8 weeks

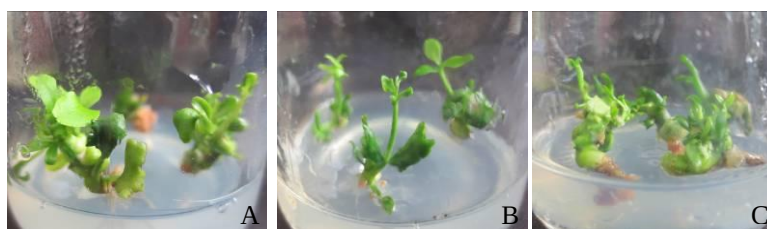


Figure 3. Plantlets regeneration from somatic embryos of *Citrus Mandarin var Batu 55*. A. Planlet regenerated from embryos incubated at room temperature; B-C. Plantlet regenerated from embryos incubated at 4 °C for 2 and 8 weeks.

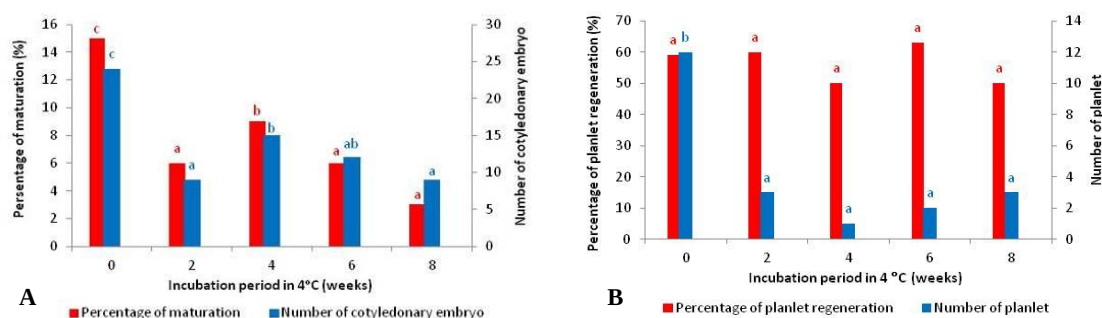


Figure 4. The percentage of somatic embryo maturation and the percentage of planlet regeneration of *Citrus Keprok Batu 55*. A. Percentage of maturation; B. Percentage of plantlet regeneration. The same letter for each maturation and plantlet regeneration percentage showed no significantly difference between means by Duncan test 5%.

There was no significant difference in planlet regeneration percentage between embryos incubated at room temperature and embryos at 4 °C. Planlet regeneration percentage of embryos incubated at room temperature and embryos incubated at 4 °C was less than 60%.

Although there was no significant difference of planlet regeneration percentage, but there was a significant difference between numbers of plantlet. Incubation of somatic embryos at 4 °C temperature decreased the number of plantlet, number of plantlet regenerated from embryos incubated at room temperature was reach 12, whereas number of plantlet regenerated from embryos incubated at 4 °C less than 3 (Fig. 4B).

Incubation at 4 °C temperature during somatic embryo multiplication step inhibited growth and

development of somatic embryos. This may occur because 4 °C temperature cause physiological stress that interfere with cell metabolism such as photosynthesis, respiration, water content, and the stability of the membrane, cell ultrastructure damage. Furthermore it led to decreased cell division and differentiation and embryogenesis [9]. Low temperature treatment causes changes in wall and cell membrane interactions, which cause any changes in gene expression and proteins synthesis that play a role in the development of somatic embryos. In addition, these proteins may be a key protein that induces maturation and germination of somatic embryos resulted an abnormal embryos [10]. Abnormal embryos morphology tends to be slow to form plantlets because it can lead to blocking the

development of the embryo in the early stages of somatic embryo development. Plantlets formed from abnormal embryos showed gigantic growth followed by none formation of buds, but capable of forming roots [11].

Eventhough low temperature caused abnormalities of somatic embryos, low temperature was also essential for maturing somatic embryo and germination in *Vitis vinifera* x *Vitis rupestris*. Globular stage embryo culture of grape (*Vitis vinifera*) was treated with 4°C temperature for two weeks, showed increasing in somatic embryo maturation percentage [12].

CONCLUSION

Incubation at 4°C temperature affected growth and development of somatic embryo in Citrus Mandarin var Batu 55. Incubation at 4°C decreased somatic embryo fresh weight and percentage of maturation. Somatic embryo incubated at room temperature and embryos incubated at 4°C able to regenerated into planlet. Although there was no affect on plantlets regeneration percentage, but number of planlet regenerated from embryo incubated at 4°C was lower. The lowest fresh weigh, maturation and plantlets regeneration percentage showed by embryos incubated at 4°C temperature treatment for 8 weeks, in addition regenerated plantlets showed stunted growth.

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Detection of VipAlbumin® Effect in CD34 and SDF-1 Mobilization in Streptozotocin-induced Diabetes Mellitus Mice

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Abstract

Diabetes mellitus is a disease in which the body loses its ability to provide tight regulation and maintain a dynamic interaction between the tissue sensitivity and insulin secretion by β cells. The impact of this dysfunctional mechanism is uncontrolled blood glucose levels that lead to hyperglycemia condition. Highly reactive free radicals have a strong involvement in the pathogenesis of diabetes mellitus, where one of its forming process may be triggered by hyperglycemia condition. Patients with diabetes mellitus itself vulnerable to endothelial dysfunction, which is caused by a decrease in circulating endothelial progenitor cells, and also a decrease in chemokines which play a role in affecting the activities of these cells. Hyperglycemia condition and free radical activity is a major cause of these endothelial progenitor cells dysfunction. The purpose of this study was to determine the role of VipAlbumin®, a supplement derived from *Channa striatus* albumin extracts in inhibiting the action of free radicals that are formed due to hyperglycemia condition, which can affect the increase in endothelial progenitor cells relative amount. This study used BALB/C mice that induced to undergo diabetes mellitus through streptozotocin injection intraperitoneally at 5-day old. Mice who have reached 4 week old and positive to diabetes mellitus (blood glucose levels $> 200 \text{ mg.dl}^{-1}$) will be administered with VipAlbumin® orally for 14 days. VipAlbumin® dosage was divided into 4 groups: positive control (without VipAlbumin®); 1st dose ($0.01664 \text{ mg.gr}^{-1} \text{ BW}$); 2nd dose ($0.416 \text{ mg.gr}^{-1} \text{ BW}$); 3rd dose ($10.4 \text{ mg.gr}^{-1} \text{ BW}$). The last step was flow cytometric analysis to determine the development of endothelial progenitor cells relative amount, which isolated from bone marrow. The variables measured in this study were the relative amount of CD34^+ and SDF-1. Based to flow cytometric analysis, mice with VipAlbumin® administration did not show any significant improvement in CD34 relative amount when compared to the positive control. Relative amount of Chemokine SDF-1 itself, although only occur at the 3rd dose of VipAlbumin® treatment, has increased and significantly different from the positive control.

Keywords: CD34, diabetes mellitus, free radicals, hyperglycemia, SDF-1, streptozotocin, VipAlbumin®

INTRODUCTION

CD34 is a transmembrane glycoprotein sized 115-kD which is strongly expressed on progenitor /hematopoietic stem cell (HSPC), and progressively decreased when HSPCs differentiate [1,2]. CD34 is a wide used marker to detect hematopoietic progenitor in human. Several researches show that antigen CD34 has signal with transduction capacity and is involved in cell adhesion, which resulting polymerization of actin and homotypic adhesion on cells of CD34^+ and KG1a [3-7]. Circulation of immature cells derived from bone marrow, one of which is CD34^+ cells, contributes in holding vascular homeostasis and repair, and play an important role in maintaining vascular endothelial function [8].

The amount of CD34^+ cells, as the part of endothelial progenitor cells, are lower in diabetic

patients compare to those with normal glucose tolerance [9]. This leads to the reinforcement of the disease in triggering the endothelial dysfunction and the other forms of complication.

The high-level of blood glucose and free radicals activity on diabetic patients also can influence the reduction of SDF-1, a chemokine functioned to stimulate the mobilization of endothelial progenitor cells from bone marrow [10]. Stromal cell-derived factor-1 (SDF-1) or CXCL12 is a chemokine for CXC subfamily which is generally characterized as the pre-B cell stimulation factor and cloned from bone marrow cells supernatant [11]. SDF-1 is chemotactic factor for T cells, monocyte, pre-B cell, dendritic, and hematopoietic progenitor cells, and also functioned to support proliferation of B and CD34^+ progenitor cells. SDF-1 gave its effect by binding with CXCR, a member of G protein-coupled receptor superfamily [14,15].

Responding to ischemia, SDF-1 regulation is usually increased, and through its binding with CXCR4, will stimulate the bone marrow to release EPC (one of them is CD34^+ cells) which will later

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be recruited in ischemic area [10]. In diabetic animal, the deformed response of SDF-1 to ischemia correlates with the decline of progenitor cells release from bone marrow and will cause post-ischemic angiogenesis deformation [16].

To figure out the effect of blood glucose level increase towards cells development which expresses CD34 and SDF-1, mice models of diabetes mellitus are used. The mice are injected by streptozotocin, a molecule of 2-Deoxy-2-([methyl (nitroso) amino] carbonyl) amino) - β -D glucopyranose, that produce selective toxic toward β cells and inducing diabetes mellitus in most of laboratory animals. High dose of β cells toxin such as streptozotocin and alloxan inducing insulin deficiency and type 1 diabetes with ketosis. But a precise dose calculation would partially destruct β cells mass and conduct mild insulin resistance that characterize type 2 diabetes [17].

The increase of blood sugar level through the injection of streptozotocin would trigger highly reactive radicals formation. In this research, VipAlbumin®, a supplement which extracted from *Channa striatus*, is used to obstruct free radicals impact on the endothelial progenitor cells activity. Several researches show that the snakehead murrell (*Channa striatus*) has positive effect as anti-inflammatory agent [18] reviewed from the high arachidonic acids level and important amino acids such as aspartic, glycine, and glutamic acid [19]. It also becomes the key factor in polypeptide formation which takes role in growth and wound healing [20,21]. The other functions are due to substance conductor, osmotic pressure regulator, platelets and anti-thrombotic formation hindrance, increase cells permeability, and as antioxidant [22,23].

MATERIALS AND METHODS

This research was conducted from November 2014 to May 2015 in Laboratory of Animal Physiology, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya.

Research design

The research was categorized into experimental research, in which the researcher intentionally manipulating the treatment to see the emerging effect. This research used *in vivo* procedure. There were 5 different treatment groups, i.e. negative control (healthy mice without streptozotocin injection and

VipAlbumin® administration), positive control (diabetes mellitus model mice without VipAlbumin® administration), and diabetes mellitus model mice with VipAlbumin® administration on 3 different doses. Each treatment were divided into 5 repetition.

In vivo Procedure

Diabetes mellitus model-mice induction through streptozotocin injection

Healthy mice were induced to have diabetes mellitus through streptozotocin injection intra-peritoneally at a dose of 100 mg.kg⁻¹ BW in the age of 5 days. Each mice in this research was assumed to weigh 2.5 g, thus, the proper dose of streptozotocin is 50 μ l. This amount derived from the mixture of 0.005 g of streptozotocin powder with 1 ml of citrate buffer. Successfully injected mice were then nurtured in sterile environment. The measurement of blood glucose level was conducted when the mice were reaching 4 week-old, by using glucometer. Mice was diagnosed undergo diabetes mellitus if the blood glucose levels were more than 200 mg.dl⁻¹.

VipAlbumin® administration on various doses

Oral administration of VipAlbumin® was done daily for 14 days to the diabetic mice. This administration were divided into 3 treatment doses: 0.01664 mg.g⁻¹ BW (dose 1); 0.416 mg.g⁻¹ BW (dose 2); 10.4 mg.g⁻¹ BW (dose 3). These three doses were derived from the albumin dose conversion for 1 kg of human BW i.e. 33.3 mg.kg⁻¹ with the assumption that the normal weight of adults was 60 kg. In order to figure out VipAlbumin® effect in decreasing blood glucose level, the measurement was done regularly in every 3 days.

Cell isolation from bone marrow

Mice were dislocated and dissected. Afterward, the femur and tibia part of the mice were flushed with PBS by using 1 ml spuit at one end. The obtained suspension is later put into propylene tube and centrifuged on 2500 rpm, 10°C temperature, for 5 minutes. The pellet formed was then resuspended with 1 ml PBS. The resuspension then taken 5 μ l, put in microtube, and added with 95 μ l evans blue. Pipetting is done for homogenization. The amount of successfully isolated cells was later counted with haemocytometer under microscope.

Antibody staining and Flowcytometric analysis

The staining process were divided into two phases based on the antibody type used. The first phase was extracellular antibody staining. 250 µl cell suspension isolated from bone marrow was put into microtube and centrifuged on 2500 rpm speed, 10°C temperature, for 5 minutes. Supernatant was eliminated and the pellet was added with 1 µl antibody which has being liquified with 50µl PBS dan 10% FBS. The suspension was later incubated in 4°C ice box for 20 minutes. The staining combination used was FITC-conjugated rat anti-mouse CD34.

The second phase was intracellular antibody staining. After conduct the procedure of extracellular antibody staining, cells were then given with 100 µl fixative solution cytofix/cytoperm and incubated 20 minutes in ice box. Mixed the cells with 500 µl washperm in order to clean the fixative solution remains. The suspension was re-centrifuged on 2500 rpm, 10°C temperature, for 5 minutes, whereas the pellet was then stained with intracellular antibody and incubated for 20 minutes in ice box. The staining combination used is PE/Cy5-conjugated SDF-1. The cells which were incubated by antibody staining is added with 300-500 µl PBS, and then moved into cuvette and being operated in flowcytometry machine.

Flowcytometric Data Analysis

The data obtained from flowcytometry then analyzed with BD Cellquest Pro™ software, and continued with the statistical analysis using SPSS version 16 for Windows. The statistical analysis

used was one way ANOVA parametric analysis with $p = 0,05\%$, followed by Tukey test.

RESULT AND DISCUSSION

Comparison of blood glucose levels on each treatment

The comparison of mice blood glucose levels on each treatment was measured regularly once in 3 days, for 15 days (Fig. 1). The positive control group (diabetic mice without VipAlbumin® administration) has significantly higher blood glucose levels than other treatments. Although it is not as high as the positive control, 1st dose (0.01664 mg.g⁻¹ BW) of VipAlbumin® also has an increase in blood glucose level along with the age. Both 2nd (0.416 mg.g⁻¹ BW) and 3rd dose (10.4 mg.g⁻¹ BW) of VipAlbumin® tend to have fluctuated blood glucose levels, which was able to increase or decrease depends on measurement days. However, the blood glucose levels from both treatments were significantly lower than positive control. The negative control has the lowest sugar blood level compared with the other treatments.

Flow cytometric analysis of CD34⁺ relative amount

We figured out that the relative amount of CD34⁺ cells in diabetic mice has a significant decrease compared with the normal mice (Fig. 2). The healing effort through VipAlbumin® administration has not given a notable change yet, whereas none of the three doses of VipAlbumin® treatment can increase CD34 expression and significantly different from the positive control (Fig. 3).

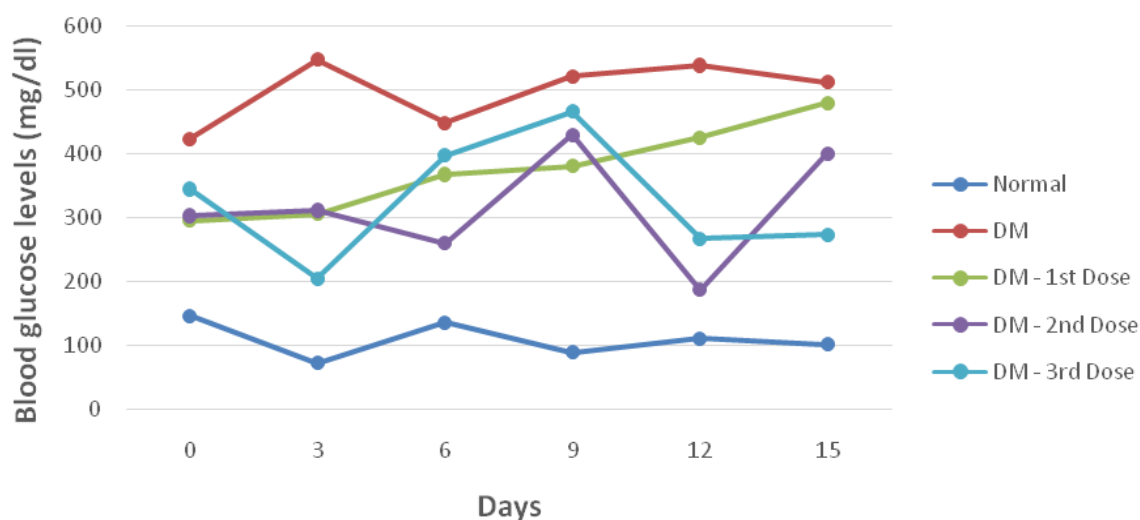


Figure 1. The Comparison of Blood Glucose Levels on Each Treatment

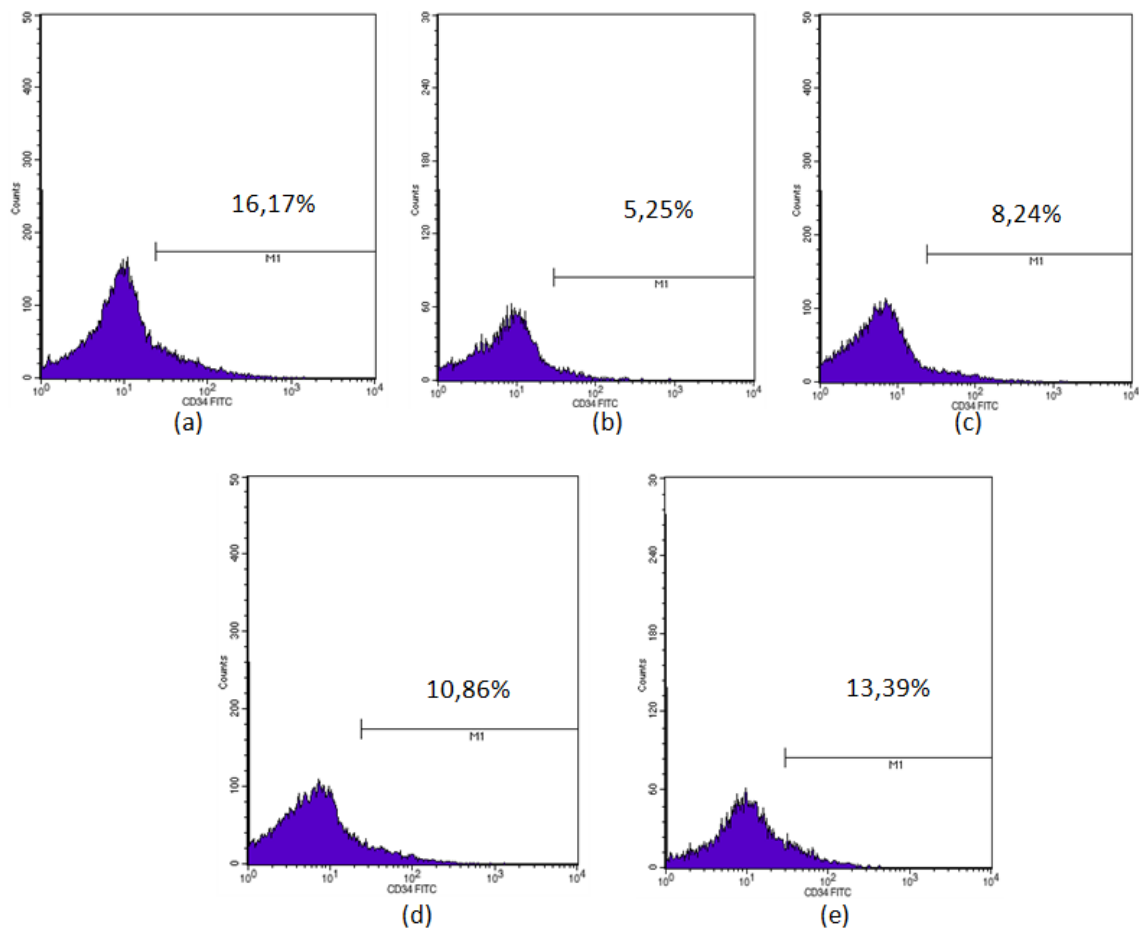


Figure 2. The Profile of CD34⁺ through flow cytometric analysis
(a) negative control, (b) positive control, (c) VipAlbumin® 1st dose, (d) VipAlbumin® 2nd dose, (e) VipAlbumin® 3rd dose

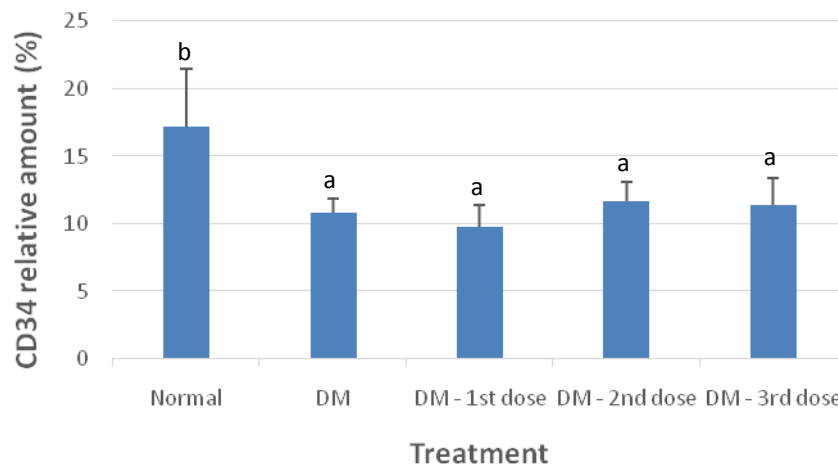


Figure 3. The comparison of CD34 relative amount on each treatment

It has been elucidated that diabetic patients experiencing the decrease of circulated progenitor. Although without complication, the amount of progenitor cells majority on diabetic patients still significantly lower than healthy control. The endothelial progenitor cells reduction can also be happened on patients with

high blood glucose level and HbA_{1c} (glycated hemoglobin) [24]. The previous research mentioned that the amount of CD34⁺, one type of the immature cell which was circulated in blood and becomes the part of endothelial progenitor cells (EPC), was lower in diabetic

patients compared with subjects having normal tolerance of blood glucose level [9].

