Anti-hyperglycemic and Immunomodulatory Activity of a Polyherbal Composed of *Sesbania grandiflora*, *Salacca zalacca* and *Acalypha indica*

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Abstract

Diabetes has become a serious global public health problem due to its high prevalence and mortality. Unfortunately, current anti-diabetic drugs are having some limitations and adverse effects. Therefore, searching for a new anti-diabetic agent is an urgent challenge. In this research, we examined the effectiveness of a traditional anti-diabetic polyherbal composed of *Sesbania grandiflora* seeds, *Salacca zalacca* leaves and *Acalypha indica* roots (2:1:1). The study was aimed to explore the anti-hyperglycemic effect of the polyherbal in STZ-induced diabetic mice and to investigate the immunomodulatory activity involved in the process of controlling hyperglycemia. Our results showed that the polyherbal water extract (150 mg.kgBW^{-1}) could suppress blood glucose elevation and preserve pancreatic islet of diabetic mice. Moreover, the polyherbal treatment could normalize the relative amount of activated $CD4^+CD62L^-$ and $CD8^+CD62L^-$ T cells. The polyherbal extract also stimulated the production of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) which is known to play an important role in diabetes control. In addition, polyherbal treatment also increased the relative amount of anti-inflammatory cytokines IL-10 and TGF-β. These results revealed that the polyherbal extract has an anti-hyperglycemic and immunomodulatory activity that may provide beneficial function in diabetes healing.

Keywords: Antidiabetes, Immunomodulator, Polyherbal, Regulatory T cell.

INTRODUCTION

Diabetes is a disease characterized by hyperglycemia [1]. It occurs due to inadequate peripheral tissue response to levels of insulin in the body, and/or by overall insufficient insulin production [2]. Diabetes can cause some serious health problems including of the damage of blood vessels, heart, kidneys, eyes, nerves, as well as increases the risk of stroke. Diabetes and related complications are also a prime cause of death [3]. The International Diabetes Federation (IDF) estimated that the total number of people with diabetes in the world was 415 million in 2015 and predicts this figure will increase to 642 million people in 2040. Because of its high rate of prevalence and mortality, diabetes has become one of the global health emergencies of the twenty-first century [4].

Currently, there are some approved drugs available to clinically treat diabetes. However, the use of those drugs has been reported to lead to undesirable side effects [5]. Moreover, these medicines do not significantly improve β cell function, so they cannot cure diabetes completely in a patient [6,7]. Contrastingly, many people have claimed to have recovered from diabetes by consuming plant-based herbal medicines as prescribed in traditional remedies. Unfortunately, most of their claims have not yet been supported by scientific evidence. Therefore, it is necessary to scientifically investigate anti-diabetic activity resulting from plant-based herbals used in traditional diabetes remedies. This investigation is essential if there are to be discoveries of potential anti-diabetic agents that are more effective and safer than drugs available today.

Studies on diabetes have revealed the close link between immune system and diabetes pathology. It has been reported that pro-inflammatory markers were increase in diabetic patients [8,9]. Inflammatory cytokines also contributes to the onset of insulin resistance [10] and diabetic complications [11]. Based on these findings, a new stategy for diabetes treatment namely immunomodulatory theraphy was developed by using anti-inflammatory or other immunomodulatory agents [12]. Recent data revealed that immunomodulatory treatments could give beneficial effects on glycemia, β-cell function, and insulin resistance [13]. In accordance with this research, compounds

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contained in traditional anti-diabetic herbals could be expected to have immunomodulatory properties that may modulate immune system and further ameliorate diabetes symptoms.

In this study, we investigated scientific evidence of the effectiveness of an Indonesian traditional polyherbal composed of *Sesbania grandiflora* seeds, *Salacca zalacca* leaves and *Acalypha indica* roots (2:1:1) which has been used to combat diabetes by local people in Malang City, Indonesia. Originally, this polyherbal mixture was made by a traditional herbal practitioner who has more than 20 years of experience in composing various plant-based medicines. The study was aimed to investigate the anti-hyperglycemic effects of the polyherbal treatment in STZ-induced diabetic mice and to analyze the immune system modulation involved in the process of controlling hyperglycemia.

