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Antihyperglycemic Effect of *Sesbania grandiflora* Seed Decoction on Streptozotocin-induced Diabetic Mice: Inflammatory Status and the Role of Interleukin-10

Ahmad Zamroni[1, a)], Simon B. Widjanarko[2, b)], Muhaimin Rifa'i[3, c)] and Elok Zubaidah[2, d)]

[1]*Department of Technology of Agricultural Product Processing, Samarinda State Polytechnique of Agriculture, Jalan Samratulangi, Samarinda, Indonesia*
[2] *Technology of Agricultural Product Department, Brawijaya University, Jalan Veteran, Malang 65145, Indonesia*
[3] *Biology Department, Brawijaya University, Jalan Veteran, Malang 65145, Indonesia*

Corresponding author:
[a)]zam.ahzami@gmail.com, [b)]simonbw@ub.ac.id
[c)]rifa123@ub.ac.id, [d)]elzoeba@yahoo.com

Abstract. Diabetes is one of the fastest growing diseases in the world: its prevalence is estimated to reach 642 million people, or one-tenth of adults will have diabetes by 2040. Traditional herbal exploration and investigation are needed in order to discover medicines that have potential anti-diabetic activity, with no or lower side effects than the medicines clinically used today. In this research, we investigated the anti-hyperglycemic activity of an aqueous decoction of *Sesbania grandiflora* seeds in streptozotocin-induced diabetic mice, and analyzed the immune responses that occurred during the counter balance process to reach blood glucose homeostasis. Our results revealed that administration of the aqueous decoction (2.5 g/kg BW) could lower the blood glucose levels of diabetic mice from an initial blood glucose level of 435 mg/dl to 213 mg/dl within 18 days of treatment. Analysis of inflammatory markers showed that there was no significant difference in the relative amounts of $CD4^+CD62L^-$, $CD8^+CD62L^-$, TNF-α or IFN-γ between the experimental groups, which revealed that there were no pro-inflammatory responses involved either in hyperglycemia or in the blood glucose lowering process. On the other hand, an increased amount of interleukin-10 in diabetic mice treated with an *S. grandiflora* seed decoction indicated a role for IL-10 in maintaining blood glucose homeostasis.

INTRODUCTION

Diabetes is a metabolic disorder characterized by hyperglycemia[1] due to insufficient production of insulin or inadequate peripheral tissue response to physiological levels of insulin.[2] The International Diabetes Federation (IDF) estimated that the total number of people with diabetes in the world is 415 million in 2015 and that it will increase to 642 million people in 2040.[3] Over time, diabetes can damage the heart, blood vessels, eyes, kidneys, nerves, and increase the risk of stroke. Diabetes and its complications are also a major cause of mortality.[4]

Several anti-diabetic drugs have been developed, but most drugs clinically used today tend to cause undesirable side effects.[5] Furthermore, there have been no reported cases of complete recovery from diabetes.[6] On the other hand, many people claim to have recovered from diabetes by using traditional remedies. Unfortunately, their claims have not been supported by scientific evidence. Therefore, there have been increased attempts to search for effective and safe anti-diabetic agents mainly from herbal plants used in traditional medications. Recently, the World Health Organization (WHO) recommended the use of medicinal plants for the management of diabetes mellitus (DM) and further encouraged expanding the scientific evaluation of hypoglycemic properties of diverse plant species.[7]

Over the last decade, much evidence has shown a close link between metabolism and immunity.[8] Inflammation has been indicated to be involved in insulin resistance[9] and several diabetic complications.[10] Also, pro-inflammatory

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cytokines, such as IL-6, TNF-α, IL-18, and IFN-γ, were found at a higher level in diabetic patients than in healthy people.[11-12] The emerging understanding of correlations between the immune system and the pathogenesis of diabetes brings a new approach in diabetes treatment, named immunomodulatory therapy.[13]

Among several immunomodulatory agents tested, interleukin 10 (IL-10) was assessed as a cytokine that could be used in the treatment of diabetes. IL-10 has a protective effect in type 1 diabetes[14-15] and type 2 diabetes.[16] Therefore, searching for herbal remedies capable of accelerating IL-10 production could be a rational strategy in the immunomodulatory therapy of diabetes treatment.

S. grandiflora seeds have been used as an anti-diabetic medicine by a traditional herbal practitioner who has been more than 20 years of experience in composing traditional herbal medicines. In this research, we examined the anti-hyperglycemic activity of an *S. grandiflora* seed decoction in streptozotocin (STZ)-induced diabetic mouse. In order to evaluate the mechanism, we investigated changes that occurred in the immune system along with a decrease in blood glucose. Specifically, the analysis focused on inflammation markers and IL-10 relative amounts. To our knowledge, this is the first publication that showed the anti-diabetic activity of *S. grandiflora*.

MATERIALS AND METHODS

Material

S. grandiflora seeds were obtained from a local medicinal herbal practitioner who has experienced making various traditional herbal medicines for more than 20 years in Malang City, East Java Province, Indonesia. Streptozotocin was purchased from BioWORLD (GeneLinx International, Inc., USA). The glucometer and its test strips were manufactured by General Electric (GE), USA.