The increase of blood glucose level (hyperglycemia) became the main factor affecting endothelial dysfunction, which was the first criterion of atherosclerosis pathogenesis [25,26]. Endothelial dysfunction takes important role in the development of atherosclerosis, and the circulation of endothelial progenitor cells derived from the bone marrow participating in the repair of vascular endothelial cells and defending the function of endothelial. On the patients with diabetes, it is reported that there was decreasing amount and dysfunction of circulated EPC, contributing to the diabetic microvascular complication [27].

Various research using animal model showed that the circulated EPC cells contribute to re-endothelialization and/or neovascularization therapy [28,29]. Several evidence indicates that various chemokine, cytokine, growth factor and their specific receptors can regulate the mobilization and recruitment of EPC from the bone marrow to the peripherals area, and in the process of proliferation and differentiation [30,31]. The chemokine (motif C-X-C) receptor 4 (CXCR4), receptor for SDF-1, play important role in the mobilization of cells from bone marrow and also in regulating the mobilization and recruitment of EPC [32-36].

One alternative way to prevent complication caused by hyperglycemia condition and the formation of free radicals is through induction of endothelial progenitor cells activation; in which this research using VipAlbumin® administration to conduct that process. Although the result shows that there was no significant increase in relative amount of CD34 compared with the positive control, it does not eliminate the potential function of albumin. Albumin functioned as the antioxidant to obstruct the free radicals ROS/NOS movement, maintaining the extracellular redox equilibrium, and playing role in the transportation process of various molecules such as fatty acid, nitric oxide, hemin, and drugs [37,38]. Some other researches show that in culture condition, albumin can be used as the inhibitor of apoptosis process for macrophages, neutrophil, lymphocyte, and endothelial cells [39-42]. In primary structure form, albumin contains 34 cysteine residues contributing with 17 disulfide bridges to form whole tertiary structure with one free cysteine residue (Cys34) which plays important role in various functions of albumin mentioned before. This very active residue that containing

80% (500 $\mu\text{mol.L}^{-1}$) total thiol in plasma is the primary scavenger of reactive oxygen and nitrogen in plasma [43].

Another strategy also proposed to improve the healthy condition from diabetic patients with hyperglycemia by using propolis treatment [44]. This material is known to contain high-level of nutrient factor such as vitamins, polyphenols, and amino acids, that expected to improve insulin sensitivity and suppress the action of inflammatory molecules. This suppression activity had a high relationship with the ability of T regulatory cells (especially on $\text{CD4}^+\text{CD25}^+$ population), while the increase number of this cells can induced by propolis intake. Highly reactive T cells could induce insulin resistance by its pro-inflammatory action, but T regulatory cells can prevent this effect by producing IL-10 and TGF- β . This cells can also prevents inflammatory condition being wide-spread, by reversing the activated memory T cells become naive type [45].

Flow cytometric analysis of SDF-1 relative amount

Flow cytometric data analysis shows that relative amount of SDF-1 on diabetic mice was significantly decrease compared to the normal control (Fig. 4). Two given doses of VipAlbumin® i.e. 1st dose (0.01664 mg.g^{-1} BW) and 2nd dose (0.416 mg.g^{-1} BW) did not show meaningful increase of SDF-1 expression. The significant increase compared with the positive control only occurred in the 3rd dose (10.4 mg.g^{-1} BW) of VipAlbumin®, although the relative amount has not yet reached the normal condition (Fig. 5).

It was reported that diabetic patients will experience the decrease of SDF-1, a chemokine that stimulates the endothelial progenitor cells mobilization (EPC) derived from the bone marrow [10]. The decreased ability of EPC mobilization from the bone marrow becomes one of the mechanism resulting the low amount of circulated EPC [28,36]. It has been known that SDF-1 and the expression of its receptor (CXCR4) play an important role in the regulation of mobilization and recruitment of progenitor cells, so that the reduction of the molecules estimated to be the cause of low amount of circulated EPC [32,34,36]. The cells expressing CXCR4 correlates positively with the progenitor cells amount on normal individual, while on diabetic patients, there is no correlation. Other research indicated that there is significant decrease of CXCR4 expression in PBMC of type 2 diabetic patients [24].

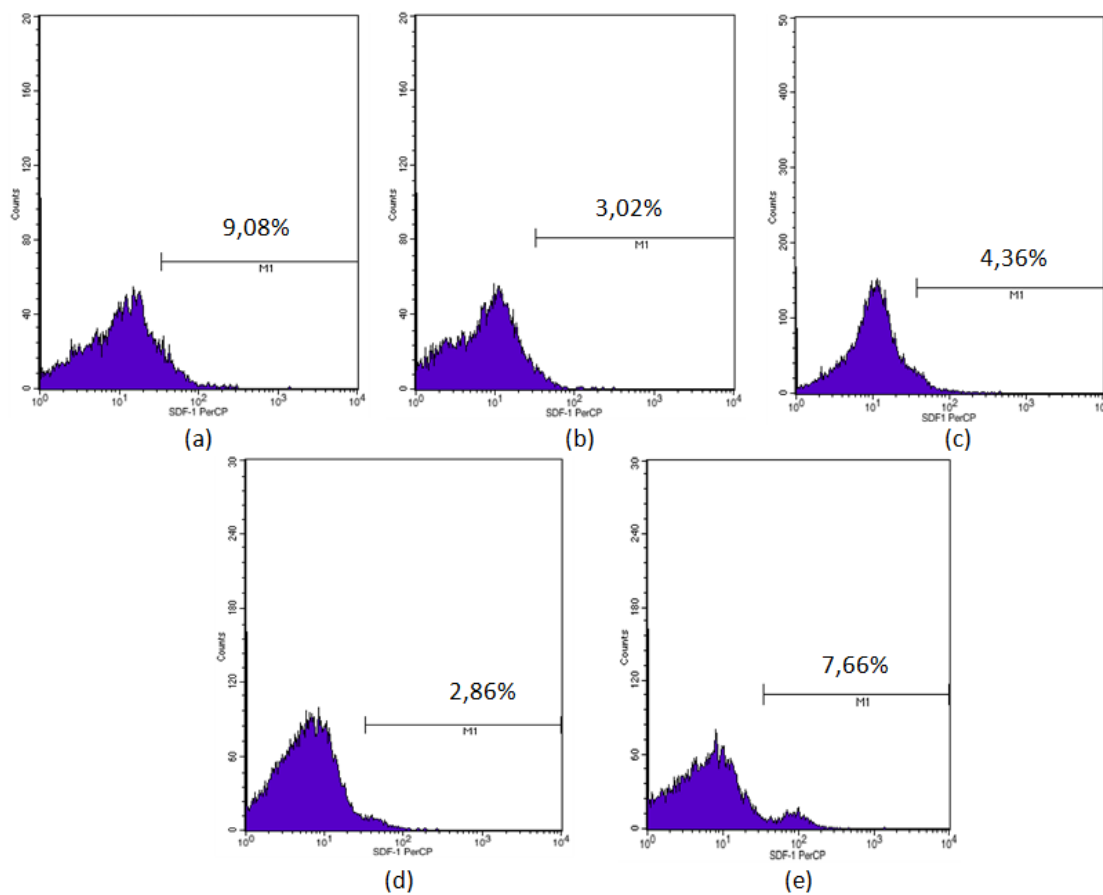


Figure 4. The Profile of SDF-1 through flow cytometric analysis; (a) negative control, (b) positive control, (c) VipAlbumin® 1st dose, (d) VipAlbumin® 2nd dose, (e) VipAlbumin® 3rd dose

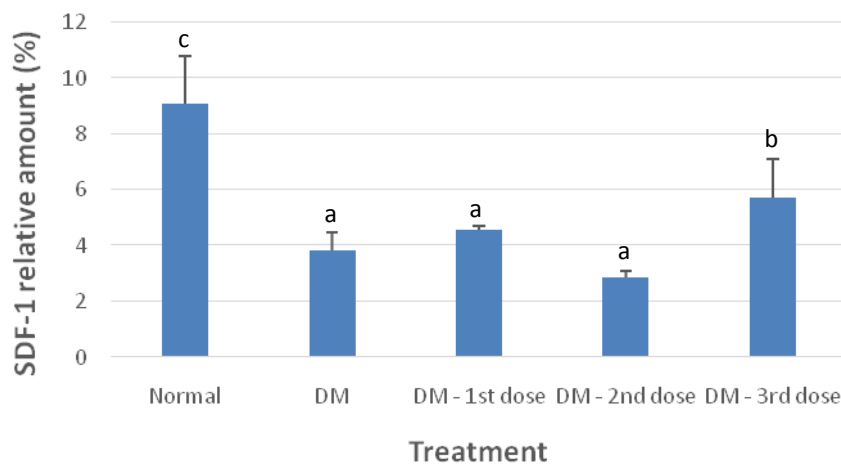


Figure 5. The comparison of SDF-1 relative amount on each treatment

The decrease of circulated EPC amount is affected by the low ability of EPC cells in responding to SDF-1 [36]. It was also reported that on diabetic patients, there is decrease in ability of CD34⁺ cells in migrating, which is affected by the disability in responding the SDF-1 performance [46]. The genotype of SDF-1 can

influences insulin in mobilizing mature progenitor cells on type 2 diabetic patients [47].

CONCLUSION

In this research, the administration of three doses of VipAlbumin® were not capable to increase CD34 relative amount, one of

endothelial progenitor cells marker derived from bone marrow. 1st and 2nd dose of VipAlbumin® also has no ability to stimulate the development of SDF-1 relative amount. The development of this molecule only occur at the 3rd dose of VipAlbumin® (10.4 mg.g⁻¹ BW), and was significantly different from positive control.

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The Effect of Gibberellin on Somatic Embryo Growth and Maturation and Plantlet Regeneration of Tangerine (*Citrus reticulata* Blanco.) var. Batu 55

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Abstract

The effect of gibberellin at multiplication stage on somatic embryo growth and maturation and plantlet regeneration of tangerine (*Citrus reticulata* Blanco.) var. Batu 55 was assessed. Somatic embryo at globular phase was cultured on MT media + 30 gL⁻¹ sucrose and various concentrations of gibberellin (0, 2, 4, 6, and 8 ppm). The somatic embryo was matured on MT media + 500 ppm malt extract + 73 mM sorbitol + 73 mM galactose. Cotyledonary somatic embryo was regenerated into plantlet on MT media + 500 ppm malt extract + 30 gL⁻¹ sucrose + 2 ppm GA₃. The results showed that the addition of gibberellin in somatic embryo multiplication stage increased somatic embryo growth and maturation and plantlet regeneration of tangerine. Optimum concentration of gibberellin needed for somatic embryo growth was 4 ppm which yielded two-fold fresh weight compared to control. The percentage of maturation was very low below 5%. The addition of gibberellin in media at embryo multiplication stage slightly increased the percentage of maturation about 1-2%. Thirty percent of cotyledonary embryo was able to regenerate into plantlet. The addition of gibberellin in media at embryo multiplication stage increased the regeneration percentage, even the addition of 8 ppm gibberellin yielded regeneration percentage up to 70%.

Keywords: gibberellin, growth, maturation, plantlet regeneration, somatic embryo

INTRODUCTION

Tangerine var. Batu 55 is one of local citrus varieties that has several special characters such as sweet, easy to peel, and almost seedless. These characters cause the high demand of tangerine, but it is still unfulfilled by national production. The fulfillment of national tangerine need was constrained by the procurement of large amount of good seeds. The effort to handle this problem was already conducted by using tissue culture technique through somatic embryogenesis method. Unfortunately, the plants regenerated from somatic embryo had long juvenile period [1]. Along with this reason, the endeavor to multiply seeds with shorter juvenile period was important.

Several tissue culture-derived plants with shorter juvenile period such as *Phoenix dactylifera* [2,3] and *Cymbidium niveo-marginatum* Mak [4] were successfully regenerated by means of media modification. The media modification used was the addition of gibberellin. Gibberellin is a plant growth regulator that plays role in flowering. According to

Blazques [5], gibberellin is able to shorten the juvenile period because it activates *LEAVY* promoter, gene that responsible for flowering.

In somatic embryogenesis, gibberellin was evidenced to trigger embryo maturation of *Medicago sativa* [6] and plantlet regeneration of *Vitis vinifera* [7], but it inhibited somatic embryo maturation of *Daucus carota* [8]. Therefore, it was important to assess the effect of gibberellin on somatic embryo growth and maturation and plantlet regeneration of Tangerine var. Batu 55.

MATERIALS AND METHODS

Induction and multiplication of somatic embryo

Somatic embryo was obtained from previous study [9]. Embryogenic calli were multiplied through subculture every six weeks and cultured on MT media + 30 gL⁻¹ sucrose. After three times subcultures, the somatic embryo was used for gibberellin treatment.

Gibberellin treatment

Somatic embryo at globular phase was cultured on MT media + 30 gL⁻¹ sucrose + various gibberellin concentrations (0, 2, 4, 6, and 8 ppm). Every treatment was repeated five times (5 bottles) and every bottle was filled with one clump of embryo. The embryo was maintained for two months. The morphology and the fresh weight were observed.

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Somatic embryo maturation and plantlet regeneration

Somatic embryo maturation was carried out on MT media + 500 ppm malt extracts + 73 mM sorbitol + 73 mM galactose. Maturation culture was maintained in room temperature for three months. Percentage of maturation was calculated by using this formula:

$$\% \text{ maturation} = \frac{\text{the amount of cotyledone embryo}}{\text{the amount of total embryo}} \times 100 \%$$

Cotyledone embryo as the result of maturation was regenerated into plantlet by culturing it on MT media + 30 gL⁻¹ sucrose + 500 ppm malt extract + 2 ppm GA₃. Percentage of regeneration was determined by using this formula:

$$\% \text{ regeneration} = \frac{\text{the amount of plantlet}}{\text{the amount of cotyledone embryo}} \times 100 \%$$

Data analysis

Data were analyzed using ANOVA. If there were any significant differences, analysis was continued using Duncan test (α : 0.05).

RESULT AND DISCUSSION

Effect of gibberellin on the growth of somatic embryo

Somatic embryo of Tangerine var. Batu 55 was friable and yellowish white. The growth of somatic embryo increased with the addition of gibberellin. The clumps of somatic embryo cultured in gibberellin media were apparently bigger compared to control (Fig. 1B-E). The addition of gibberellin in media increased the fresh weight of somatic embryos. The fresh weight of somatic embryo cultured in gibberellin media was significantly higher compared to control (Fig. 2). Optimum gibberellin concentration for somatic embryo growth was 4 ppm, giving the highest fresh weight (0.71 g), almost two-fold compared to control (0.37 g).

The proliferation of somatic embryo is related to the maintenance of embryogenic competence, in this term assumed as the growth of somatic embryo. Gibberellin caused various effects on the growth of somatic embryo in different species. In *Medicago sativa*, gibberellin enhanced somatic embryo growth [6], while in *Centaurea erythraea* it caused contrary effect [10]. The different result showed that beside plant regulator, the genotype was also a determining factor for somatic embryogenesis.

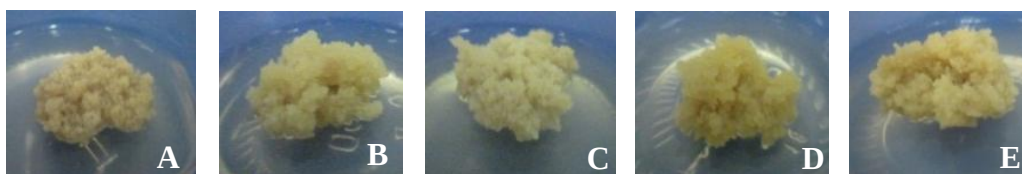


Figure 1. The growth of somatic embryo cultured in gibberellin media

A. Gibberellin 0 ppm (control); B. Gibberellin 2 ppm; C. Gibberellin 4 ppm; D. Gibberellin 6 ppm; E. Gibberellin 8 ppm.

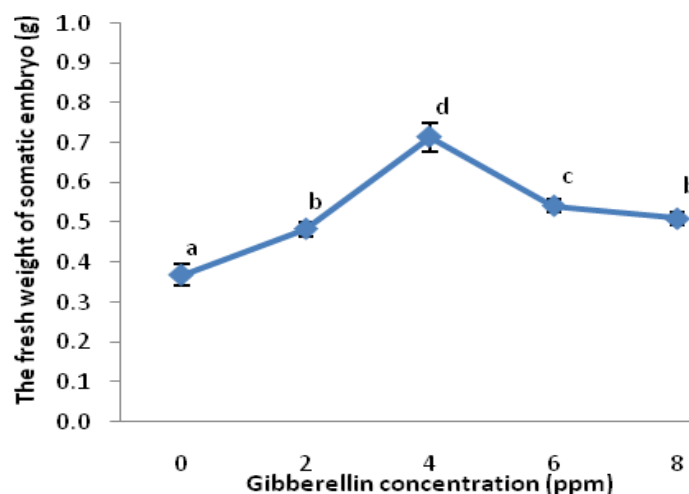


Figure 2. The effect of gibberellin on the fresh weight of somatic embryo.

The same letter did not show significant difference using Duncan's test (α ; 0.05).