MATERIAL AND METHOD

Materials

Sesbania grandiflora (local name: turi, English name: hummingbird tree) seeds were purchased from a local agricultural seed supplier. *Salacca zalacca* (local name: salak, English name: snake fruit) leaves were taken from private local gardens in Pasuruan City, Indonesia (-7°38'43.08" S 112°54'27.00" E). *Acalypha indica* (local name: anting-anting, English name: Indian nettle) roots were taken from local private gardens in Malang City, Indonesia (7º55'23.61" S 112º38'4.62" E). The plant names of materials used in this study have been checked with plant list species provided in http://www.theplantlist.org.

Animals

Animals used in this experiment were 10-weeks old male BALB/c mice (*Mus musculus* Linnaeus) with an average body weight of 30 $\pm$ 2 g. The mice were contained in individual cages at the animal experimentation laboratory of Biosains Institute, Brawijaya University.

Induction of Diabetes

Induction of diabetes was conducted by the method of Furman [14] with modification at the dose of STZ. The healthy non-diabetic mice were injected intraperitoneally with streptozotocin solution (Bioworld, USA) in a dose of 140 mg.kg^{-1}. STZ solution was prepared immediately before injection by dissolving an appropriate amount of STZ powder with 10 mM Na-citrate buffer (pH 4.5) at a final concentration of 20 mg STZ mL^{-1}. In order to optimize STZ absorption, the mice were fasted for 4 hours prior to injection. At six days post-injection, blood glucose levels of the mice were measured with a glucometer device. Mice with glucose levels higher than 200 mg dl^{-1} were considered as diabetic mice.

Preparation of Water Extract of Polyherbal

The polyherbal mixture ingredients were washed with fresh water and left to dry outdoor for 24 hours. The materials were then dried in an oven (set at 120°C) for 1 hour, and then each component was ground finely into a powder. The powders were then mixed in the ratio of *S. gradiflora*: *S. zalacca*: *A. indica* = 2:1:1 as prescribed in the traditional polyherbal preparation.

Traditionally, the polyherbal decoction was prepared by pouring hot water into the powdered polyherbal mixture, and, once the mixture had cooled and the solid materials had settled, the decoction was consumed immediately. In this experiment, the polyherbal extract was prepared by dissolving the polyherbal powder into boiled water (at a ratio of 1:10) and then kept at room temperature for 24 hours. The solid sediment was then separated from aqueous extract. The liquid extract was then evaporated using a freeze dryer.

Treatments

Animals were divided into three groups. The first group was comprised of only normal mice (Non-diabetic Mice, NDM), the second group contained diabetic mice who were not being given treatment (Untreated Diabetic Mice, UTDM), and the third group was made up of diabetic mice who were administered with the polyherbal extract (Polyherbal-treated Diabetic Mice, PTDM). Six experimental mice were used for each group. The extract was administered once a day for 24 days orally using the gavage technique at a dose of 150 mg kg^{-1} BW.

Blood Glucose Measurement

The blood glucose level was measured with a glucometer (General Electric, USA) every six days. At the blood glucose readings, the tip of each mouse's tail was carefully snipped and then massaged until a small bead of blood had formed. The blood was then put on the test strip which was inserted into the glucometer device.

Histology of Pancreatic Islet

On day 25 of the study, the mice were sacrificed, then their pancreas was harvested and fixed in 10% formalin solution. The fixed pancreas specimens were then immediately sliced, processed and embedded into paraffin

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blocks. The blocks were cut into 4 µm paraffin sections by a rotator microtome and stained with Hematoxylin and Eosin (H&E) [15]. The histological observation was done by using a light microscope.

Isolation of Splenocytes and Flow Cytometry Analysis

Isolation of splenocytes and flow cytometry analysis were performed according to the method of Rifa'i and Widodo [16] with modification in the type of antibodies. Harvested mouse spleens were washed with sterile PBS twice and placed on a petri dish containing additional sterile PBS. The spleens were then pressed using a syringe holder. A single cell solution was filtered with a sterile wire and placed into a 15 mL polypropylene tube. PBS was added to this suspension till the 10 mL mark and then centrifuged at 2,500 rpm at 4°C for five minutes. The supernatant was then discarded, and the obtained pellet was resuspended in 1 mL of sterile PBS. The single cell suspension containing around 2-3 × 10^6 cells was washed with PBS and stained with FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD8, PE-conjugated anti-mouse CD25, PE/Cy5-conjugated anti-mouse CD62L.

