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 ± 2.5 g body weight. Mice were taken care in free pahtogen animal chamber, Departement of Biology, Faculty of Mathematic and Natural Sciences, University of Brawijaya, Malang, East Java, Indonesia.

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

Induction of Diabetes mellitus
Neonatal d-5 Balb/C mice was intraperi-toneally injected with 50 µL 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. Poly-herbal EMSA Eritin was derived from Royal Medicalink Pharmalab Company, Makassar, Indo-nesia.

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 glucostick 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 poly-propylene 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 resuspensed 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 µL 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 µL cytofix buffer and incubated for 20 minutes in dark condition, at 4°C. Sample then resuspended with 500 µL 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 µL of PBS, transferred into flow cytometry cuvette and ready for running with BD Biosciences FACS Calibur^{TM} nozzle flow cytometry using BD CellQuest software settings PRO^{TM}.

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 homeostatis. 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^{-1}BW) showed a fluctuated blood glucose levels. The blood glucoe level was decreased from the start and increased at the 12th day of measurement, as well as the high dose (31.25 mg.g^{-1}BW) 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^{-1}BW) 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].
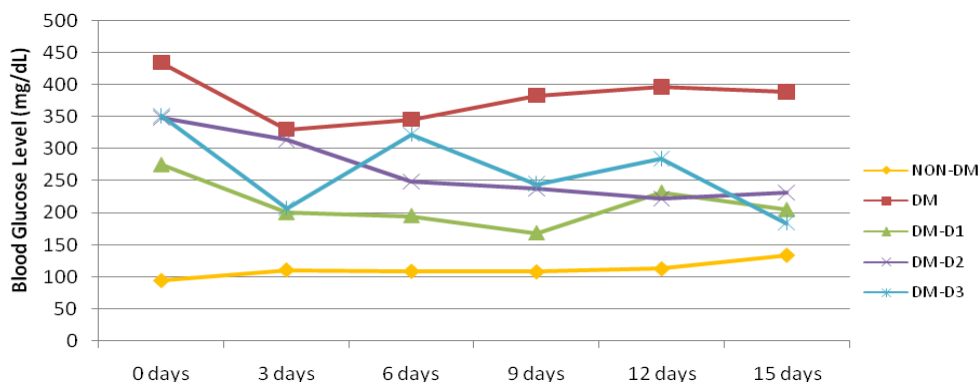
Figure 1. Measurement of blood glucose level. **(NON-DM)** nondiabetic mice as negative control group without any treatment. **(DM)** diabetic mice as positive control group, treated with injection of Streptozotocin 100 mg.kg^{-1}. **(DM-D1)** DM mice treated with EMSA Eritin at low dose 0.3125 mg.g^{-1} 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.
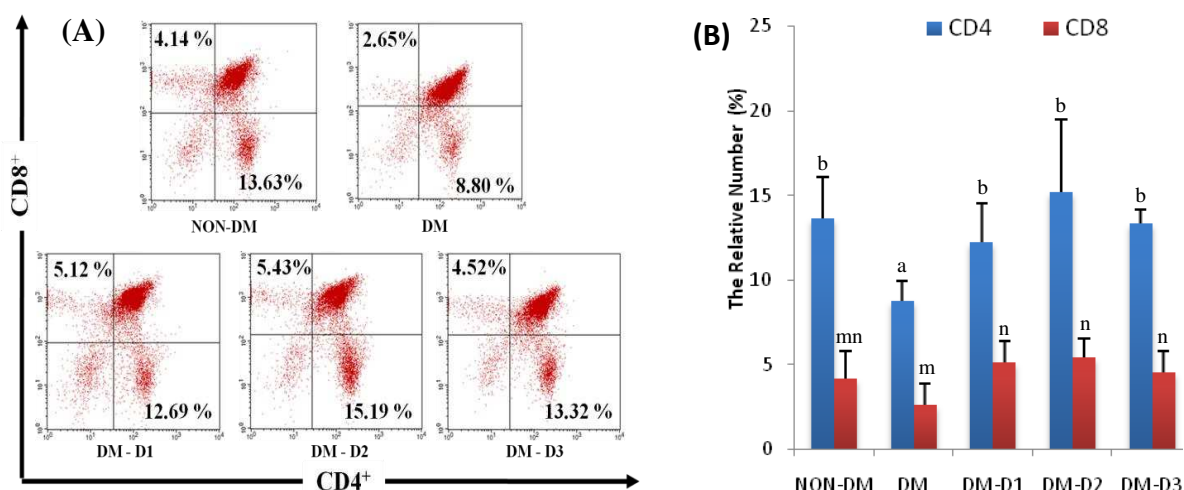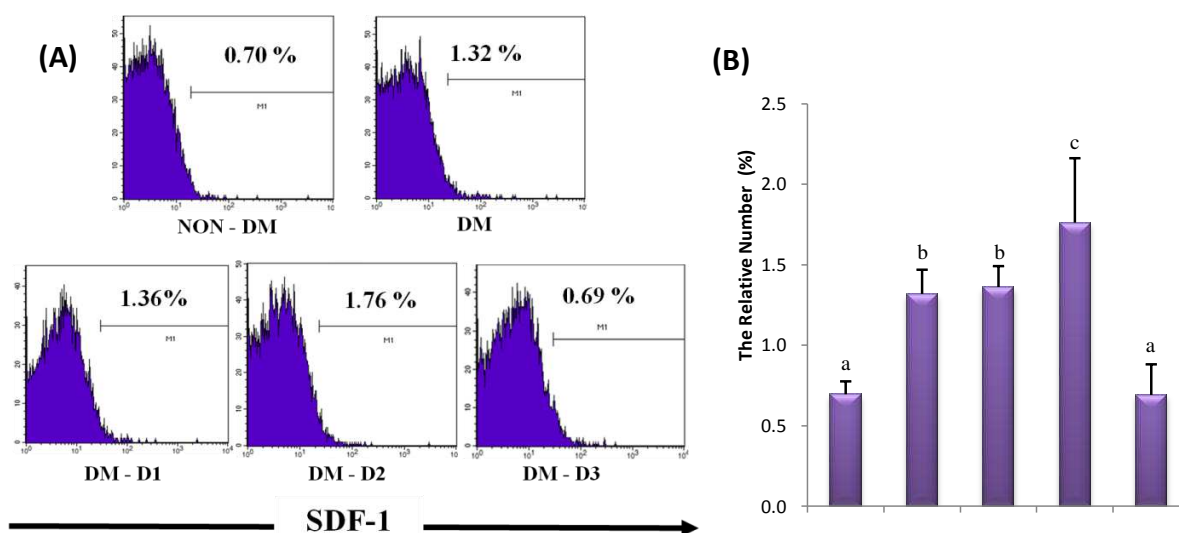

Figure 2. Cells population of CD4$^+$ and CD8$^+$ T cells were increased in all treatment group. **(A)** The results of flow-cytometry analysis **(B)** Mean of percentage relative number from five group. Differences notation indicates significant difference ($p<0.05$). **(NON-DM)** nondiabetic mice as 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 tisue 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 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.

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 mg.g^{-1} BW and supress SDF-1production in high dose (31.25 mg.g^{-1} 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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