Effect of gibberellin on somatic embryo maturation and plantlet regeneration

The maturation of somatic embryo indicated the cessation of somatic division and occurrence of differentiation. Somatic embryo maturation in tangerine was observed one month after the globular somatic embryo was transferred into maturation media. The markings of maturation were the change of shape and color of embryo that previously yellowish white globular embryo (Fig. 3A) into the next phase that greenish in color (Fig. 3B). The embryo maturation consisted of several phases: globular, heart, and cotyledone. Unfortunately, the torpedo phase was rarely seen. The embryo that already entered cotyledone phase was considered mature (Fig. 3C). The mature embryo was transferred into regeneration media to yield plantlet. The obtained plantlets were about 1.5-2 cm with primary roots and leaves (Fig. 3D).

The maturation of somatic embryo was very low. The maturation percentage was only below 5%, but the addition of gibberellin in media at embryo multiplication stage slightly increased the maturation. Somatic embryo cultured on media that contained gibberellin up to 4 ppm could yield higher maturation percentage. However, the maturation was inhibited in embryo that multiplied on media containing higher gibberellin concentration (Fig. 4).

The addition of gibberellin in media at embryo multiplication stage not only increased somatic embryo maturation but also the plantlet regeneration. The significant increment of percentage regeneration was obtained from somatic embryo that multiplied in media containing gibberellin of 8 ppm. It contributed to the highest regeneration percentage (70%), almost twice as control (36%) (Fig. 4).

Somatic embryo maturation was still a crucial problem of Tangerine var. Batu 55 somatic embryogenesis. The low conversion rate in many species from globular somatic embryo to the next

phases might be caused by the low quality of somatic embryo or the somatic embryo had developed tolerance toward desiccation [11]. Whereas, maturation should not only be synthetic phase and accumulation of food reserve, but also the end of desiccation tolerance. Thus, desiccation played important role in transition period between embryogenic phase and germinative phase.

The effect of gibberellin that increase the growth and maturation of somatic embryo was also observed in *Tylophora indica* (Burm. f.) Merrill somatic embryo [12]. Different result was obtained in *Arabidopsis* somatic embryo in which gibberellin inhibited maturation of somatic embryo [13].

The improvement of regeneration of plantlet by gibberellin was related to the activation of α -amylase genes. This activation caused activity acceleration of the α -amylase activity and the enhancement of starch hydrolysis into sugar occurred [14]. Hence, the plantlet development by means the elongation of the embryos axis took place [15]. The regeneration plantlet was also affected by the quality of the morphology cotyledonary embryo. Cotyledonary embryo morphology was important for plantlet regeneration [16]. The regeneration could occur only if the embryo had good polarization: a root axis and well-developed shoot (a hypocotyl and two cotyledons).

Exogenous plant growth regulator could be viewed as supplement for endogenous hormone, or conversely, it could be destructive for hormonal balance [8]. According to the result of this study, it could be noted that the gibberellin concentration used had already proper to support the balance of endogenous hormonal of Tangerine somatic embryo.

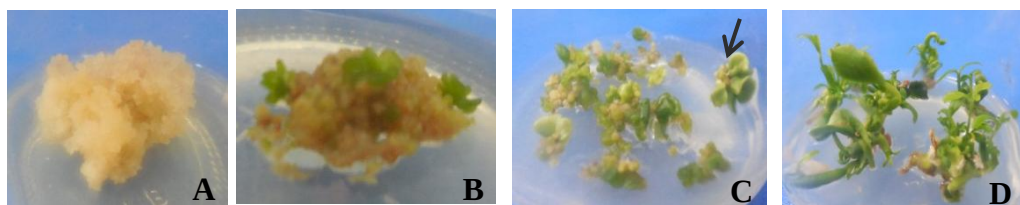


Figure 3. Steps of somatic embryo maturation and plantlet regeneration of Tangerine
A. Clump of globular somatic embryo, B. Somatic embryo showing the markings of maturation, C. Advanced maturation, D. Plantlet. Arrow showed cotyledon phase.

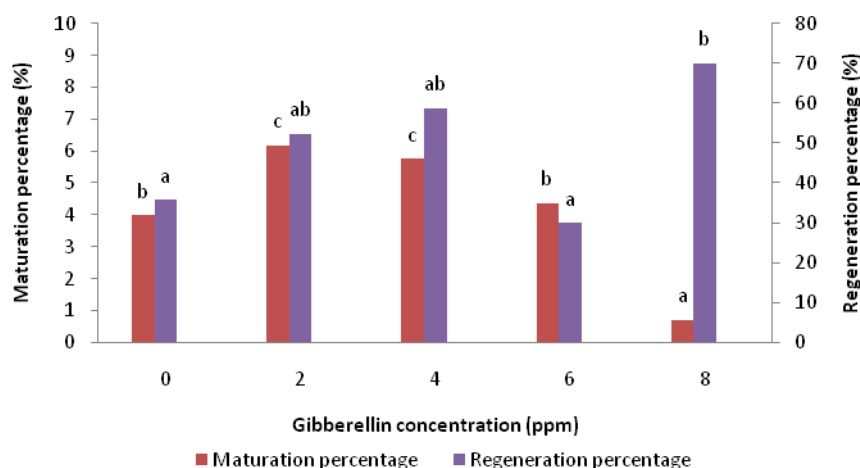


Figure 4. The percentage of somatic embryo maturation and plantlet regeneration of tangerine var. Batu 55. The same letter at the same bar did not show any significant different using Duncan's test (α : 0.05).

CONCLUSION

Gibberelin increased somatic embryo growth and maturation and plantlet regeneration of Tangerine var. Batu 55. The highest fresh weight was reached by the addition of 4 ppm gibberellin in culture media, almost twice as control. The percentage of maturation was very low below 5%. The addition of gibberellin in media at embryo multiplication stage slightly increased the percentage of maturation about 1-2%. Thirty percent of cotyledonary embryo was able to regenerate into plantlet. The addition of gibberellin in media at embryo multiplication stage increased the regeneration percentage, even the embryo cultured on media containing 8 ppm of gibberelline yielded the highest regeneration percentage.

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EMSA Eritin Blocks Nuclear Factor-Kappa B in Diabetes Mellitus Mice Model

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Abstract

Diabetes mellitus is one of the common metabolic disorders with increasing prevalence during recent years with hyperglycemia as its characteristic. DM has been shown to be a state of free radicals over production resulted from hyperglycemia that can activate cellular signaling pathways transcription of factor NF- κ B which stimulates the production of several inflammatory mediators and lead to chronic inflammation. Chronic inflammation is implicated in β -cell damage and function and promotes apoptosis. EMSA Eritin is a polyherbal consisting of soy bean extracts, coconut water extract and red rice extract that assumed to be antidiabetic and anti-inflammatory. This study will assess the effectiveness of EMSA Eritin against inflammation in diabetes mellitus by measuring levels of NF- κ B produced by immunocompetent cells in DM mice model. Streptozotocin 100 mg.kg⁻¹ BW is used to induce diabetes mellitus in mice. Oral administration of EMSA Eritin was given for 14 days with dose of 0.3125 mg.g⁻¹ BW, 3.125 mg.g⁻¹ BW and 31.25 mg.g⁻¹ BW. Data were analyzed using One-way ANOVA ($p < 0.05$) and Duncan test using SPSS 16.0 for Windows. The results showed that EMSA Eritin can be used as an alternative therapy for the treatment of DM. The level of NF- κ B in diabetic mice significantly decreased when the mice received EMSA Eritin.

Keywords: Diabetes Mellitus, EMSA Eritin, NF- κ B, ROS

INTRODUCTION

Diabetes mellitus is one of the most common metabolic disorders with increasing prevalence during recent years. Changes in lifestyle, diet and environment becomes the causes of the increasing prevalence of this disease. Prevalence occurred in Asia and Africa, as a result of urbanization trend and lifestyle changes [1]. Statistical data confirmed that diabetes has reached epidemic proportions. More than 346 million people world wide have diabetes and it is predicted to be the seventh leading cause of death in the world by 2030 [2]. Meanwhile, the number of patients with DM in Indonesia reached 8.6 million in 2000 and will increase to 21.3 million by 2030. Due to the high number of deaths, Indonesia was ranked 4th in the world after United States, India and China [3].

Diabetes mellitus characterized by hyperglycemia that occurs because the body is unable to secrete insulin in sufficient amounts or is unable to use insulin effectively or both [4,5,6]. DM can be a consequence of the reduction in the number of insulin receptors or failure in the

insulin-receptor binding, and failure in the transport of glucose into cells by the protein glucose transporter (GLUT) [7]. Previous research suggested that oxidative stress and inflammation play an important role in the development of DM [8].

Among various factors that are being involved in the progression of DM, oxidative stress could be typically induced by excessive nutritional factors like glucose and free fatty acids (FFA) which increasing formation of free radicals, auto oxidation of glucose and lipid peroxidation. In the process of oxidative stress, excessive reactive oxygen species (ROS) such as superoxide anion, hydroxyl radicals and hydrogen peroxide were produced by mitochondria and plasma membrane [9]. ROS can activate cellular signaling pathways such as nuclear factor- κ B (NF- κ B), which stimulates the production of several inflammatory mediators. Recent finding suggest that proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and TNF- α , play important roles in the pathophysiology of DM [7,9]. NF- κ B first became a main suspect in the development of insulin resistance and diabetes after the milestone discovery that the anti-inflammatory agent, aspirin inhibits NF- κ B and prevents degradation of the NF- κ B inhibitor, I κ B [10]. NF- κ B which is involved in proinflammatory cytokines production and in the

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induction of ROS-mediated NF- κ B activation, may be important in etiology of DM and related complications [11].

Appropriately prescribed drugs such as sulfonylureas or thiazolidinediones as monotherapy or add-on treatment are essential to cure the disease. However, many patients still cannot achieve satisfactory glycemic goals and even experienced serious side effects. Therefore, searching for alternative protective strategies becomes great interest. Recently, interests have been growing in the application of natural components as antidiabetic agents which have fewer side effects, cheap and easy to obtain [12,13]. EMSA Eritin is a dietary supplement that consisting of soy bean, coconut water and brown rice extract. Polyphenol compounds such as isoflavones and flavonoids, found in soy and red rice and mineral from coconut water known have antioxidant activity, anti-inflammatory, and anti-diabetic to prevent β -cell apoptosis, increasing β -cell proliferation, secretion and activity of insulin [13,14,15]. Based on these references, poly herbal EMSA Eritin is believed to be useful in the process of DM treatments. Therefore, this study will assess the effectiveness of EMSA Eritin towards inflammation in DM by measuring levels of the transcription factor of inflammatory, NF- κ B produced by immunocompetent cells in diabetes mellitus mice model with streptozotocin induced.

MATERIALS AND METHODS

Animal and research protocol approval

Healthy Balb/c mice (25-35 g) were purchased from the Central Animal Room, Cellular and Molecular Biology Laboratory, University of Brawijaya. The mice were maintained in a temperature and humidity-regulated room with controlled lighting (12-h light/dark cycle). The animals were given standard pellets and tap water was provided ad libitum.

Experimental design

Total of 25 Balb/c female mice, between the ages of 4-6 weeks were used and divided into 5 groups of treatment include negative control (healthy mice and without EMSA Eritin administration) and positive control (diabetic mice with EMSA Eritin administration). EMSA Eritin was grouped into three doses: Low dose (0.3125 mg.g⁻¹ BW), optimum dose (3.125 mg.g⁻¹ BW) and high dose (31.25 mg.g⁻¹ BW). Each treatment was repeated 5 times.

Drugs solution

Streptozotocin was dissolved in 0.1 M citrate buffer (pH 4.5). EMSA Eritin was dissolved in aquades.

Induction of diabetes

Streptozotocin (100 mg.kg⁻¹, i.p.) was administered to induce diabetes to 5-day-old female mice [4]. The mice were grown, fed and water by ad libitum. Mice were weaned at 4 weeks old and selected for screening by glucometer glucose tolerance test. Mice with glucose level $\geq$ 200 mg.dl⁻¹ were considered to be diabetic and included in the research.

Oral administration of EMSA Eritin

Positive diabetic mice were given EMSA Eritin orally at a dose of 0 mg.g⁻¹ BW (N); 0.3125 mg.g⁻¹ BW (D1); 3.125 mg.g⁻¹ BW (D2); and 31.25 mg.g⁻¹ BW (D3). The dose is obtained from the conversion of the human dose that is 250 mg.kg⁻¹ BW. EMSA Eritin of all doses was freshly prepared twice a day before administration and given orally by syringe at 9:00 a.m. and 03.00 p.m. Administration was conducted for 14 days. Blood glucose levels were measured every 3 days during the administration of EMSA Eritin to determine the change of blood glucose level.

Lymphocyte isolation

Mice were sectioned after 14 days treatment. Spleen were isolated and crushed in petri dish containing PBS. Lymphocyte homogenates were filtered using BD nylon cell strainerTM (100 μ m) and transferred into a new propylene tubes. Homogenates were centrifuged at 2500 rpm, 4°C for 5 min. Supernatant was removed and pellet resuspended in 1 mL PBS.

Antibody staining and flowcytometry analysis

Pellet stained with antibodies with extracellular and intracellular staining. The following antibodies were used for extracellular staining are fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4, phycoerythrin (PE)-conjugated anti-mouse CD8, and fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD68. Antibodies used for intracellular staining is PE-Cy5 conjugated anti-mouse NF- κ B.

For extracellular staining, cells were stained with 1 μ L of extracellular antibody that had been diluted with 50 μ L of PBS, and then incubated in ice box (4°C) for 20 minutes. For intracellular staining, cells were stained with extracellular antibody first and incubated in ice box (4°C) for

20 minutes. After that, cells were added with 50 μ L fixative solution (cytofix/cytosperm) and incubated again in ice box (4°C) for 20 minutes. Cells were added with 500 μ L washperm to removes the fixative solution and centrifuged at 2500 rpm, 4°C for 5 min. Pellet was stained with intracellular antibodies and then incubated in ice box (4°C) for 20 minutes. Suspension then was added with 300 μ L PBS, transferred to flow cytometry cuvet and ready for analyzed with flow cytometry.

Statistical analysis

The data were evaluated by one-way ANOVA using the SPSS 16.0 program for Windows. The differences between the means assessed using Duncan's multiple range test. Statistical significance was considered at $p < 0.05$.

RESULTS

EMSA Eritin suppressed NF- κ B in T lymphocyte

Nuclear translocation of NF- κ B in response to inflammatory insults is the primary regulatory step for its activation. NF- κ B showed high expression in diabetic mice compared with normal mice (Fig. 1). STZ injection in the diabetic group (DM) can significantly increase the relative number of CD4^{+} T cells NF- κ B $^{+}$ $0.06 \pm 0.01\%$

compared to the healthy group (N) 0.04%. EMSA Eritin which administered in diabetic mice showed immunomodulatory activity as immune-supresant of NF- κ B which produced by CD4^{+} lymphocyte T cells. The relative number of $\text{CD4}^{+}\text{NF-}\kappa\text{B}^{+}$ T cells in EMSA Eritin treatment group (D1-DM, D2-DM, and D3-DM) was significantly decrease ($p < 0.05$) compared with control group (N). The low dose, normal dose and high dose of EMSA Eritin can suppress the level of $\text{CD4}^{+}\text{NF-}\kappa\text{B}^{+}$ with the relative number 0.02%, 0.04% and 0.01%, respectively.

EMSA Eritin also showed suppression activity of NF- κ B that produced by CD8^{+} T cells (Fig. 2). The relative number of NF- κ B in negative control (DM) is 0.04% and increased after STZ injection 0.11%. Relative number of $\text{CD8}^{+}\text{NF-}\kappa\text{B}^{+}$ T cells in all of dose of EMSA Eritin treatment group was lower than negative control group (DM), with the value of each are 0.05%, 0.05% and 0.08% ($p > 0.05$). NF- κ B that produced by CD4^{+} and CD8^{+} T cells was decreased in all doses of EMSA Eritin treatments. The relative number of NF- κ B in treatment groups did not show a significant difference with healthy group. This result showed that the treatment group of diabetic mice had returned to normal condition or became healthier.

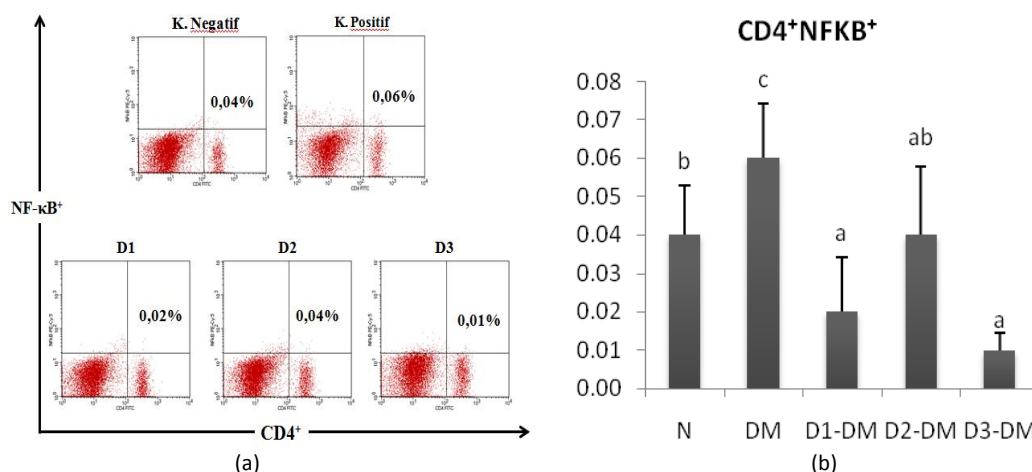


Figure 1. EMSA Eritin decrease relative number of NF- κ B on CD4^{+} T cells.

Description:

N: healthy mice group,

DM: diabetic mice group were treated with EMSA Eritin $0 \text{ mg} \cdot \text{g}^{-1} \text{ BW}$;

D1-DM = diabetic mice group were treated with Emsa Eritin low dose $0.3125 \text{ mg} \cdot \text{g}^{-1} \text{ BW}$;

D2-DM = diabetic mice group were treated with Emsa Eritin normal dose $3.125 \text{ mg} \cdot \text{g}^{-1} \text{ BW}$;

D3-DM = diabetic mice group were treated with Emsa Eritin high dose $31.25 \text{ mg} \cdot \text{g}^{-1} \text{ BW}$.

(a) The percentage of CD4^{+} T cells expressing positive NF- κ B using flow cytometry.

(b) The bars are the number of CD4^{+} T cells expressing positive NF- κ B on the spleen cells of mice after EMSA Eritin treatment.

The data are mean $\pm$ SD in each group with p value < 0.05 .

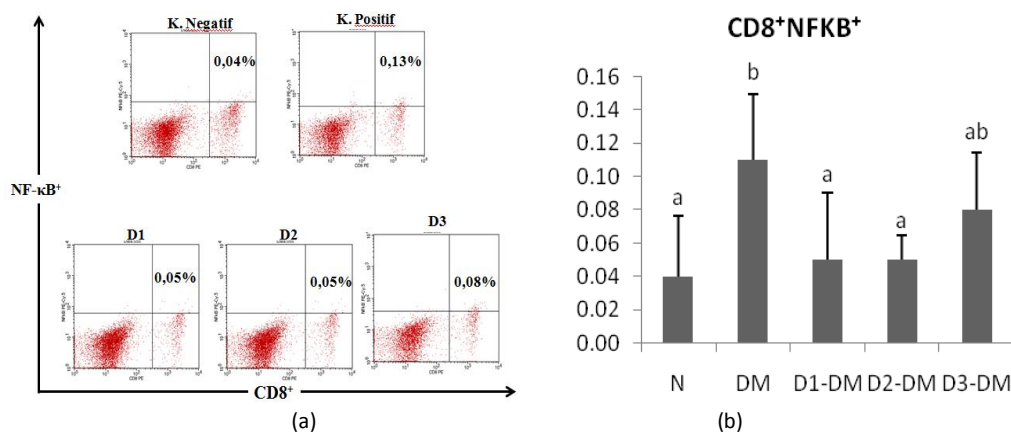


Figure 2. EMSA Eritin decrease relative number of NF-κB on CD8⁺ T cells

Description:

N: healthy mice group

DM: diabetic mice group were treated with EMSA Eritin 0 mg.g⁻¹ BW;

D1-DM = diabetic mice group were treated with Emsa Eritin low dose 0.3125 mg.g⁻¹ BW;

D2-DM = diabetic mice group were treated with Emsa Eritin normal dose 3.125 mg/gr BW;

D3-DM = diabetic mice group were treated with Emsa Eritin high dose 31.25 mg.g⁻¹ BW.