Intracellular cytokine staining was performed with a Cytofix/Cytoperm kit (BD-Biosciences Pharmingen) according to the protocol provided by the manufacturer. Pellets with approximately 2-3 × 10^6 cells were stained with FITC-conjugated anti-mouse CD4 and PE-conjugated anti-mouse CD25 for 30 min. After incubation, the suspension was washed, and the pellet was resuspended in cytofix buffer (200 µL) for 20 min in the dark at 4°C, then resuspended in 1 mL wash-perm and centrifuged again at 2500 rpm at 4°C for 5 min. The supernatant was discarded, and the obtained pellet was subjected to intracellular staining with PerCP anti-mouse FoxP3, PerCP anti-mouse interleukin-10 and PE/Cy5 anti-mouse TGF-β for 30 minutes.

Data Analysis

The data were analyzed using the one-way analysis of variance (ANOVA) to determine the significance of the difference between the means of the groups. A post hoc analysis was conducted according to Fisher's Least Significant Different (LSD) test at 95% of confidence level. The ANOVA and LSD tests were performed using software Genstat 18th Edition (VSN International Ltd., UK).

RESULT AND DISCUSSION

Effect of Polyherbal Administration on Blood Glucose of Diabetic Mice

Figure 1 showed that the blood glucose of untreated diabetic mice increased to 487 ± 38 mg.dL^{-1} after 24 days of treatment. In the diabetic mice receiving polyherbal treatment, the blood glucose level was 313 ± 19 mg.dL^{-1} which were very significantly lower than that in diabetic control. This result indicates that polyherbal administration was able to prevent the blood glucose elevation in diabetic mice.

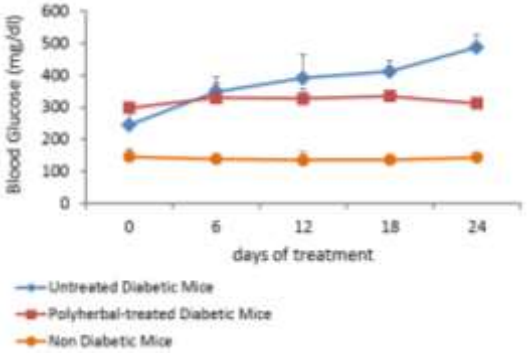

Figure 1. Blood Glucose (mg.dL^{-1}) of Experimental Mice after 24 Days of Treatment.

The anti-hyperglycemic activity of polyherbal extract showed in this study was also supported by the histological observation of the pancreatic islet. The pancreas samples of normal mice showed a granular islet cells with a smooth edge (white arrow), while untreated diabetic mice showed an irregular shaped of a damaged islet which was red in color due to blood infiltration. On the other hand, the pancreas of diabetic mice receiving polyherbal extract treatment was partially recovered. A granular islet can be seen in the pancreas samples from polyherbal-treated mice, though the size was smaller than those from normal mice (Fig. 2).

Figure 2. Photomicrograph of the Pancreas Sections. (A) Non-diabetic Mice, (B) Untreated Diabetic Mice and (c) Polyherbal-treated Diabetic Mice, stained by Hematoxylin and Eosin.

The ability of the polyherbal extract to prevent blood glucose elevation might be resulted from its immunomodulatory properties

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that contribute to pancreatic islet preservation after beta cells was destroyed by STZ toxicity. Previous studies showed that STZ could destroy pancreatic islet through necrosis mechanism [17, 18] followed by islet-specific autoreactivity after cellular debris from β cells were presented on antigen presenting cells [19]. Interestingly, immunomodulatory therapy could restore self-tolerance, causing the suppression of islet-specific autoimmune responses and prevention of β-cell destruction [20]. Moreover, therapy with immuno-modulatory agents is considered as a new prospective strategy that could give beneficial effects in the diabetes treatment [21, 12,13]. The immunomodulatory activity of polyherbal extract in this experiment are discussed in more detail in the subsections below.

Effect of Polyherbal Administration on Relative Amount of Activated T Cells in Diabetic Mice

The relative amount of $CD4^+CD62L^-$ and $CD8^+CD62L^-$ T cells in the diabetic mice were 73.55% and 71.24%, respectively (Fig. 3). These values were significantly higher than that in the healthy mice (52.29% and 47.69%, respectively). The increase of $CD4^+CD62L^-$ and $CD8^+CD62L^-$ T cells relative amount in the diabetic mice indicates that hyperglycemia stimulates activation of both T helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) which were characterized by lost of their L-selectin ($CD62L^-$) from their surface. This result is similar to that found by Rifa'i and Widodo [16] showing an increase of activated T cells in the diabetic mice group. In addition, it has been found that diabetic patients have more pro-inflammatory markers than healthy people [10,11].