(a) The percentage of CD8⁺ T cells expressing positive NF-κB using flow cytometry.

(b) The bars are the number of CD8⁺ T cells expressing positive NF-κB on the spleen cells of mice after EMSA Eritin treatment. Data are mean±SD in each group with p value < 0.05.

EMSA Eritin suppressed NF-κB in macrophage cells (CD68⁺)

We also found that administered EMSA Eritin decreased the level of NF-κB that is produced by CD68 macrophage cells (Fig. 3). NF-κB was increased significantly (p<0.05) after mice got induction of STZ (0.26%) compared to healthy mice (0.11%) in Fig.3. The relative number of NF-κB in all dose treatment showed significant

difference compared to negative control group (DM) with the value of 0.17%, 0.20% and 0.14%, respectively. It showed that the treatment of EMSA Eritin improve the diabetic condition to be better. Increased levels of NF-κB would lead to proliferation of CD4 T cells, CD8 T cells and CD68 macrophage cells to produce proinflammatory cytokines that essential in the development of diabetes mellitus.

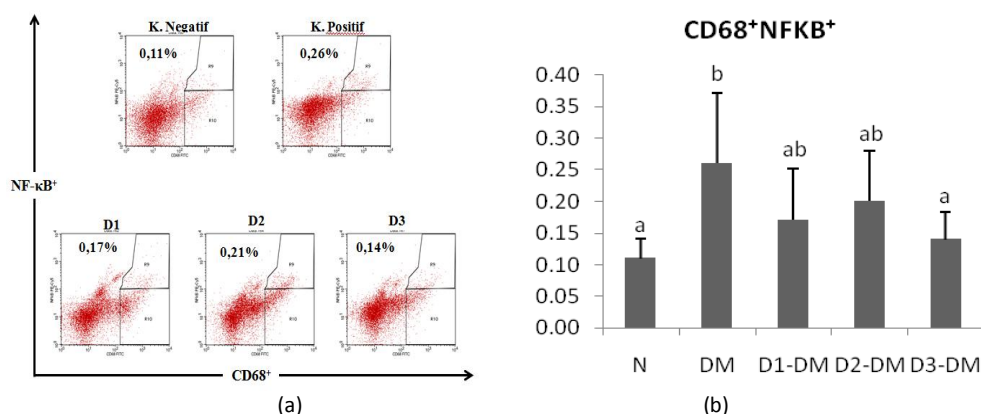


Figure 3. EMSA Eritin decrease relative number of NF-κB on CD68⁺ macrophage cells

Description:

N: healthy mice group,

DM: diabetic mice group were treated with EMSA Eritin 0 mg.g⁻¹ BW;

D1-DM = diabetic mice group were treated with Emsa Eritin low dose 0.3125 mg.g⁻¹ BW;

D2-DM = diabetic mice group were treated with Emsa Eritin normal dose 3.125 mg.g⁻¹ BW;

D3-DM = diabetic mice group were treated with Emsa Eritin high dose 31.25 mg.g⁻¹ BW).

(a) Bars are number of CD68⁺ macrophage cells expressing positive NF-κB on spleen cells of mice after EMSA Eritin treatment.

(b) The percentage of CD68⁺ macrophage cells expressing positive NF-κB using flow cytometry.

The data are mean±SD in each group with p value < 0.05.

DISCUSSIONS

Diabetes mellitus has been shown to be a state of free radicals over production resulting from hyperglycemia and FFA. Factors that attributed to the formation of free radicals may include not only elevated non-enzymatic and autooxidative glycosylation, but also metabolic stress as a result of alterations in energy metabolism, inflammatory mediators levels and the status of antioxidant defense [16]. In the process of oxidative stress, ROS primarily produced by mitochondria excessively. ROS activates cellular signaling pathways nuclear factor- κ B (NF- κ B) which stimulates the production of several mediators of proinflammatory cytokines such as IL-1, IL-6 and TNF- α [9].

The novel finding of the current study is that NF- κ B is involved in the development of inflammation-associated metabolic diseases such as diabetes mellitus. NF- κ B, or nuclear factor κ B, is a nuclear transcription factor found in all cell types. It is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, bacterial or viral antigens and activation of the adaptive immune receptors like TLR4 in response to FFA [10,17]. NF- κ B promotes immunity by controlling the expression of genes involved in inflammation. NF- κ B drives expression of target genes that mediate cell proliferation and release of cytokines to activate the immune response [17].

NF- κ B is a transcription factor composed of p50 and p65 subunits (heterodimer) and expressed in the cytoplasm of virtually all cell types. Its activity is controlled by a family of regulatory proteins, called inhibitors of NF- κ B (I κ B). In the non-active condition, NF- κ B in the cytoplasm constitute as heterodimer that binds to I κ B which responsible for the activation of NF- κ B. NF- κ B activity is under the control of signaling from extracellular stimuli. Cytokine or PAMP ligation of cell surface receptors initiates signaling cascades that converge on the activation of the inhibitor of κ B kinase (IKK) complex. The IKK complex consists of three subunits: the catalytic subunits IKK α and IKK β , and the regulatory subunit IKK γ . IKK phosphorylation of I κ B molecules promotes their degradation and releases heterodimers p65 and p50 NF- κ B dissociation from IKK α [17]. NF- κ B binds to NF- κ B binding domain on gene promoter and regulate the transcription of genes involved in innate immunity and inflammation. [18]. NF- κ B signaling pathway can be activated by many proinflammatory stimuli such as TNF- α which is

not only being the activator of NF- κ B but also a regulation product of NF- κ B [7].

EMSA Eritin showed anti-inflammatory activity when administered to the lymphocytes in mice model diabetes mellitus. EMSA Eritin reduced the level of NF- κ B that produced by CD4 T cells, CD8 T cells and CD68 macrophage cells. EMSA Eritin is a polyherbal that consists of soy bean extract, coconut water extract and red rice extract. This composition seem to be effective to suppress the transcription factor of inflammation, NF- κ B. Soy isoflavone genistein is one of the active compound on EMSA Eritin and has been reported to be protective agent for diabetes by regulation of oxidative stress and inflammation as it decreases lipid peroxidation, inhibits cyclooxygenase expression and myeloperoxidase activity as well as reacts with free radicals and neutralizes their effects [16]. Anthocyanin, especially C3G (cyanide-3-O- β -glucoside) is red rice pigment had antioxidative and anti-inflammatory activity [19]. Meanwhile, coconut water extract contains L-arginine, ascorbic acid, minerals such as calcium, magnesium and potassium which have hypolipidemic and antidiabetic activity [15].

Previous researches reported that genistein and anthocyanin can interfere NF- κ B signaling by blocking translocation NF- κ B p65 to nucleus, reduce JNK activation and reduce I κ B α phosphorylation, thus interfere transcription genes that role in the inflammation [20,21]. Biological activity of genistein and anthocyanins directly inhibit p50-p65 NF- κ B binding to the target of gene promoter NF- κ B, stabilization of I κ B α by inhibit the phosphorylation of I κ B α by IKK- β . Thus the translocation of NF- κ B into the nucleus can be blocked and transcription process will not occur [18]. In addition, L-arginine can inhibit the reaction of non-enzymatic glycosylation of proteins and the formation of AGEs by inhibiting the covalent modification proteins or AGEs formation [22]. AGE will increases ROS and triggers the MAPK and NF- κ B signaling pathway. MAPK activation will trigger macrophage activity to synthesizes cytokine pro inflammation [23]. Inhibition of AGEs by L-arginine will reduce NF- κ B activation.

CONCLUSION

The major findings of our study are: (i) NF- κ B expressions that produce by CD4 T cells, CD8 T cells and CD68 macrophage cells are increased in diabetic mice and (ii) treatment with EMSA Eritin could decrease the NF- κ B level. This findings

suggest that NF- κ B might be an important molecule contributing to diabetes mellitus and its complication.

Acknowledgement

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Ethics

This study has received ethical eligibility certificate (Ethical Clearance) from The Research Ethics Committee (Animal Care and Use Committee) Brawijaya University No. 384-KEP-UB.

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Minimizing Antioxidant Damage of Purple Sweet Potato var Antin 3 in Vacuum Packaging

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Abstract

The aim of this research is to find out the best immersion time in liquid nitrogen and the thickness of the packaging plastic to minimize the antioxidant damage of purple sweet potato during storage process. This research is conducted by using a factorial experiment in a randomized block design with two factors. In this research, first factor is the immersion time (0; 50; 60; 70 seconds), the second factor is the thickness of plastic (0.07 and 0.10 mm). The result of this research showed that the best immersion time is 70 seconds, while the best plastic thickness is 0.1 mm. The interaction of these two factors is the most effective to prevent antioxidants damage of purple sweet potato chips during 1 month storage.

Keywords: antin 3, antioxidants, multilayer, vacuum packaging

INTRODUCTION

Sweetpotato includes as tubers that has a high potential because its high domestic demand. Based on Indonesian Ministry of Agriculture, on 2013, the national wide of sweetpotato harvest area is 161,850 hectar with 2,386,729 ton of national production, while the total export of sweetpotato is 9,796.844 ton [1]. Thuse, most of sweetpotato production is used for domestic need.

Purple sweet potato is a kind of sweet potato that has much benefit, especially for health because its antioxidant substance. That character is strongly related to the purple pigment that represents anthocyanin substance as antioxidant. Purple sweet potato contains compounds that function as antioxidants, such as phenolic compounds and anthocyanins [2]. The content of phenolic compounds in purple sweet potato is 4.9 to 6.7 higher than the yellow and white potatoes [3] and from 2.5 to 3.2 higher than blueberries [4].

Purple sweet potato antin 3 varieties have purple pigment from the main tuber to the peel. For that reason, this variety can be processed as antioxidant-beneficial products such as natural dye, supplement for health, flour, or as intermediate product [5].

The obstacle in processing purple sweet potato is the difficulty in maintaining antioxidant substance (anthocyanin) as purple pigment in purple sweetpotato. Physical and enzymatical damage in purple sweet potato will influence antioxidant substance damage. Freezing is one of method to prevent the damage of food product during storage. There are many kinds of freezing method for food product which is used based on the product. One of them is fast-freezing-process using liquid nitrogen [6].

The growth of ice crystals is one of the factors that influence the quality of frozen foods. If freezing occurs slowly then allow the ice crystals grow so that the cells become damaged and the tissue can not be thawed back again like the original because the formed ice crystals will penetrate and injure the tissue material. Rapid freezing using liquid nitrogen resulted in formation of less ice crystals both in the extracellular and intracellular regions. Thus prevent the damage on nutritions, one of which is the antioxidant content [6]. Liquid nitrogen (boiling point -196°C) has become very important lately due to its role in quick freezing food (rapid freezing). This method reduces the oxidation on the surface of the food that is not packaged and the loss of water from the food material [7].

Stored food product, especially purple sweet potato has high risk of contamination. The contamination of other substance will damage the antioxidant. Therefore, packing system is needed which functions as protector for food

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product from physical damage and obstruct the quality damage [8].

Damaging prevention of antioxidant substance, especially for purple sweet potato is not widely observed. Therefore, this research was aimed to assess the combination between immersion time in nitrogen liquid and thickness of packing plastic. It is expected to minimize the damage of antioxidant substance in purple sweetpotato.

MATERIALS AND METHODS

In this research, purple sweet potato tuber was cut into rounded shape chips with thickness of ± 1 cm. In order for the purple sweet potato chips are not damaged during storage, then the multilayer plastic packaging used with two levels of thickness: 0.07 mm and 0.10 mm. After packaging, freezing was done quickly with the treatment of immersion in liquid nitrogen at four levels of times, i.e. 0 seconds, 50 seconds, 60 seconds, and 70 seconds. The treatment was done with three replications. Thereafter, purple sweet potato chips stored in a freezer at a temperature of -25°C for one month.

This research used quantitative method. The measuring parameters are antioxidant activity used DPPH method [9], while total anthocyanin content used differential pH method [10]. Total phenol content used Follin-Ciocalteu method [11]. Total dissolved solids content used *refractometer* based on AOAC [12], and hue saturation value used color reader [13]. The measuring parameter procedures were:

Measuring antioxidant activity with DPPH

Purple sweet potato chip was crushed and weighed about 20 g, taken into volumetric flask 100 mL, extracted by additional HCL 1% in methanol until it reached the limit mark. After homogenized, the sample was shook for 4 hour and filtered by filter paper, and filtrate was produced. The filtrate centrifuged for 10 min at 4,000 rpm.

Total 0.1 mL filtrate was measured, then added methanol 96% as much as 5 ml and then performed vortex to dissolve the sample. Extracts were centrifuged at a speed of 4,000 rpm for 10 min to separate the extract. A total of 4 mL supernatant was taken and added to 1 mL of 1.1 diphenil-2-picrylhydrazil (DPPH) 0.2 mMol, set for 10 min. Then the absorbance was measured at a wavelength of 517 nm, but previously control absorbance was measured. Control is made by added 1 mL of 1.1 DPPH 0.2 mMol into 4 mL of

methanol. Free radical scavenger activity as DPPH color presentage deficiency, calculated with the formula:

$$\text{Antioxidant activity} = 100 \times 1 - \frac{\text{sample absorbance}}{\text{blank absorbance}}$$

Anthocyanin measurement with differential pH

Results of filtrate pipetted 1 mL, then put 10 mL volumetric flask, and then diluted with a buffer solution of pH 1.0 to mark boundaries. Measured absorbance of each solution at a maximum wavelength and 700 nm, measured with distilled water as a blank. Dilution factor appropriate to the sample was determined by dissolving the sample with a buffer of pH 1.0 to obtain maximum wavelength.

Furthermore 1 mL of solution preparation results were taken then put in a 10 mL volumetric flask and diluted with a buffer solution of pH 4.5 to mark boundaries. Absorbance of each solution at a maximum wavelength and 700 nm is measured with distilled water as a blank. Maximum wavelength is the maximum wavelength of cyanidin-3-glucoside, while the wavelength of 700 nm to correct the sediment was still present in the sample. If the sample was absolutely clear that the absorbance at 700 nm is 0. Absorbance of the sample has been dissolved (A) is determined by the formula:

$$A = [(A_{\text{max}} - A_{700}) \text{ pH } 1,0 - (A_{\text{max}} - A_{700}) \text{ pH } 4,5]$$

Anthocyanin content in the sample is calculated using the formula:

$$\text{Anthocyanin (mg.100g}^{-1}\text{)} = \frac{A \times MW \times DF \times 1000}{\epsilon \times 1}$$

Description:

A = Absorbance

MW = the molecular weight (expressed as cyanidin-3-glucoside), i.e. 449.2

DF = Dilution Factor

ϵ = Absorbitivity molar coefficient: 26900 (expressed as cyanidin-3-glucoside)

Total Phenol Measurement

The sample extract of purple sweet potato chip to be tested is volumed of 1 mL. Na_2CO_3 was added with a solution of 75 g.L^{-1} 4 mL and Follin-Ciocalteu reagent (diluted 1:10) 5 mL and homogenized by vortex. The solution Incubated for an hour at room temperature with dark conditions.

Total 2 mL of the extract was taken and put in a cuvette. Absorbance was measured at a wavelength of 765 nm. It was calibrated with a standard curve of gallic acid to obtain total phenols in ppm GAE. Total phenol calculated by the formula:

$$C = \frac{CGAE \times V}{G}$$

Description:

C = total phenol content (ppm GAE)
CGAE = levels of total phenols in the form of gallic acid equivalents (ppm)
V = the resulting extract volume (mL)
G = the mass of material (g)

Measurement of Total Dissolved Solids

Sample of the purple sweet potato chip extract was prepared, and the prism refractometer was cleaned with distilled water, and then dried with tissue. Samples were taken with a pipette and dropped into the field prism, and we observed the number that passed the boundary of dark and light on the refractometer. The number showed the total dissolved solids in the sample.

Measurement of Hue Value

Samples of the fresh purple sweetpotato tuber were prepared and color reader was turned. Then the target reading L, a*, b* was determined and measuring color. Then the color scale parameter was read L* (lightness) for brightness and a*, b* for chromaticity value. Changes color (hue) expressed in ΔH can be calculated using the formula:

$$\Delta H = \Delta E^2 - \Delta L^2 - \Delta C^2$$

$$\begin{aligned} \text{Where: } \Delta L &= L^*0 - L^* \\ \Delta E &= \Delta L^2 + \Delta a^2 + \Delta b^2 \\ \Delta C &= C^*0 - C^* \end{aligned}$$

Description:

ΔH = changes color for a certain time
 ΔE = changes value of L, a, b for a certain time
 ΔL = changes value of L for a certain time
 ΔC = changes value of C for a certain time
 L^*0 = L values of samples at the initial condition
 L^* = L value of sample for a certain time
 Δa = changes value of a* for a certain time
 Δb = changes in the of b* for a certain time
 C^* = sample saturation value for a certain time
 C^*0 = saturation value on initial conditions

Data Collection Method

This research used sampling with Randomize Block Design as data collection method. The reaserch use factorial experiment with two factors. The first factor is the time of immersion

in liquid nitrogen in three levels (0; 50; 60; 70 sec), and the second factor is the thickness of plastic packaging with three levels (0.07 mm and 0.10 mm). This research resulted in 8 combinations with 3 repetitions, thus there are total 24 samples.

ANOVA was used in this research as data analysis method. Furthermore, if there is a significant result, the data will be analyzed by DMRT. The data werere analyzed with SPSS program. The best treatment will be explained by descriptive method.

RESULT AND DISCUSSION

The result included antioxidant activity, anthocyanin level, total phenol content, total dissolved solids content, and color (hue saturation value) of purple sweet potato antin 3 variety chips. Based on those parameter of physical and chemistry characters, the best treatment will be chosen.

Antioxidant Activity

The observation result of purple sweet potato antin 3 varieties chips with combination treatment of immersion time in liquid nitrogen and thickness of packing plastic showed that antioxidant activity was 24.83% - 45.33% (Fig. 1). Combination treatments caused different antioxidant activity level in each concentration. There was degradation of antioxidant activity after one month of storage.

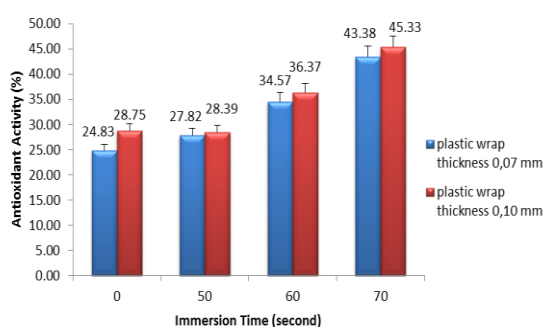


Figure 1. Average antioxidant activity of purple sweet potato var Antin 3 chips after one month of storage.

Antioxidant activity is strongly related to the anthocyanin substance which is found in purple sweetpotato. In this case, we measured antioxidant activity by DPPH method. In addition, the principal of antioxidant activity in sample changes the DPPH liquid color in methanol, the color chage from dark purple to pale orange.

That phenomenon shows the sample ability to muffle the free radical activity which is represented by DPPH [5,11]. The antioxidant substance is anthocyanin, therefore, the higher anthocyanin substance the higher antioxidant activity.

The best immersion time showed that the immersion to liquid nitrogen process can maintain the anthocyanin substance as antioxidant. Freezing with liquid nitrogen will retain important compounds in frozen products [6]. While, the plastic wrap thickness factor which is not significantly influence on that case may be caused by the thickness level (which is not significantly different). However, previous study found the difference of plastic wrap thickness level will affect the ability of the plastic in protecting physical and chemical characteristic of wrapped-material [14].

Total Anthocyanin Content

The result of multi-analysis showed that the immersion time factor, the thickness of plastic wrap factor, and the interaction between those factors significantly influenced anthocyanin content. It indicated that the longer immersion time and the thicker plastic-wrap will increase the level of maintaining anthocyanin substance ability. The reduction of anthocyanin substance was 1.90-50.25% (Fig. 2).

The highest reduction of anthocyanin level was 50.25% which found in 0.00 second of immersion time and 0.07 mm of plastic-wrap thickness. Whereas, the smallest reduction of anthocyanin level was 1.90% found in 70 second of immersion time and 0.1 mm of plastic-wrap thickness. Those indicated that the longest of immersion time and the thinnest plastic-wrap, the damage of anthocyanin substance was smallest.

The beneficial influence of immersion time (in liquid nitrogen) on anthocyanin level is strongly influenced by fast freezing process which produces micro crystal. Therefore, tuber tissue in purple sweetpotato is not damage after thawing process. Concerning the previous case, the thickness of plastic-wrap also gives significant effect on anthocyanin substance protection from contamination of other substance that damages not only the physical structure but also the chemical structure of the protected-material. Moreover, the packing method uses vacuum packing with multilayer plastic which makes potential contamination is very small because multilayer plastic does not have pore as contamination trigger [15].

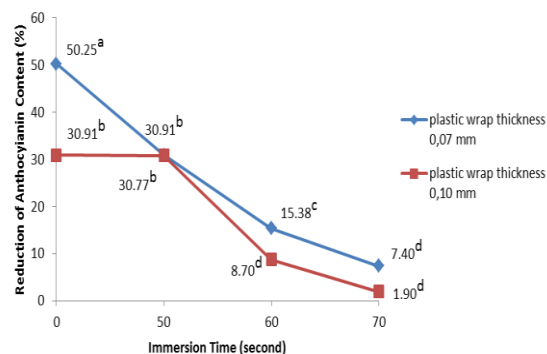


Figure 2. Reduction of anthocyanin content on purple sweet potato var Antin 3 chip after 1 month storage.

Total Phenol Content

The result of multi-analysis showed that immersion time factor, thickness of plastic wrap factor, and interaction between those factors significantly influenced the total phenol content. It was also indicated that the longer immersion time and the thicker plastic-wrap will increase the ability in preventing damage on the phenol substance.

After one month storage, the total phenol content was reduced as the effect of phenol substance degradation during the storage. The reduction of total phenol content was 4.54 – 53.18% (Fig. 3).

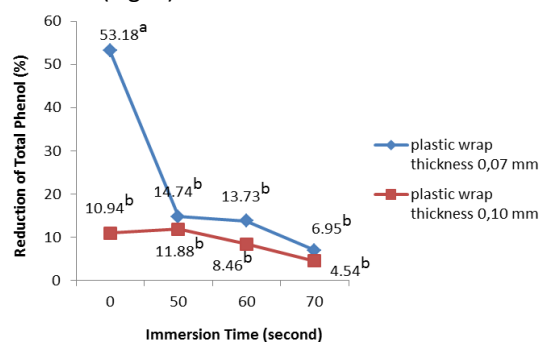


Figure 3. Reduction of total phenol content on purple sweet potato var Antin 3 chip after 1 month storage.

The highest reduction of total phenol content was 53.18% which was found in 0.00 second of immersion time and 0.07 mm of plastic-wrap thickness. While, the lowest reduction of total phenol content was 4.54% which was found in 70 second of immersion time and 0.1 mm of plastic-wrap thickness. These results showed that immersion on liquid nitrogen will maintain physicochemistry characteristic (one of them is phenol substance) of the material. Previous study reported on freezing process used liquid nitrogen

in some level which were applied to cherry and apricot juice. The result showed that it can maintain the physicochemistry character of the materials [16].

In other case, the plastic-wrap thickness also influenced the total phenol contain. It showed that the thicker plastic-wrap, the higher ability in maintaining total phenol contain of wrapped-material. The process will be better if the wrapping process uses vacuum method because it can maintain the physico-chemistry character of the material well. Related to this matter, Dewandari and Mulyawati [14] stated that the application of plastic to protect the wrapped-material from light, air or oxygen, heat transmission, contamination, and contact with other chemical substance which makes the food material/product maintain its water, fat and other beneficial substance.

Total Dissolved Solid

The result of total dissolved solid test after one month of storage showed that the reduction of total dissolved solid was 23.08-56.41% (Fig. 4). The highest reduction level of total dissolved solid by 56.41% found in 0.00 second of immersion time and 0.07 mm of plastic-wrap thickness. Otherwise, the lowest reduction level of total dissolved solid by 23.81% found in 70 second of immersion time and 0.1 mm of plastic-wrap thickness. Previous study found that storage at freezing temperatures decrease the minimum content of total dissolved solids [17].

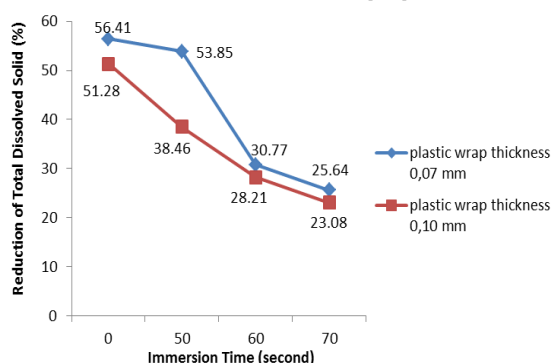


Figure 4. Total dissolved solid reduction of purple sweet potato var Antin 3 chip after 1 month storage.

Fast freezing process also reported to influence the ability of cherry in maintaining its total dissolved contain [18]. A study on the total dissolved contain in slices of pineapple Smooth Cayenne and Red Spanish which were stored in a -18°C , found that effective freezing process will increase the ability in maintaining total dissolved

solid [17].

Other study reported the total dissolved solid of Puree Mango tended to decrease during 6 month storage when it wrapped by polyethylene plastic and freeze by immersion to liquid nitrogen [14]. The reduction of total dissolved solid in frozen product is the effect of the loss of beneficial components during freezing and storage process. Moreover, dehydration and reduction of sugar contain may also increase the reduction of total dissolved solid.

Hue Saturation Value

The observation result of hue saturation value after one month storage concluded that hue saturation value was 15.830 – 24.450 (Fig. 5). Based on Figure 5, hue saturation value is change after 1 month of storage. In this research, hue saturation value is related to red intensity which shows anthocyanin color in purple sweet potato. The higher hue saturation value showed the higher red intensity of purple sweet potato. This means that degradation levels of anthocyanins contained in the purple sweet potato was getting smaller. According to Winarno [19], the pigment concentration in a material determines the hue level.

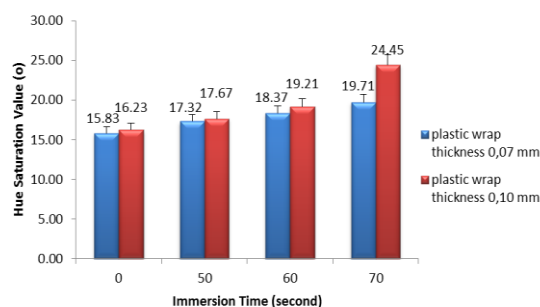


Figure 5. Average hue saturation of purple sweet potato var Antin 3 chip after 1 month storage.

Immersion with liquid nitrogen can maintain the anthocyanin substance which is known as color determiner of purple sweetpotato. Freezing with liquid nitrogen in whole fruit may damage the product, but for sliced-fruit that process can maintains the fruit appearance during 1 month of storage [6].

In the same case, plastic-wrap thickness also influences the ability in maintaining substances which are found in wrap material. The thicker plastic wrap, the higher ability in preventing the damage of material [14], e.g. maintaining the anthocyanin substance as antioxidant.

Determination of the best treatment

The best treatment of this experiment was determined by comparing observed-parameters of each treatment combinations (Table 1). The treatment combination which have highest value in each treatment is the best treatment.

The average of highest anthocyanin content was 151.90 mg.100g⁻¹ which was found in combination of immersion time 70 second and plastic-wrap thickness 0.1 mm. Next, the highest total phenol content was 4646.60 ppm GAE which was found in 70 seconds of immersion time and 0.1 mm of plastic-wrap thickness. The highest antioxidant activity was 45.33% which was found in 70 seconds of immersion time and 0.1 mm of plastic-wrap thickness. The highest level of total dissolved solid is 5.00% brix found in 70 seconds of immersion time and 0.1 mm of plastic-wrap thickness. The highest hue saturation value is 24.45° hue found in 70 seconds of immersion time and 0.1 mm of plastic-wrap thickness.

Tabel 1. Highest value of each parameter after treatment

Parameter	Result	Treatment
Antioxidant Activity (%)	45.33	immersion time: 70 s plastic-wrap thickness: 0.1 mm
Anthocyanin (mg.100g ⁻¹)	151.90	immersion time: 70 s plastic-wrap thickness: 0.1 mm
Total Phenol (ppm GAE)	4646.60	immersion time: 70 s plastic-wrap thickness: 0.1 mm
Total Dissolved Solid (%brix)	5.00	immersion time: 70 s plastic-wrap thickness: 0.1 mm
Hue (°hue)	24.45	immersion time: 70 s plastic-wrap thickness: 0.1 mm

Those parameters showed that 70 seconds of immersion time and 0.1 mm of plastic-wrap thickness is the best treatment combination in this research because all five parameters shows the highest value in the combination of 70 seconds of immersion time and 0.1 mm of plastic-wrap thickness. The best treatment combination (immersion time and plastic-wrap thickness) was expected to become reference for further research on purple sweet potato chip products.

CONCLUSION

The best immersion time on liquid nitrogen was 70 seconds and the best plastic-wrap thickness was 0.1 mm. Moreover, combination of between the factors is the most effective in preventing actioxidant damage of purple sweet

potato chip during storage. Further research is needed on ready to serve purple sweet potato chip which through some process after fast freezing and storage.

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Technique of Overlap Extension by Polymerase Chain Reaction for Splicing Cauliflower Mosaic Virus (CaMV) 35S Promoter and *DhPEX11-Like*

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Abstract

The promoter plays an important role in the regulation of gene expression. The problem is some of binary vector that absence from promoter at cloning site. The cauliflower mosaic virus (CaMV) 35S promoter is a strong and constitutive promoter that widely used to produce transgenic organisms. In this experiment the cauliflower mosaic virus (CaMV) 35S promoter was spliced at upstream of *DhPEX11-like* for driving downstream transgenes *DhPEX11-like* expression used the technique of Overlap Extension by The Polymerase Chain Reaction. In gene splicing, internal primers are used to amplify some overlapping regions of both genes and then these internal primers are combined with the external primers in PCR process which allows amplification of the entire region. In the experiment, the recombinant PCR successfully spliced the 35S-*DhPEX11* gene. This method is simple, rapid and reduced reagents used because it does not need many vector constructions.

Keywords: *DhPEX11-Like*, Gene splicing, Oligonucleotide, PCR, Promoter

INTRODUCTION

Promoters are one of the essential constitute of gene, as they are required to drive expression of both the selectable marker gene and the gene of interest in transgenes [1]. The problem is some of binary vector is absence from promoter at cloning site. Combining genes or regulatory elements to make hybrid genes is a widely used methodology throughout the biological sciences [2]. One of the techniques for synthesis of artificial genes is called gene splicing, in which segments of DNA are joined together to create a new genetic combination [3]. Gene splicing by overlap extension is a technique for combining the DNA molecule from two genes on the short nucleotide sequences that have been recombined in precise junctions without using restriction endonucleases or ligase [4]. The polymerase chain reaction (PCR) has greatly enhanced the field of molecular biology by making numerous regions of the genome (coding and noncoding), in both extant and extinct taxa, accessible for detailed analysis [5]. Previous research demonstrated that the PCR recombinant can be used to remove selectable markers or other introduced transgenes that are no

longer desired and therefore can be a useful tool for genome engineering in plants [6]. The ability to fuse two DNA fragments by overlap extension can be exploited further to splice two or more DNA fragments from different genes [7]. Overlap extension-PCR also can be used as a means for site directed mutagenesis, introducing desired mutations to the final hybrid gene [2]. Under PCR conditions, the common sequence allows strands from two different fragments to hybridize to one another, forming an overlap [4]. Initial PCRs generate overlapping gene segments that are then used as template DNA for another PCR to create a full-length product [8].

Overlap extension by recombinant PCR proved to be an effective technique for providing adequate amounts of spliced DNA for cloning purposes, having established the appropriate conditions [9]. Technique overlapping gene segments by recombinant PCR is a simple, versatile technique for gene splicing [8]. This powerful and technically simple approach offers many advantages over conventional approaches for manipulating gene sequences [4]. Splicing by overlap extension by the polymerase chain reaction (SOE by PCR) can be used to splice promoter and interest gene [10]. In this paper, we describe the use of gene splicing technique for splice the cauliflower mosaic virus (CaMV) 35S promoter and *DhPEX11-like*.

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MATERIALS AND METHODS

35S promoter and DhPEX11-like gene

The 35S promoter and *DhPEX11-like* gene were used in this research. The promoters that have been most commonly used in the transformed plants so far, include the CaMV 35S, ubiquitin 1 and actin promoters [11]. Cauliflower Mosaic Virus (CaMV) and the closely related Figwort Mosaic Virus are circular duplex DNA viruses which replicate via transcription of a full-length (35S) genomic RNA intermediate [12]. The cauliflower mosaic virus (CaMV) 35S promoter is a strong and constitutive promoter that widely used for production purposes [13, 14].

Plasmid preparation

To get the promoter and the gene for splice process, first was needed to extract plasmid that contained the genes from *E. coli*. The transformed white single colony *E. coli* was picked out from a petri dish and was transferred to small volume of LB-medium 5 mL containing the ampicillin antibiotic (0.2 mg/ml) and shaken at 37°C and 200 rpm for 16-24 h. Plasmid Miniprep Purification Kit (GeneMark) was used to extract the plasmid from *E. coli* culture, according to the manufacturer's manual. Cell pellet was collected in a 1.5 mL Eppendorf tube (1-3 ml of cells) by centrifugation of *E. coli* culture at 14,000 rpm for 1 min.

The supernatant was decanted and the pellet was resuspended in 200 µl Solution I by pipetting or vortexing. And 200 µl Solution II was added into the tube and the mixture was inverted 5 times. Next, 200 µl Solution III was also added and the tube was inverted again (5 times) to lyse cells. The lysate was centrifuged at 14,000 rpm for 5 min and the supernatant was transferred into a spin column combined with collection tube. The flow-through in the collection tube was discarded. The spin column was added 700 µl Wash Solution and centrifuged at 14,000 rpm for 1 min. The washing step was repeated for one more time. The spin column was centrifuged for 5-10 min at top speed to remove residual trace of ethanol. After ethanol removed completely, the spin column was then transferred into a new 1.5 mL Eppendorf tube and was added 30-100 µl of Elution Solution. The spin column was centrifuged at 14,000 rpm for 1 min to elute plasmid DNA. The yield of plasmid DNA was around 2-30 µg in 1-3 ml of cells culture which had the purity of ratio of 1.8-2.0 of 260/280 nm (ND-1000 spectrophotometer, NanoDrop, USA).

Gene splicing

Gene splicing by overlap extension consists of three times PCR process. In the first stage reactions produced the two DNA fragments, and the first products to be used as template in the second stage [4,8,9,15]. Four primers is needed consisting of two flanking and hybrid primers to one construct [9]. SOE by PCR (Fig. 1) was used to combine 35S Promoter and *DhPEX11-like* into expression cassette.

PCR amplification

The PCR were performed in 25 µl reaction volumes with 1 µl of template DNA. The PCR reaction mix contained 0.5 µl 10 mM Deoxyribonucleotide triphosphate (dNTP), 0.25 µl of *Taq* Phusion High-Fidelity DNA Polymerase, 5 µl of 5X Phusion HF reaction buffer and 1 µl primer mix (10 µM each). PCR amplification was performed initialized at 94°C for 3 min, followed by 28 cycles (94°C for 45 s, 60°C for 30 s, and 72°C for 30 s), with a final extension of 5 min at 72°C.

Gel agarose preparation

For preparing 1% gel, 0.25 g of agarose powder (Seakem, Marine Colloids, Inc.) dissolved in 25 ml 0.5X Tris acetate EDTA buffers (20 mM Tris acetate, 0.5 mM EDTA). The mixture was heated in a microwave oven for about 1-2 min to dissolve agarose. The solution was cooled for 4 min and poured into electrophoresis apparatus. After gel has solidified, 0.5X TAE buffer was poured into gel box, and comb was removed after solidified. A dye solution was added at DNA samples prior to electrophoresis. DNA molecular weight marker was used in each size marker lane of the electrophoresis gel. Electrophoresis was performed at 100 V for 35 minutes or until the dye neared the bottom of the gel. The gel was then placed in a solution of ethidium bromide in water and stained for 2 minutes. About 10 minutes the gel was rinsed in water. The gel is illuminated with UV transilluminator then photographed.

PCR recombinant [16]

Directly after PCR amplification the reaction (50 µL) was heated at 95°C for 20 minutes. Fifteen µL of dATP (from a mM stock) and 5 units of *Taq* DNA Polymerase were added. The reaction was incubated at 70°C for 15 minutes. Following electrophoresis, the DNA band of interest quickly was located by illuminating the gel on UV transilluminator for purification of PCR

fragments. The gel was sliced using a clean razor blade and was excised with the Wizard DNA Clean-Up system kit (Promega). Gel slice was transferred in a 1.5-ml microcentrifuge tube (Eppendorf) and 10 μ l Membrane Binding Solution was added per 10 mg of gel slice. The gel was incubated at 50-65°C until gel slice is completely dissolved.

Dissolved gel mixture was transferred to the Minicolumn assembly and then centrifuged at 14,000 rpm for 1 min. Following centrifugation flow-through was discarded and the minicolumn was washed with 700 μ l Membrane Wash Solution (ethanol added) then centrifuged at 14,000 rpm for 1 minute. The washing step was repeated with 500 μ l Membrane Wash Solution and centrifuged at 14,000 rpm for 5 minutes. The column was recentrifuged for 1 min with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol. Fifty μ l Nuclease-Free Water was added to the minicolumn and centrifuged at 14,000 rpm for 1 min. For further confirmation these PCR products were sequenced.

Confirmation of DNA fragments

Confirming DNA fragments of CaMV35S promoter, *DhPEX11-like* and 35S/*DhPEX11* we did PCR, enzyme digestion and sequence analysis. For the digestion reaction includes 1.5 μ g of plasmid DNA, 2 μ l of 10X buffer, 0.2 μ l of 100x BSA, 0.5 μ l of restriction enzyme, and sterile water to 20 μ l. The reaction was incubated for 1 h at the temperature specific for the enzyme

used. Digested products were electrophorated on 1 % agarose gels.

DNA Sequencing

After PCR and enzyme digestion the transformed strains of plasmids were delivered to Mission Biotech Company (Taipei, Taiwan) for sequencing. DNA sequence data were analyzed using the National Centre of Biotechnology (NCBI) web site (<http://www.ncbi.nlm.nih.gov>) for alignment by the program nucleotide blast (BLASTn).