Mature T cells are developed in the thymus which is immunologically considered as naïve T cells [22]. After naïve T cells are released from the thymus gland, they circulate between the blood and lymph, making intermediate stops in the secondary lymphoid organs such as the spleen and lymph nodes. In secondary lymphoid organs, naïve T cells will be activated when they encounter mediators for T cell activation. Once T cells are activated, they lose their adhesion molecules L selectin (CD62L) from their surface and induce other immune cells leading to inflammatory state [23,24,25,26].

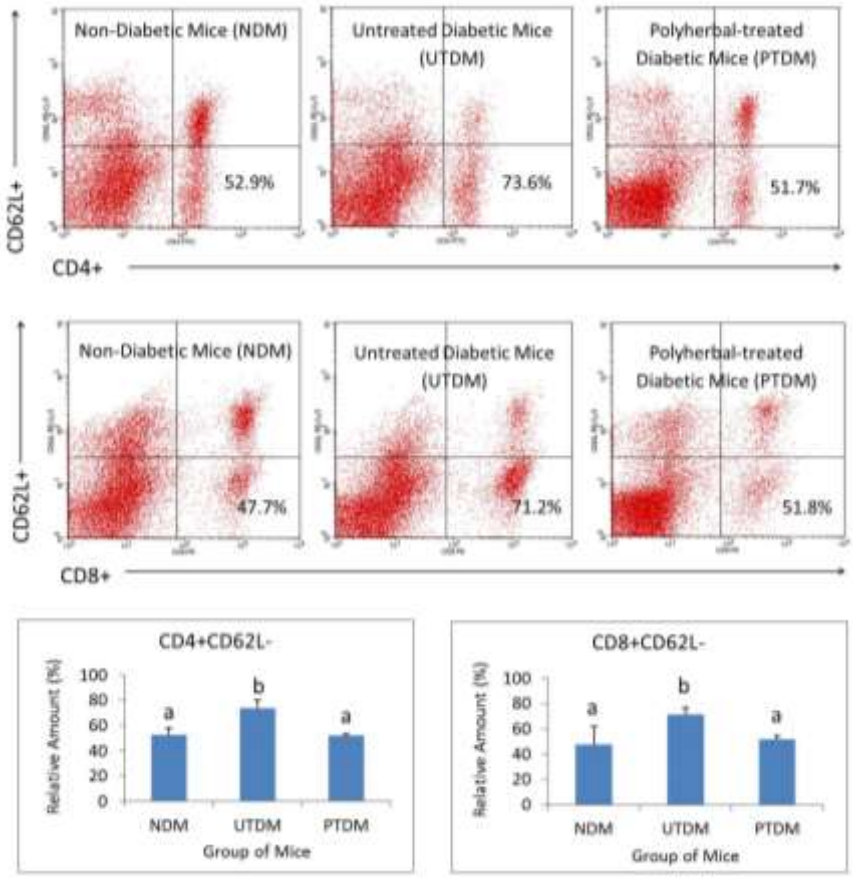

Figure 3. Relative Amount of Activated $CD4^+$ and $CD8^+$ T Cells in Experimental Mice after 24 Days of Treatment

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In the diabetic state, T cells might be activated through hyperglycemia-induced oxidative stress [27] or by exposure of islet-specific antigens [19,28]. Although the precise mechanism is debatable, involvement of T cells auto-reactivity leading to β cell self-destruction is evidenced both in mice and in human [29]. Based on this understanding, some trials have been conducted by targeting of T cells to induce β cell-specific tolerance for diabetes treatment [29,30].

Interestingly, administration of the polyherbal extract could lower the population of activated T cells in diabetic mice to as low as the numbers in healthy mice (Fig. 3). This finding indicates that the polyherbal extract used in this study is a potential source of an immunomodulator, which may lead to benefit in the treatment of diabetes. Possibly, polyherbal suppress T cell activation by decreasing oxidative stress facilitated by antioxidant compounds contained in the polyherbal extract. Alternatively, compounds in the polyherbal extract might stimulate the production of endogenous biological substances that act as immune suppressors such as regulatory T cell (Treg) and anti-inflammatory cytokines.