RESULT AND DISCUSSION

First Stage PCR

In the first stage PCRs produces two DNA fragment with the sequence 5' and 3'to the splice point (1)–(2) in Fig. 1. However, since the hybrid oligonucleotides have the splice regions, products in the first stage will splice at the short sequences derived from the other (2) in Fig. 1. Therefore when the two products are combined they can partially anneal and used two external primers in the second stage PCR to get the final result (3–4) in Fig. 1 [9].

This technique uses 2 steps PCR, first step is to get two kind of template that the promoter and gene sequence are joined use internal Oligonucleotides. Second step used two kind of template with external Oligonucleotides (Table 1). The oligonucleotides design (Table 1) were based on the full-lengthed nucleotide sequence of 35S promoter in NCBI (<http://www.ncbi.nlm.nih.gov>) and *DhPEX11-like* [17].

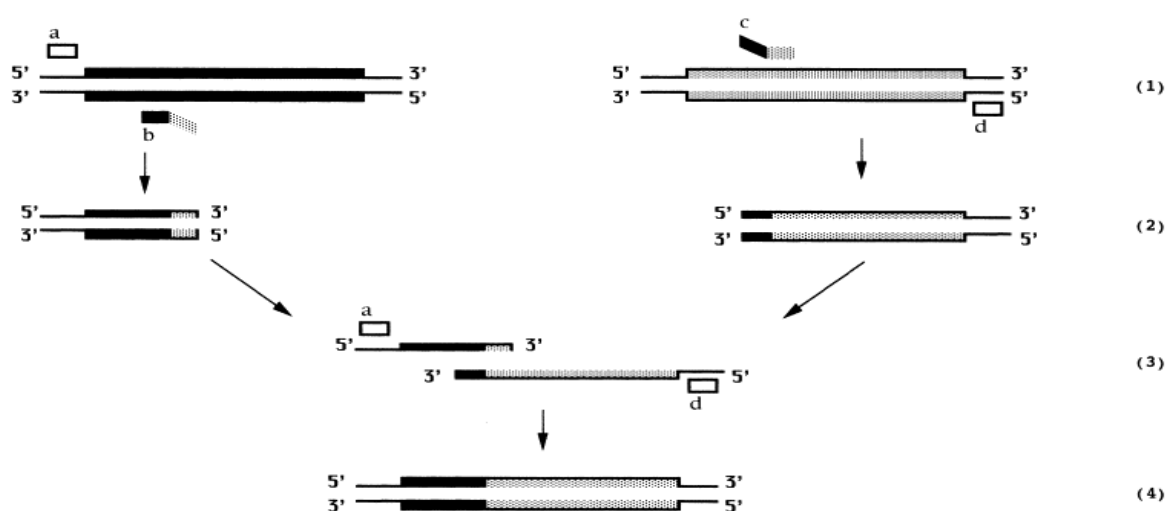


Figure 1. Gene splicing by overlap extension by polymerase chain reaction [9]

Promoter and gene illustrated in the form of bars. Promoter illustrated in the form of solid shading bars, while gene illustrated with lightly stippled. Oligonucleotides shown by the label a lower case letter (a,b,c,d). If shown above the promoter and gene, their orientation is 5' to 3' (left to right), and 3' to 5' (right to left) if shown below it.

Table 1. Oligonucleotides for cloning CaMV 35S Promoter and *DhPEX11-like* and splicing CaMV35S Promoter and *DhPEX11-like* to generate *35S/DhPEX11*

Gene	Oligonucleotide sequences 5'-3'
(A) First	
35S Pro	
35S-F	GCGAATTCATGGAGTCAAAGATTCAAATAGAGGACCTAACAG
35S-R	GCTATTCATCCTGGGTGTCATGGTCAAGAGTCCCCGTGTT
<i>DhPEX11</i>	
PEX11-F	AACACGGGGGACTCTTGACCATGACCCAGGATGAAATAGC
PEX11-R	CCGATCCTCATAATCATTAGGAGGAGGAGCAGCTGCTTC
(B) Second	
35S-F	GCGAATTCATGGAGTCAAAGATTCAAATAGAGGACCTAACAG
PEX-R	CCGATCCTCATAATCATTAGGAGGAGGAGCAGCTGCTTC

The extracted plasmid DNA from pCAMBIA 1302 was used as template in PCR with prime pair of 35S-F and 35S-R for cloning CaMV 35S Promoter, as well as the *DhPEX11-like* fragment was cloned from the transformed pMETB Plasmid using the prime pair of PEX11-F and PEX11-R [17]. The secondary PCR spliced the two DNA fragmented of CaMV 35S promoter and *DhPEX11-like* to become to the recombinant of *35S/DhPEX11* gene, using outprimer pair of 35S-F and PEX11-R. The hybrid oligonucleotides are designed from the known nucleotide sequences to generate fragments that will have overlapping sequence [9]. They had 20 bp overlap which sequences were underlay at 5'end of 35S

promoter and 3'end of *PEX11-like* gene (Fig. 2). The availability of thermostable DNA polymerases with a much lower tendency to add a non-templated nucleotide to DNA fragments [18] may decrease the rate of mutation further and the need to blunt end intermediate products [9].

The first PCR cloned the 35S promoter and the second PCR cloned the *DhPEX11-like* gene, their sizes were confirmed by analysis on 1% gel electrophoresis with expected with expected DNA fragments of 573 bp (Fig. 2A) and 579 bp (Fig. 2B), respectively. The size of the recombination had expected band and sequenced being 1051 bp. (Fig. 2C).

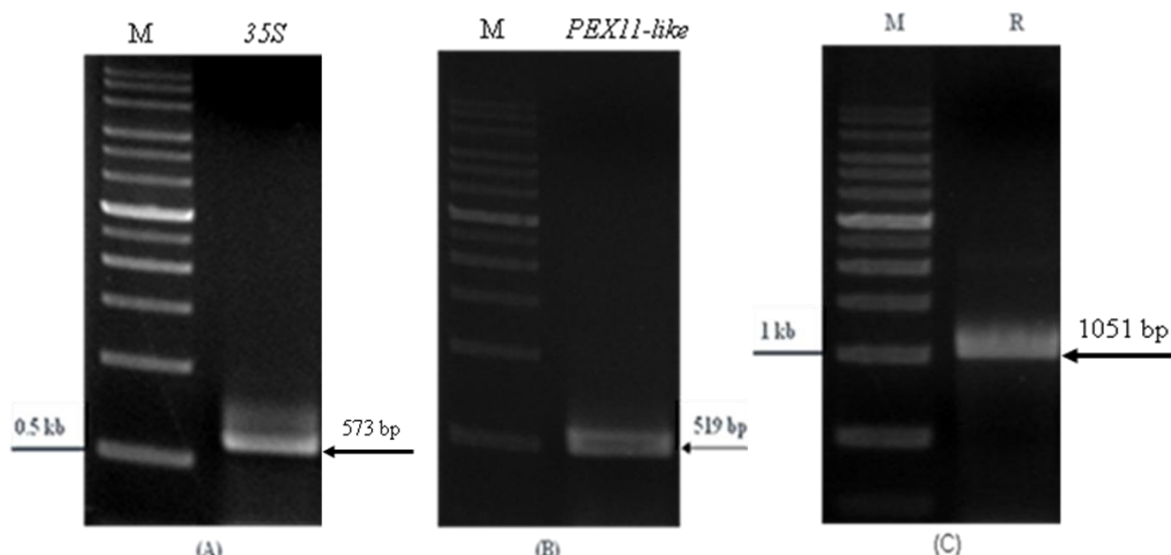


Figure 2. Recombinant PCR for 35S/PEX11

(A) PCR analysis of 35S promoter. The figure showed the expected size of 35S promoter was 573 bp.

(B) PCR analysis of *PEX11-like* gene. the expected size for *PEX11-like* was 519 bp.

(C) PCR analysis of 35S/*PEX11* recombinant, the 1051 bp band was shown.

M: 1-kb DNA markers. 35S: 35S promoter.

PEX11-like: *PEX11-like* gene.

R: 35S/*PEX11* recombinant.

PCR recombinant product

PCR recombinant product was ligated to be cloned into pGEM-T Easy vector and infected to *E. coli* which was spanned on LB-agar medium containing X-gal, IPTG and ampicillin. The white colonies was the host of transformant vector which was confirmed by PCR. Its reactant were run on 1% agarose gel and distinct band of 1051 bp revealed under the UV light (Fig. 3A). It presented the DNA fragment of 35S/PEX11 in the transformant plasmid.

Confirmed DNA fragments

The expression cassette of 35S/PEX11 that released from the pGEM plasmid DNA digested with *Bam*HI and *Hind*III enzymes, the reactant was run on 1% agarose gel which appeared both 1051 bp fragment and 3 Kb of the vector backbone (Fig. 3B). The final product that has been amplified by flanking primers also can be obtained restriction enzymes sites to insert it into an expression vector for cloning step [8].

DNA sequencing

The identity value of alignment results of the original sequence of 35S/PEX11-like and the sequence of 35S/PEX11-like fragment cloned from p-GEM T-easy vector was 98%.

In the experiment, the recombinant PCR successfully spliced the 35S-DhPEX11 gene which appeared strong expression of transformant. SOE by PCR is rapid because it does not need many vector constructions which are a time consuming process. This method is simple and widely applicable approach has significant advantages over standard recombinant DNA techniques [4]. Moreover, the recombinant might be amplified and cloned into expression vector, and it is readily applied in plant transformation. The 35S promoter of cauliflower mosaic virus (CaMV) able to confer high-level gene expression in most organs of transgenic plants [19].

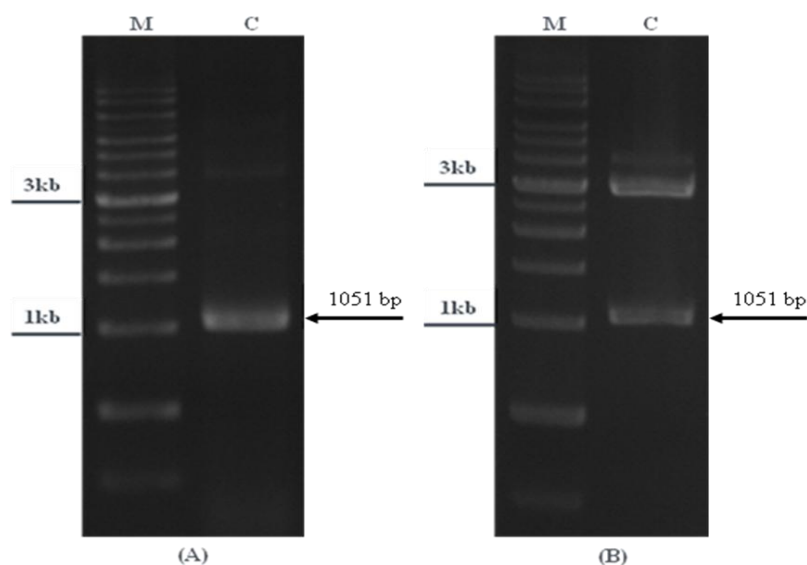


Fig. 3. Gel checking of the DNA fragments inserted into pGEM-T Easy vector. **(A)** Polymerase chain reaction (PCR) analysis. The figure showed the expected size of 35S/PEX11 cloned into vector. **(B)** The constructed 35S/PEX11/pGEM-T was digested by *Eco*RI and *Spe*I enzymes for double checked before sequencing, the 1051 bp band and the 3.0 kb band were shown. M: 1-kb DNA markers. C: Constructed 35S/PEX11/pGEM-T.

CONCLUSION

Recombinant PCR in this study successfully spliced the 35S-DhPEX11 gene. SOE by PCR did not need many vector constructions thus less time consuming. This simple and widely applicable method of approach has significant advantages for standard recombinant DNA. The use on 35S promoter of CaMV confer high-level gene expression for transgenic plants as in this study.

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The Potential of EMSA Eritin to Modulate T Cells (CD4⁺ and CD8⁺) in Balb/C Mice Model of Diabetes Mellitus

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Abstract

EMSA Eritin is a medicinal polyherbal contains with soy bean, coconut water and red rice extract. In several studies of this individual components have proven the potential to modulate lymphocyte cells that indirectly lowering blood glucose levels in diabetes meliitus. This study was aimed to analyzing the effect of polyherbal EMSA Eritin on the expression of CD4⁺, CD8⁺ and SDF-1 cells population in mice model of diabetes mellitus. Neonatal d-5 mice was intraperitoneally injected with Streptozotocin (STZ) 100 mg.kg⁻¹ BW. Four weeks post injection the mice was treated with EMSA Eritin in three doses 0.3125 mg.g⁻¹ BW, 3.125 mg.g⁻¹ BW and 31.25 mg.g⁻¹ BW for 14 days. Cells Profile of T lymphocyte (CD4⁺ and CD8⁺) and chemokine SDF-1 were analyzed by Flow cytometry. Data were analyzed with One-way ANOVA (p<0.05) and Duncan test using SPSS 16.0 for Windows. The result showed that EMSA Eritin significantly increased T lymphocyte (CD4⁺ and CD8⁺) in optimum dose 3.125 mg.g⁻¹ BW and suppress SDF-1 production in high dose (31.25 mg.g⁻¹ BW) as well as the blood glucose level measurement. This study suggest that polyherbal EMSA Eritin has a potential to decreased blood glucose level in mice model of diabetes mellitus.

Keywords: CD4⁺, CD8⁺, diabetes mellitus, EMSA Eritin, SDF-1

INTRODUCTION

Diabetes mellitus (DM) has become one of the major causes of morbidity and mortality in the world with increasing prevalence where 3.4 million people died in 2004. It is predicted to be doubled in 2030 which makes this metabolic disorder one of the leading death cause in the world. Diabetes mellitus is a metabolic disease characterized by hyperglycemia, caused by the loss of glucose homeostasis in blood [1,2].

Diabetes has a several complications that can cause systemic disorders in some organs, including cellular immune system, e.g. lymphocytes, monocytes, neutrophils and macrophages that can lead to cell death [3]. Some studies suggest that patients with diabetes have an increased apoptotic cell death in lymphocyte from the peripheral blood vessels, and other pathogens. Influence exerted by reduced the number of CD4⁺ and CD8⁺ T cells and also affecting lymphopoiesis [4,5,6]. CD4⁺ is a marker in T cell known as T-helper cell that can help activate B cells to

secrete antibodies and macrophages to destroy foreign substance, and also activated CD8⁺ known as T-cytotoxic cell to kill infected target cells. CD4⁺ T cells along with CD8⁺ T cells make up the majority of T-lymphocytes and plays important roles in immune system [7]. Immune system dysfunction in diabetic patients also occurs in polymorphonuclear cells (PMN) such as chemotaxis and phagocytosis as well [8]. Chemotaxis as a chemoattractant are postulated to help direct lymphocyte traffic into sites of inflammation and into lymphoid and non-lymphoid tissues during recirculation. Stromal cell-derived factor-1 or SDF-1 is known to be a highly efficacious chemoattractant for T lymphocytes and has a complex effects on migration, proliferation, and differentiation of leukocytes [9]. Reduced number of T lymphocytes in patients with diabetes mellitus caused by hyperglycemia that increase reactive oxygen species (ROS) and excess produce of superoxide in the body. This immune disorders are known to be a stressor that affects the immune modulation and play an important pathogenic role in immunodeficiency [10,11].

In traditional medicine, a mixture of several species of herbs known as poliherbal has comprising prescriptions that can improve body defense mechanisms [12]. EMSA Eritin is a mixture of three edible medicinal herbal, consist

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of soybean (*Glycine max*), water coconut (*Cocos nucifera*) and red rice (*Oryza sp.*) extract. Several studies of EMSA Eritin individual components have proven they have a potential to lowering blood glucose levels in diabetes mellitus [13-16]. This study was aimed to determine the expression of CD4⁺, CD8⁺ and SDF-1 cells population in thymus of mice (*Mus musculus*) model of diabetes mellitus after EMSA Eritin administration.

MATERIALS AND METHODS

Mice

In this study we used a total 25 neonatal 5-day-old Balb/C mice with $\pm$ 2.5 g body weight. Mice were taken care in free pathogen animal chamber, Departement of Biology, Faculty of Mathematic and Natural Sciences, University of Brawijaya, Malang, East Java, Indonesia.

This research has obtained an ethical clearance certificate from Brawijaya University Research Ethics Committee. No: KEP-385-UB.

Induction of Diabetes mellitus

Neonatal d-5 Balb/C mice was intraperitoneally injected with 50 μ L Streptozotocin 100 mg.kg⁻¹ BW (0.005 mg STZ was dissolved in 1 mL of 0,1 M citrate buffer pH 4.5) [17]. Mice were divided into five experiment group; three treatment group (low, optimum and high doses) and two control group (positive and negative). Mice from all treatment group and positive control group were injected with STZ. Blood glucose level then measure when mice at the ages 4-5 weeks post injection.

Administration of EMSA Eritin

Polyherbal EMSA Eritin was given orally for 14 days in three group doses: low dose (0.3125 mg.g⁻¹ BW), optimum dose (3.125 mg.g⁻¹ BW) and high dose (31.25 mg.g⁻¹ BW). Determination of EMSA Eritin doses for each treatment group were based on the human consumption of 60 kg BW that consumed about 15 g of EMSA Eritin. Polyherbal EMSA Eritin was derived from Royal Medicalink Pharmed Company, Makassar, Indonesia.

Blood Glucose Measurement

Measurement of blood glucose levels was done every three days with *One Touch Ultra@* glucometer. Blood sampling was taken from the mice tails, blood was dropped in the glucometer and wait for the value appear on the glucometer screen. Mice with blood glucose levels over than

200 mg.dl⁻¹ is declared as a type 2 diabetes mellitus [18].

Cell Isolation from Thymus

Lymphocyte and chemokine cells was isolated from thymus. Mice were killed by cervical dislocation and surgery with an incision in the abdominal region of mice. Thymus was cleaned, crushed and filtered with PBS to separate from fat tissue. The suspension then put into polypropylene tube and added with PBS until 10 mL and put it in centrifuge (2500 rpm for 5 min at 4°C). The supernatant was discarded and the obtained pellet was resuspended with 1 mL PBS, and put into micro tube, added PBS and centrifuged again (2500 rpm for 5 min at 4°C). The suspended cells ready to proceed for intracellular staining.

Intracellular Staining and Flowcytometry

Intracellular staining was performed from the suspended isolation cells and incubated with 40 μ L antibody (FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD8) Catalogue No. 100531, 100708, BioLegend, San Diego, CA, USA for 20 minutes. After incubation, resuspended using 100 μ L cytofix buffer and incubated for 20 minutes in dark condition, at 4°C. Sample then resuspended with 500 μ L wash-perm and centrifuged (2500 rpm at 4°C for 5 min). The supernatant was discarded and pellet was added with PE-Cy5-conjugated anti-mouse SDF-1 (Catalogue No. IC350C, BioLegend, San Diego, CA, USA) and incubated for 20 minutes. Sample then added with 300 μ L of PBS, transferred into flow cytometry cuvette and ready for running with BD Biosciences FACS CaliburTM nozzle flow cytometry using BD CellQuest software settings PROTM.

Statistical Analysis

Data from this study were analyzed statistically by One way ANOVA (Analysis of Variance) with $p < 0.05$ to assess the statistical difference between experiment group and continued with Duncan test using SPSS 16.0 for Windows.