Effect of Polyherbal Administration on Relative Amount of Regulatory T Cells in Diabetic Mice

Recently, regulatory T cells (Tregs) have become widely accepted as a new tool for understanding DM1 pathogenesis as well as giving new prospects in prevention and treatment of the disease [31]. Earlier studies [32,33] have shown that the balance between effector Th cells and Tregs plays a role in diabetes progression. After the onset of diabetes, autoimmunity progression continuously increases as the increase of ratio between effector Th cells and Tregs occurs within an inflamed pancreas [34].

Tregs are characterized by a high constitutive surface expression of the IL-2 receptor alpha chain (IL2RA), which is also commonly called Cluster of Differentiation (CD)25. In addition, the expression of intracellular forkhead box protein 3(FOXP3) transcription factor is accepted as being the best marker of Treg cells [31,35].

In this study, the polyherbal administration was found to increase the relative amount of $CD4^+CD25^+$ regulatory T cells (Tregs) in STZ-induced diabetic mice. Specifically, $CD4^+CD25^+FoxP3^+$ Tregs level increased more than two-fold when compared to the diabetic control group (Fig. 4). These results indicate that polyherbal extract could stimulate the production of Treg cells. Regarding the important role of Tregs in T1D, boosting Tregs number may provide an effective aspect of diabetes treatment [36,37].

The role of Tregs in diabetes treatment is correlated with its immunomodulatory function which suppresses the excessive responses of immune cells in both innate and adaptive immune systems. Previous research has revealed that CD^+CD25^+Treg could suppress $CD4^+$ and $CD8^+$ T cells proliferation and their pro-inflammatory cytokines production in addition to suppressing their effector activities such as $CD8^+$ T cell cytotoxicity [38]. Various immune cells that are suppressed by Tregs include T cells, B cells, natural killer, macrophage, neutrophils, dendritic cells and mast cells [39]. In this study, the contribution of Tregs to the suppression of T cell activation could be seen in polyherbal-treated diabetic mice as shown in Figure 3.

To our knowledge, this is the first study that showed stimulation of Tregs induction by using polyherbal composed of *S. grandiflora*, *S. zalacca,* and *A. indica*. However, several studies have been done on Tregs induction by using other medicinal herbals. Licorice (the root of *Glycyrrhiza* species) extract, and its two constituents, isoliquiritigenin, and naringenin have been proved to effectively promote Treg cell production both *in vitro* and *in vivo* [40]. Other studies also reported that some medicinal plants and their derivatives, including *Astragalus membranaceus*, *Pterodon emarginatus* Vogel, *Hypericum perforatum*, hyungbangpaedok-san, matrine, Bu Shen Yi Sui Capsule, resveratrol, and curcumin could induce Tregs production and increase its functional activities [41].

Effect of Polyherbal Administration on Anti-inflammatory Cytokines Production

Several mechanisms have been proposed as being explanations of the suppressive effect of regulatory T cells. One of these Tregs suppressive mechanisms is done by anti-inflammatory cytokines secretion. Based on cytokine production, Tregs have been classified as follows: (1) Th3 cells which are characterized by TGF-β production; (2) Tr1 cells which produce IL-10, and (3) Tr35 cells which produce IL-35 [42]. In this study, IL-10 and TGF-β produced by $CD4^+CD25^+$ regulatory T cells in polyherbal treated diabetic mice showed an increase relative to those in the untreated diabetic control and normal control groups (Fig. 5). This result is

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consistent with the finding of an overall increase of Tregs in polyherbal-treated diabetic mice as described above (Fig. 4).

In addition to its suppressive effect, IL-10 also plays a role in the process of cell regeneration. The ability of IL-10 to promote regenerative healing is most likely a result of its synergistic multiple actions including regulation of inflammatory response, endothelial progenitor cells, fibroblast cellular function, and extracellular matrix [43].

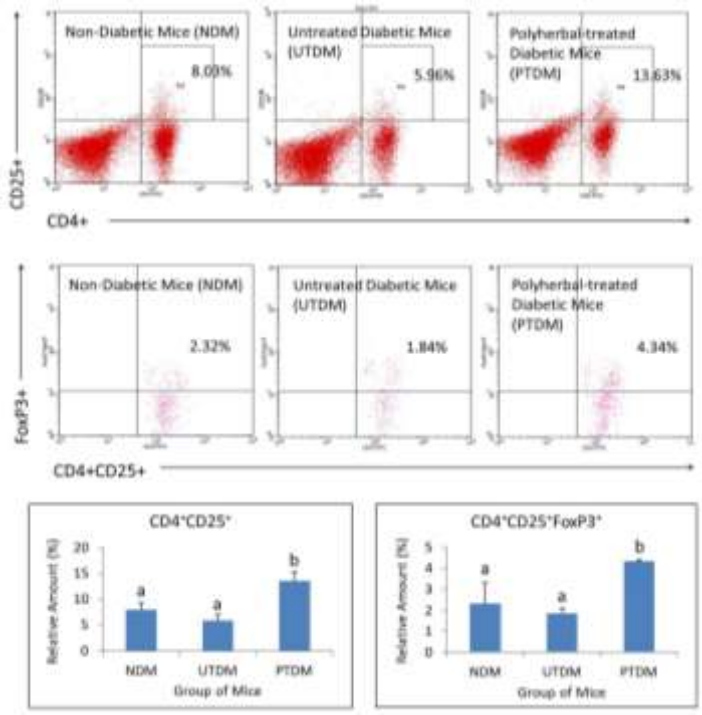

Figure 4. Relative Amount of Regulatory T Cells in Experimental Mice after 24 Days of Treatment

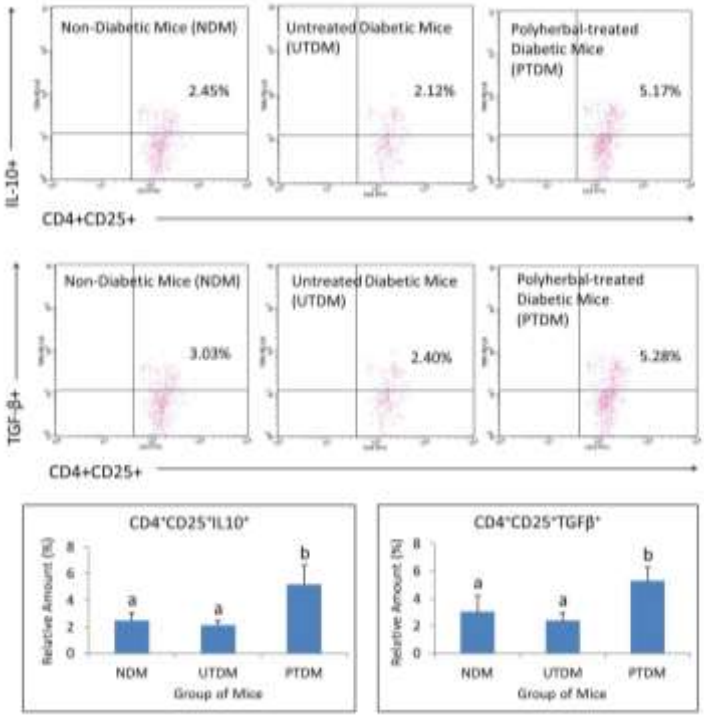

Figure 5. Relative Amount of Pro-inflammatory Cytokines in Experimental Mice after 24 Days of Treatment.

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Endogenous IL-10 limits the severity of fibrosis and glandular atrophy as well as regulates cell regeneration in experimental chronic pancreatitis [44]. Therefore, we suggest that IL-10 might help the process of pancreas regeneration after STZ destruction so that polyherbal- treated diabetic mice could partially recover their islet cells as shown in Figure 2 above. However, more in-depth research is needed to prove this speculation.

CONCLUSION

Water extract from a polyherbal mixture containing *Sesbania grandiflora* seeds, *Salacca zalacca* leaves and *Acalypha indica* roots (2:1:1) was proven to be able to prevent the progression of blood glucose elevation in STZ induced diabetic mice. The pancreatic islet of polyherbal-treated diabetic mice was also found to be partially recovered from the damage of diabetes induction. Furthermore, this study highlighted the significant increase of regulatory T cells and anti-inflammatory cytokines production after polyherbal administration which contributes to lowering the relative amount of activated T cell in diabetic mice. These results revealed that the polyherbal extracts have an anti-hyperglycemic effect and that the polyherbal treatment could modulate the immune system of diabetic mice contributing to the process of controlling hyperglycemia.

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ETHICAL APPROVAL

This experiment has been reviewed and legalized by the Ethics Committee of the Brawijaya University, Indonesia. All procedures performed in this experiment were in accordance with the guidelines and ethical standards of the Ethics Committee of Brawijaya University.

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