RESULT AND DISCUSSION

Blood Glucose level

Blood glucose level is the amount of glucose that present and transported through the bloodstream to supply energy to all the cells in our bodies. The body naturally regulates blood glucose levels as a part of metabolic homeostasis. Based on obtained data from treatment and

control group of the blood glucose levels measurements showed a varying result (Fig. 1). Blood glucose levels in NON-DM group still on the normal range between 95-134 mg.dL⁻¹ whereas blood glucose levels in DM group was approved to be a hyperglycemia because it is over than 200 mg.dL⁻¹ [18]. The treatment group at low dose (0.3125 mg.g⁻¹BW) showed a fluctuated blood glucose levels. The blood glucose level was decreased from the start and increased at the 12th day of measurement, as well as the high dose (31.25 mg.g⁻¹BW) level of blood glucose was extremely fluctuated with final measurement blood glucose level are 133.8 mg.dL⁻¹. While the treatment group at optimum dose (3.125 mg.g⁻¹BW) showed a gradually decrease blood sugar levels even though the final measurement blood glucose level is 231 mg.dL⁻¹. The mean result of statistical analysis showed there are significantly different blood glucose level between the treatment and control group ($p < 0.05$), even though there are no significant difference within the treatment group ($p > 0.05$).

The changes of blood sugar level may be influenced by several factors, for example the endurance from each mice to response free radicals that damage the pancreas due to STZ injection, hormonal factors in the body that affect insulin production and environmental factors such as stress that suffered from the treatment [19].

Profile Population of T cells CD4⁺ and CD8⁺

T cells CD4⁺ and CD8⁺ is a trans membrane protein that has a function as co-receptor on T lymphocytes with a different roles. T cells populating the peripheral blood or secondary lymphoid organs were distinguished by the expression of CD4⁺ and CD8⁺ T cells based on the use of particular monoclonal antibodies. The expression of CD4⁺ and CD8⁺ was linked to different T cell functions; in the case of CD4⁺ known as T-helper, augmenting the ability of B cells to produce antibodies and help activate other immune cell including CD8⁺ T cell that known as T-cytotoxic cell causing direct cytotoxicity of infected target cell. T lymphocyte cell derived from hematopoietic stem cell (HSC) in bone marrow (BM) and differentiated into lymphoid progenitor became precursor T cells and migrate from BM to thymus for further maturation became T cells mature single positive (CD4⁺ and CD8⁺). The development of primary CD8⁺ T cell responses is thought to be assisted directly by CD4⁺ T cells in two ways; the

activation of professional APC and the secretion of cytokines [7,8].

Based on T lymphocytes relative number from flow cytometry analysis (Fig. 2), the entire experimental group in a healthy state because CD4⁺ T cell population is higher than CD8⁺ T cell population [20]. T Cells Population of CD4⁺ and CD8⁺ in all treatment group was increased compare to DM control group, the relative number of CD4⁺ T cell treatment group as follows; DM-D1 (0.3125 mg.g⁻¹ BW) 12.69%, DM-D2 (3.125 mg.g⁻¹ BW) 15.19%, and DM-D3 (31.25 mg.g⁻¹ BW) 13, 32% has increased compare to DM group. Same condition in CD8⁺ T cell population relative number as follows; DM-D1 (0.3125 mg.g⁻¹ BW) 5.12%, DM-D2 (3.125 mg.g⁻¹ BW) 5.43%, and DM-D3 (31.25 mg.g⁻¹ BW) 4, 52% also increased from DM group. The relative number of T cell CD4⁺ and CD8⁺ population in all treatment group was significantly increased compare to DM group ($p < 0.05$), although there are no significantly differences within the treatment group.

The increasing of T cell CD4⁺ and CD8⁺ population in thymus after administration of EMSA Eritin was assumed because of the antioxidants compound such as flavonoids and anthocyanin that contained in soy beans and red rice extract. The possible mechanisms [12,13,15], antioxidant is known to have a role as an immunomodulatory that is able to induce and increase the secretion of cytokines IFN- γ to produce IL-2 that may involved in cell activation of T lymphocytes. The increased cell activation is caused by flavonol glycosides in flavonoids that can act as a mitogen and induced timosit to stimulate the secretion of cytokines which will increase the receptor expression of mature T lymphocytes (CD4⁺ and CD8⁺). Another content of active compounds such as saponins also able to play a role in triggering the activation of IFN- γ . Which in turn stimulates the expression of MHC-II, thereby increasing the CD4⁺ T cell proliferation which affect the CD8⁺ T cell activation [21,22]. Exposed naïve T antigen and antigen presented by APC will develop into a subset of T cells CD4⁺ and CD8⁺. CD4⁺ T cells will develop into Th1 and Th2 subsets, where Th1 is contributed to the production of cytokines IL-2 and IFN- γ which activate the CD8⁺ T cell immune cell function. Mechanism action of CD4⁺ as a modulator of the immune system derived from the activity of some cytokines such as IL-1, IL-2 and IFN- γ also to induce CD8⁺ T cell maturation [23,24].

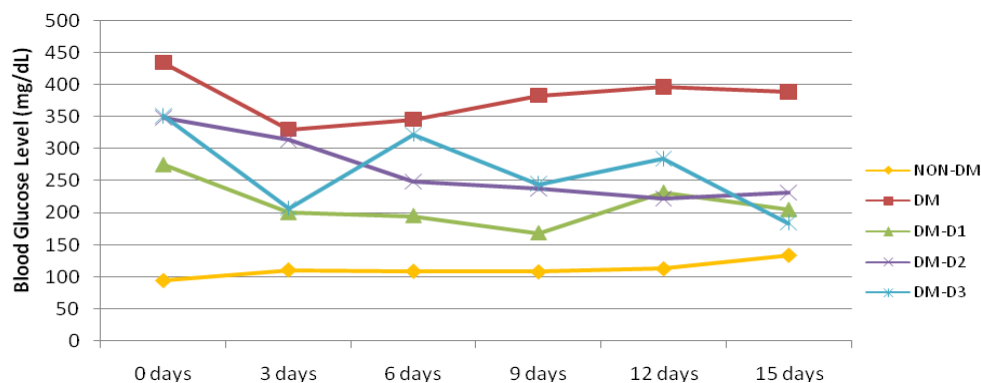


Figure 1. Measurement of blood glucose level. **(NON-DM)** nondiabetic mice as negative control group without any treatment. **(DM)** diabetic mice as positive control group, treated with injection of Streptozotocin 100 mg.kg⁻¹. **(DM-D1)** DM mice treated with EMSA Eritin at low dose 0.3125 mg.g⁻¹ BW. **(DM-D2)** DM mice treated with EMSA Eritin at optimum dose 3.125 mg.g⁻¹ BW. **(DM-D3)** DM mice treated with EMSA Eritin at high dose 31.25 mg.g⁻¹ BW.

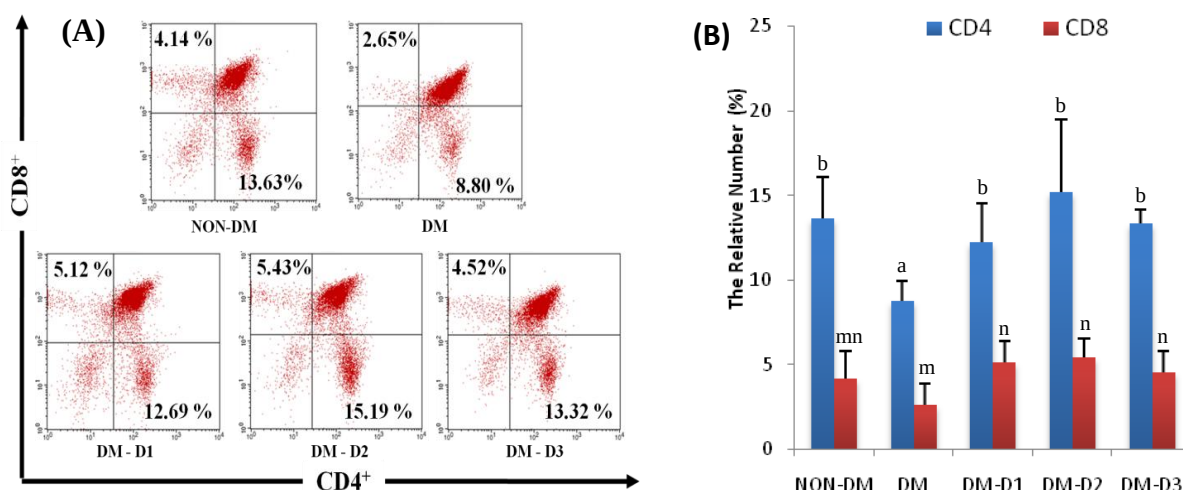


Figure 2. Cells population of CD4⁺ and CD8⁺ T cells were increased in all treatment group. **(A)** The results of flow-cytometry analysis **(B)** Mean of percentage relative number from five group. Differences notation indicates significant difference ($p < 0.05$). **(NON-DM)** nondiabetic mice as negative control group without any treatment. **(DM)** diabetic mice as positive control group, treated with injection of Streptozotocin 100 mg.kg⁻¹. **(DM-D1)** DM mice treated with EMSA Eritin at low dose 0.3125 mg.g⁻¹ BW. **(DM-D2)** DM mice treated with EMSA Eritin at optimum dose 3.125mg.g⁻¹ BW. **(DM-D3)** DM mice treated with EMSA Eritin at high dose 31.25 mg.g⁻¹ BW.

Profile Population of SDF-1 Chemokine

Stromal Cell-Derived Factor-1 (SDF-1), is a CXC chemokine stromal derived from bone marrow. Chemokines are a family on chemoattractant polypeptides that classified into 4 groups, depend on the position of conserved cysteine residues. Chemokines mediate their effect by binding to transmembrane G protein-coupled co-receptors CXCR4. Major role of chemokines is chemoattractant to guide the migration of cells. Chemokines-attracted cells follow a signal of increasing chemokine concentration towards the source. Some chemokines control mobilization cells of the immune system during processes of immune surveillance such as leukocyte subsets to inflammation sites [25,26].

Flowcytometry analysis and relative number of SDF-1 cells showed varying results between the group. SDF-1 relative number increased gradually in DM-D1 and DM-D2 but decreased in high dose (DM-D3) 0.69%. The relative number of SDF-1 cells population was significantly differences between treatment and control group ($p < 0.05$) (Fig. 3). Increased population of SDF-1 in DM-D2 (3.125 mg.g⁻¹ BW) 1.76% presumably cause by the expression of SDF-1 as chemoattractant. It released signal by injured cells or tissue in thymus lead to increased level of SDF-1 at the inflammation site. While in high dose (31.25 mg.g⁻¹ BW), expression of SDF-1 decreased probably due to recovery from the active compound flavonoid from soy bean (*G. max*) as an antioxidant at the site of inflammation [21].

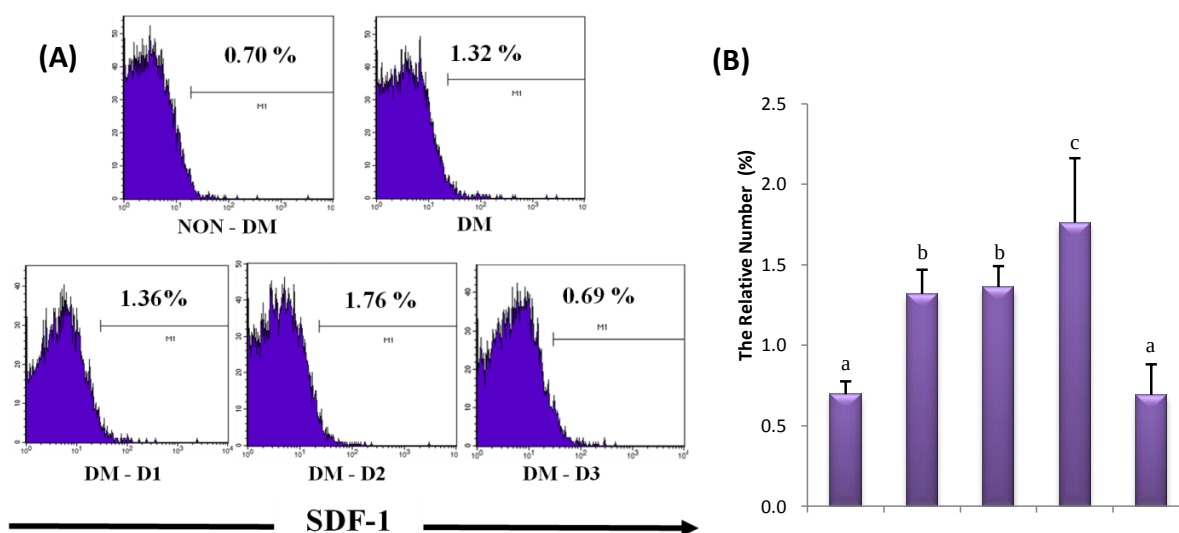


Figure 3. Cells population of SDF-1 in thymus. **(A)** The results of flow-cytometry analysis **(B)** Mean of percentage relative number from five group. Differences notation indicates significant difference ($p < 0.05$). **(NON-DM)** nondiabetic mice as negative control group without any treatment. **(DM)** diabetic mice as positive control group, treated with injection of Streptozotocin 100 mg.kg^{-1} . **(DM-D1)** DM mice treated with EMSA Eritin at low dose $0.3125 \text{ mg.g}^{-1} \text{ BW}$. **(DM-D2)** DM mice treated with EMSA Eritin at optimum dose $3.125 \text{ mg.g}^{-1} \text{ BW}$. **(DM-D3)** DM mice treated with EMSA Eritin at high dose $31.25 \text{ mg.g}^{-1} \text{ BW}$.

Polyherbal EMSA Eritin is known to have antioxidant form of isoflavones and anthocyanins derived from soy beans and red rice extract. Isoflavones are flavonoid group commonly found in soybeans and has beneficial effect on diabetic patients by eliminating the direct oxygen free radicals, increasing the concentration of antioxidant enzymes and increases serum insulin and pancreatic insulin component [13]. Genistein, a component of isoflavones, have the effect of lowering blood glucose by increasing insulin production and glucose uptake by cells [14]. Just like isoflavones, anthocyanins protect pancreatic cells from oxidative stress, lowers blood glucose levels by increasing insulin receptors work, improve antioxidant status by pressing malondialdehyde and improve the level of superoxide dismutase [16]. Compared to individual component from different studies, polyherbal EMSA Eritin can also lowering the blood glucose through the improvement of T lymphocyte cell (CD4^+ and CD8^+) and chemokine SDF-1 modulation in thymus mice model of diabetes mellitus in this experiment.

CONCLUSION

Polyherbal EMSA Eritin can modulate T lymphocytes (CD4^+ and CD8^+) in optimum dose $3.125 \text{ mg.g}^{-1} \text{ BW}$ and suppress SDF-1 production in high dose ($31.25 \text{ mg.g}^{-1} \text{ BW}$) as well as the blood glucose level measurement. This study suggest

that EMSA Eritin is a potential polyherbal that can be used as a therapy for diabetes.

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Bone Marrow Cells Lymphocyte Activity of Pregnant Mice with Therapy of *E. scaber* and *S. androgynus* Post Infection *Salmonella typhimurium*

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Abstract

Pregnancy can cause immune system changes. It is characterized by a decrease in the activity of immunocompetent cells. The use of antibiotics was intended to combat pathogenic microorganisms, but antibiotics have negative effects on pregnant women. The use of antibiotics can be replaced with extracts of *Elephantopus scaber* and *Sauropus androgynus* because both plants have chemical compounds that act as immunomodulators. This study was aimed to determine the activity of lymphocytes B220⁺, TER119⁺, and GR-1⁺ on bone marrow pregnant mice given the combination of extracts of *Elephantopus scaber* and *Sauropus androgynus* after infected with *Salmonella typhimurium*. This research uses seven treatment groups name: (K-) 0.05% NaCMC without bacterial infection; (K+) 0.05% NaCMC infected by bacteria; (P1) *E. scaber* 200 mg.kg⁻¹BW infected by bacteria; (P2) *E. scaber* 150 mg.kg⁻¹BW and *S. androgynus* 37.5 mg.kg⁻¹BW infected by bacteria; (P3) *E. scaber* 100 mg.kg⁻¹BW and *S. androgynus* 75 mg.kg⁻¹BW infected by bacteria; (P4) *E. scaber* 50 mg.kg⁻¹BW and *S. androgynus* 112.5 mg.kg⁻¹BW infected by bacteria; and (P5) *S. androgynus* 150 mg.kg⁻¹BW infected bacteria. The initial dose of *E. scaber* and *S. androgynus* was 50 mg.kg⁻¹. Each treatment has three repetitions, surgery performed on day 12th and 18th. Lymphocyte cells isolated from bone marrow, the obtained results were analyzed by flowcytometry and statistical analysis using SPSS 16.0 one-way ANOVA, Tukey test and path. Based on the results from ANOVA tables, the formulations on mice that can restore their normal conditions with B220⁺ cells is *E. scaber* 150 mg.kg⁻¹BW and *S. androgynus* 37.5 mg.kg⁻¹BW, TER119⁺ cells is *S. androgynus* 150 mg.kg⁻¹BW, while the GR-1⁺ cells affected by the surgery. Those three dose formulations can be used to obtain the optimum value which can increase the number of lymphocytes and not harmful to the developing fetus.

Keywords: Bone marrow, *Elephantopus scaber*, Lymphocyte, Pregnant mice, *Sauropus androgynus*

INTRODUCTION

Pregnancy in women will affect the physiological condition of the body. In the state, pregnant women will experience changes in their immune system, which is intended to facilitate embryo implantation, placental development, fetal tolerance initiated, as well as the defense of the maternal immune system [1]. Changes in the pregnant woman's immune system will lower the immune system [2], thus with such a situation pregnant women susceptible to disease [3].

One change that affects the immune system of pregnant women is *Salmonella* [4]. Previous study analyzed 200 blood samples and found that 129 women have positive samples of typhoid fever [5]. The ability of the bacteria causing typhoid fever because it has a Vi antigen capable of reducing the expenditure of IL-8, which has the role of neutrophils inducers [6].

Intracellular facultative bacteria live and reproduce in varies of cell types, including macrophage which leads prominent in immunity. Cellular immunity mechanism is the most appropriate mechanism to do elimination, where antibody in blood circulation unable to reach progress antigens in cells [7]. Infection in pregnant women will affect the activity of immunocompetent cells that play a role in controlling the entry of viruses, bacteria and other pathogens. Influence exerted by pathogenic microorganisms will lead to symptoms of toxicity. Delays in handling would result in perforation, perionitis, loss of consciousness and death [2]. The infection will also cause damage to erythrocytes and through lysis [8]. Infection will result in inflammatory cells and affect the polymerase and differentiation of B cells thus activity of erythrocyte disturbed [9].

The synthetic compounds administered to pregnant women to prevent immune deficiencies. However, this compound causes destructive ends effects that harm the mother and the fetus [2]. Therefore the need to look for

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alternatives with systemic effects found herbal medicine [10].

E. scaber and *S. androgynus* has been widely studied as a natural immunomodulator [11,12], but it is not known how the effect when both plants use together. Therefore, this study was aimed to determine the influence of *E. scaber* and *S. androgynus* on the activities of B220⁺ cells, TER119⁺, and GR1⁺ on pregnant mice infected by *S. typhimurium*.

MATERIALS AND METHODS

Design of Experiments

This experimental research was conducted by using seven experimental groups, i.e. two control groups and five treatment groups. Surgery performed on the 12th and 18th after the animal was assumed to be pregnant by comparing the results of observation on treatment and control, as well as between the treatment groups.

Total of 42 Balb/C mice were divided into seven treatment groups, each group consisted of three replications. Mice got the same standard treatment of eat and drink ad libitum. An object of experimentation is pregnant female mice strains balb/c obtained from PT. Galaxy science Jember. Bacteria *S. typhimurium* was injected intraperitoneal. We used strains 444-D from the collection of the Laboratory of Microbiology, Faculty of Medicine, University of Brawijaya.

Extract Preparation

Simplicia obtained from medika materia, Batu. The extract was made by maceration in 70% ethanol solution of 1:3 for 1x24 hours with occasional stirring. After 24 hours, the results were filtered using a Buchner funnel to get the plsntd extract, then evaporated at evaporator tube rotating speed of 200rpm at a temperature of 40°C waterbath. The condensed filtrate was weighed and prepared by dissolving treatment with 0.05% NaCMC and administered orally to the mice.

Treatment

Pregnant mice were given the extract of *E. scaber* and *S. androgynus* referred to the design (Table 1). The initial dose of *E. scaber* and *S. androgynus* was 50 mg.kg⁻¹BW. Each treatment contained three repetitions.

Later on 5th day after administration of the extract, the mice infected with the bacterium *S. typhimurium* concentration of 10⁷ cfu.mL⁻¹. Administration of extracts combination was

continued until surgery on the 12th and 18th day. Extracts were given by sonde daily before meal.

Table 1. Experiment design of mice groups

Mice Groups	Bacterial Infection	Therapy (mg.kg ⁻¹ BW)	
		<i>E. scaber</i>	<i>S. androgynus</i>
K -	-	-	-
K +	infected	-	-
P1	infected	200	-
P2	infected	150	37.5
P3	infected	100	75
P4	infected	50	112.5
P5	infected	-	150

Note: Control (K) were given 0.05% NaCMC

Isolation of Lymphocytes

Isolated bone marrow flushed with PBS using a syringe and placed in polypropylene tubes, whereas the isolation of peripheral blood samples were taken from the heart inserted into propylene and added PBS and centrifuged 2500 rpm for 5 min at 4°C. Pellet resuspended in 1 mL of PBS to be taken 30μL then put in a microtube containing 1 mL of PBS for re centrifuged at 1500 rpm temperature of 10°C for 5 min. Pellets then added PBS containing FITC monoclonal antibody anti-B220, anti-TER-PE 119 and PE-anti GR-1 for 50μL and incubated for 20-30 min prior to analysis using flowcytometry.

Analysis of flowcytometry

The cell suspension was transferred into a cuvet flowcytometer, added 500 mL of PBS and homogenized. The setting of flowcytometer has been on acquired by the computer. After all the instruments were ready, cuvet mounted on the nozzle BD FACS Calibur TM Bioscience flowcytometry. Data from flowcytometer was subsequently processed with software of BD CellQuest PRO™ and displayed as a histogram.

Data Analysis

The results of the relative number of B220⁺ cells, TER119⁺ and GR-1⁺ were tested its normality with SPSS 16.0. Furthermore, the data were tested by ANOVA, and if there is significance then proceed to the Tukey test.

RESULT AND DISCUSSION

B220⁺ Cells

Flowcytometry analysis of bone marrow showed the average relative number of B220⁺ cells which was significantly different (P<0.05) to the B220⁺ cell activation. The results ANOVA showed that the administration of 70% ethanol extract of the leaves of *E. scaber* and leaves of *S.*

androgynus were potential to increase the relative amount of B220⁺ cells. Figure 1 is the expression generated from flowcytometry. It described the increase in the relative number of B220⁺ cells. The real difference indicated that P2 treatment (ES 150 mg.kg⁻¹: SA 37.5 mg.kg⁻¹) with significance value of p < 0.05.

The existence of foreign objects that enter the body will be received by surface receptors [13,14]. Incoming antigens will trigger non-specific immune system by forming immuno-complex through the production of antibodies and some cytokines [15]. Other research found that flavonoid compound and the injection of *S. typhimurium* increased the proliferation of lymphocytes [9]. Thus its existence has immunostimulatory effects to stimulate the production of IL-2 [16].

Increased B220⁺ cells showed an increase in plasma cell populations. It because the B220⁺ cells are a subset of CD45R isoform predominantly expressed on all B-lymphocytes and plasma cells regulate the development. Increased plasma cells showed that the 70% ethanol extract of *E. scaber* and *S. androgynus* have immunostimulatory effects. Middleton [16] revealed the presence of flavonoids can inhibit the activity of MAPK (mitogen-Activated Protein Kinase) that causes posporilasi various transcription factor protein needed for protein synthesis. The induction of MAPK protein would activate the

transcription factor NF-kB is a transcription factor in the proliferation and differentiation of B220⁺ cells through regulation of cytokines [17].

TER119⁺ cells

The mean relative number of TER-119⁺ cells was analyzed by flowcytometry (Fig. 2.) There is a decrease compared to the control. SPSS analysis showed that the relative number of TER119⁺ cells is significantly different for the P5 treatment (SA 150mg.kg⁻¹BW).

TER-119⁺ cells are antigen expressed on erythrocytes. Viability of erythrocytes in normal mice is 42-56 days. Otherwise, 3-4 days for erythropoiesis, anemia, hypoxia and inflammation will lead to faster and release eritropoies erythrocytes in peripheral area [18]. Decreased expression of TER-119⁺ cells caused by an infection of *S. typhimurium*. Sudoyo [8] described the *Salmonella* bacteria have the ability to live well in the gastrointestinal tract and other organs, thus causing an inflammatory reaction. Inflammatory process will affect the activity of erythrocyte during oxidation will cause damage to hemoglobin. Erythrocytes will be separated from the cytoplasm, whereas macrophage cells will phagocytose abnormal erythrocyte [19]. The effect of extract gives a stable condition or equal to normal treatment. It is because pregnant mice require adequate nutrition, thus proliferation and differentiation does not work faster.

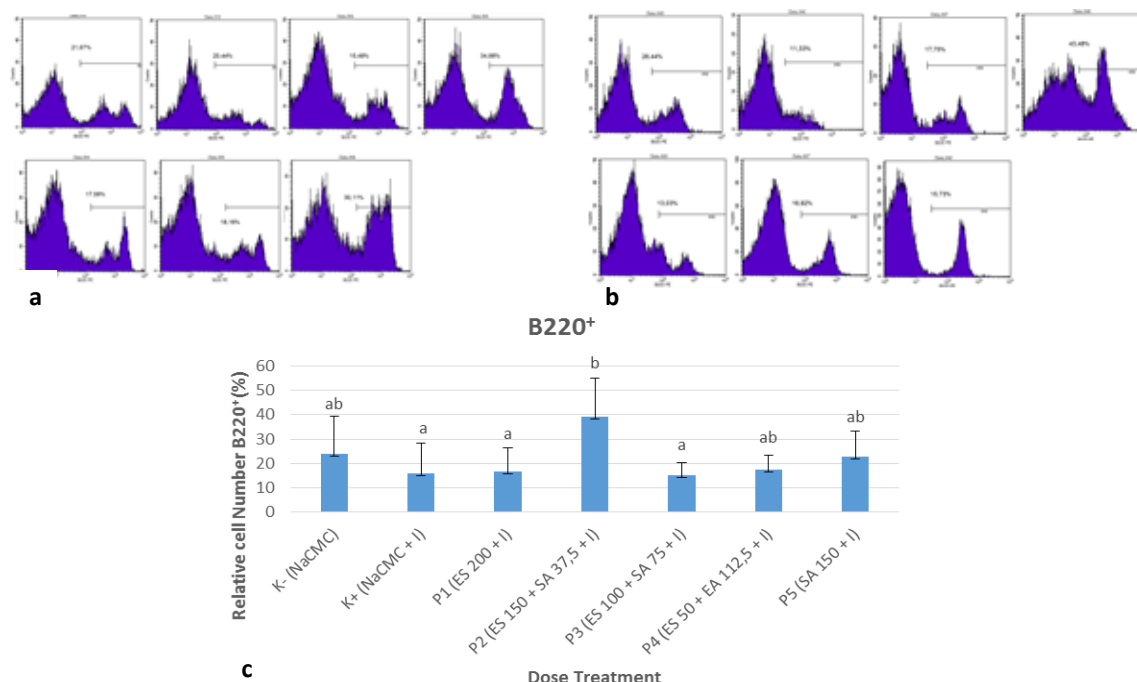


Figure 1. Expression of B220⁺ cells

Description: ES= *Elephantopus scaber*, SA= *Sauropus androgynus*, I= infected by *Salmonella typhimurium*
a. day surgery to 12th; b. day surgery to 18th; c. mean relative number of B220⁺ cells (%)

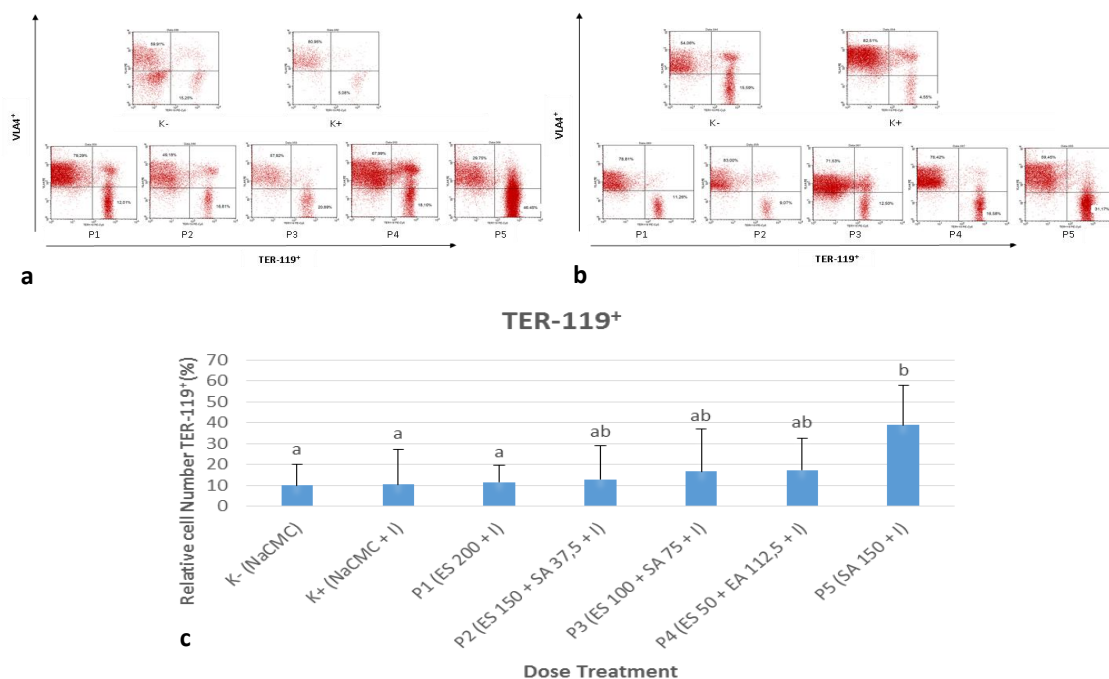


Figure 2. Expression of TER119⁺ cells

Description: ES= *Elephantopus scaber*, SA= *Sauropus androgynus*, I= infected by *Salmonella typhimurium*
a. day surgery to 12th; b. day surgery to 18th; c. mean relative number of TER119⁺ cells (%)

GR1⁺ cells

Expression of GR-1⁺ cell proliferation showed in Figure 3. Infection of *S. typhimurium* triggered cell deficiency GR-1⁺ to phagocytose cells that have been damaged or abnormal. ANOVA results showed that the 70% ethanol extract of the leaves of *E. scaber* and *S. androgynus* can increase the GR-1⁺ molecule but not significant ($p > 0.05$). Calculation continued to post hoc analysis with Tukey test. It showed that the treatment group had a significant difference to the control. Negative control group had an average increased compared to the positive control group. According to previous research, the increase in the number of neutrophils and IFN γ cells in animals affected by an infection with *Salmonella* bacteria than uninfected animals [20]. Other study stated that in Balb/C mice were given *Salmonella* infection increased the number of leukocytes including neutrophils and IFN γ significantly compared with the group treated with the standard feed [21].

Microorganisms will spur the immune system of the body's defense nonspecific starts with the way the body's defenses destroy bacteria and more complex through antibody production or manufacture of various cytokines [15]. Neutrophils (GR-1⁺) work on non-specific immune response pathway. The cytokine IFN γ will act as immunomodulator through the regulation of

gene expression to signal transduction. Neutrophil cells will express the receptor molecule with 1000 fast and stable molecule binds IFN γ [22]. After binding, there will be internalization, thus decreasing surface receptors. IFN γ regulates expression on number of genes including complement receptor regulator, B lymphocyte stimulator, dendrites chemotactic factor, chemokine receptors, neutrophil chemotactic factors and proinflammatory cytokines.

Path Analysis

Path diagram was made based on the results on statistical analysis of the correlation (Fig. 4). Proliferation and differentiation of B lymphocyte cells are affected by the *S. typhimurium* infection and extract combinations. The antigen will be responded by expressing cell surface receptors, i.e. B220⁺, TER119⁺ and Gr-1⁺. Day surgery affects the relative amount of macrophage cells as much as 13.03% and the relative number of cells suppresses TER119⁺ to 18.19%.

Infection of *S. typhimurium* will increase the number of cells of neutrophils and cytokines IFN γ [20]. Infection also affects the activity of B cells. The cells that are not growing will be phagocytosed by macrophages thus the number will be reduced. The existence of the extract will maintain the number of B lymphocyte cells in proliferation and differentiation [19].

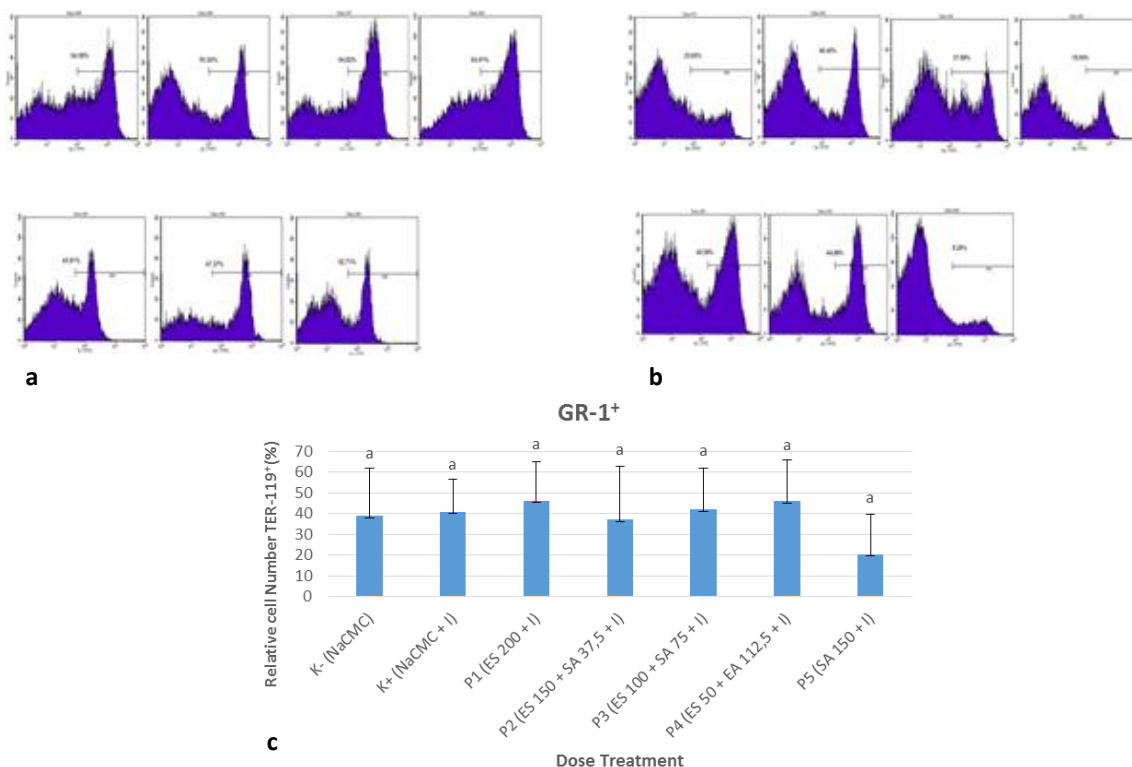


Figure 3. Expression of GR1⁺ cells

Description: ES= *Elephantopus scaber*, SA= *Sauropus androgynus*, I= infected by *Salmonella typhimurium*
a. day surgery to 12th; b. day surgery to 18th; c. mean relative number of GR1⁺ cells (%)

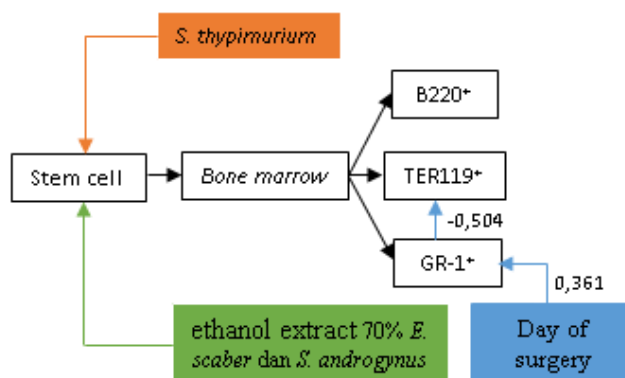


Figure 4. Development of B220⁺ cells, TER119⁺, GR-1⁺ on the organ affected Bone marrow by *S. typhimurium* infection and extract combinations of *E. scaber* and *S. androgynus* and day surgery

CONCLUSION

The combination of *E. scaber* and *S. androgynus* for *S. typhimurium* infected mice can restore the mice into their normal conditions. The best combination for B220⁺ cells is P2 treatment (150 mg.kg⁻¹BW *E. scaber* and 37.5 mg.kg⁻¹BW *S. androgynus*). While the best dose for increasing TER119⁺ cells is P5 treatment (*S. androgynus* 150 mg.kg⁻¹BW, while the GR-1⁺ cells affected by the surgery. These dose formulations can be used to optimize the number of lymphocytes and not harmful for the developing fetus.

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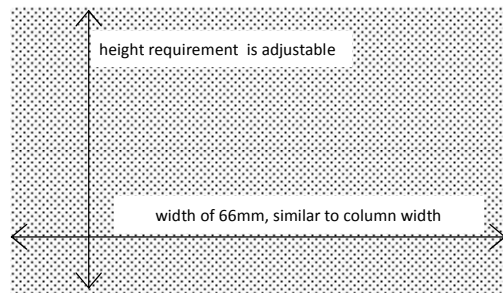


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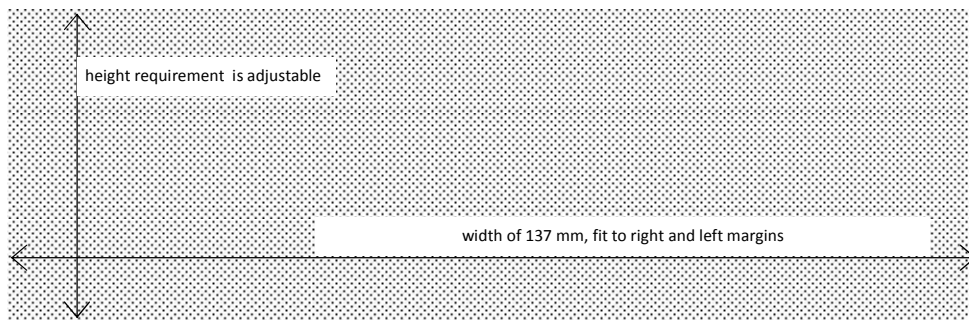


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 7. Citation in the manuscript typed only the references number (not the author and year), example: Obesity is an accumulation of fat in large quantities which would cause excessive body weight (overweight) [1]. Obesity is a risk factor of diabetic, hypertension dan atherosclerosis [2].
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CONCLUSION (Calibri 10 Bold, Left, Capslock)

Conclusion of the study's findings are written in brief, concise and solid, without more additional new interpretation. This section can also be written on research novelty, advantages and disadvantages of the research, as well as recommendations for future research. (Calibri 10 Justify)

ACKNOWLEDGEMENT (Calibri 10 Bold, Left, Capslock)

This section describes gratitude to those who have helped in substance as well as financially. (Calibri 10 Justify)

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Cover Images:

3D Structure of EGCG (*Epigallocatechin-3-Gallate*)
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