Animals and Treatment

Animals used in this research were eight-week-old female Balb/C mice, which were maintained in the pathogen-free facility, Biology Department, Faculty of Sciences, Brawijaya University, Malang, Indonesia. Mice were divided into four groups: healthy mice (negative control), diabetic mice (positive control), diabetic mice with decoction administration at 0.5 mg/g BW (dose 1) and mice with decoction administration at 2.5 mg/g BW (dose 2). The decoction was administered to the diabetic mice every day orally by force-feeding for 18 days. Blood glucose was measured every three days using a glucometer. On the 19th day, mice were sacrificed and the spleen was isolated. Cell surface molecules and intracellular cytokine of T cells were then analyzed by FACSCalibur ^{TM} flow-cytometer.

Induction of Diabetes in Mice

Induction of diabetes was done by intraperitoneal injection (150 mg/kg) of streptozotocin (STZ) into normal mice. STZ solution was freshly prepared by dissolving the appropriate amount of STZ into Na-citrate buffer (50 mM, pH 4.5) to achieve a final concentration of STZ of 20 mg/mL. The mouse was fasted for 4 h prior to injection in order to optimize STZ absorption.[17] Diabetes was confirmed three and six days after injection by measurement of blood glucose levels using the glucometer (General Electric/GE, USA) where only mice with glucose levels higher than 300 mg/dl were selected for the study.

Preparation of Decoction

The decoction was prepared based on a method commonly used by traditional medicine practitioner in Malang City, East Java Province, Indonesia. Seeds were dried in a hot air oven dryer (180ºC) for 1 h and then ground to get *S. grandiflora* seed powder. An appropriate volume of hot boiled water was added into an appropriate amount of seed powder, mixed well, and kept until the solid material settled. The aqueous phase was carefully separated from the solid material. The aqueous fraction of the *S. grandiflora* seed decoction was used directly in this study.

Isolation of Lymphoid Cells and Flow Cytometry Analysis

Mouse spleen was washed with sterile PBS twice and placed on a petri dish containing sterile PBS. The spleen was 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 up to 10 mL and then centrifuged at 2500 rpm at 4°C for five min. 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 CD62L.[18]

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 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 anti-mouse anti-IFN-γ, anti-mouse anti TNF-α and anti-mouse anti-interleukin-10 (IL-10) for 30 min.[18]

RESULTS AND DISCUSSION

Effect of *S. grandiflora* Seed Decoction on Blood Glucose of Diabetic Mice

STZ (2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is a natural compound produced by *Streptomyces achromogenes* that are widely used as the diabetogenic agent. The structure of STZ is similar to glucose, so that it is able to enter beta-cells of the pancreas through the glucose transporter GLUT 2. Once STZ enters the cells, it is metabolized, and thus produces free radicals that lead to the damage of pancreatic beta-cells.[19] In this research, hyperglycemia in Balb/C mouse was detected after a single intraperitoneal STZ injection (150 mg/kg BW) where high blood glucose (>300 mg/dl) was seen from the third day post-injection and then reached more than 400 mg/dl on the sixth day post-injection (Fig. 1). On the seventh day post-injection, diabetic mice were administered with the decoction for 18 days.

Administration of the decoction at 2.5 g/kg BW for 18 days lowered blood glucose of diabetic mice from an initial blood glucose of 435 mg/dl to 213 mg/dl. On the other hand, the diabetic control without treatment showed increased blood glucose from an initial level of 462 mg/dl to 512 mg/dl (Fig. 2). These results indicate that *S. grandiflora* has anti-hyperglycemic activity and it could potentially be used in the treatment of diabetes.

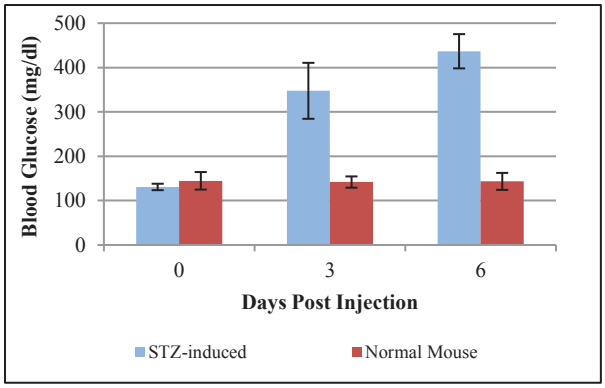

FIGURE 1. Blood Glucose (mg/dl) of female Balb/c Mouse after STZ Injection at 150 mg/kg BW

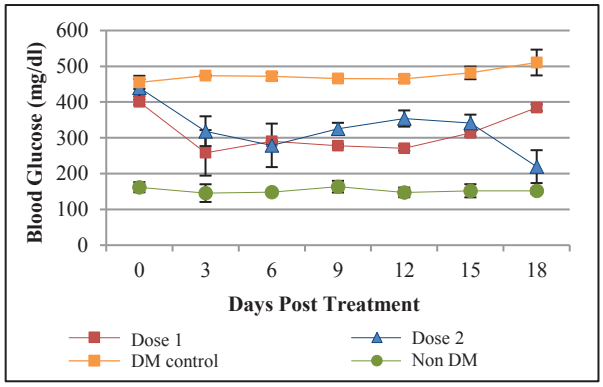

FIGURE 2. Blood Glucose (mg/dl) of STZ-induced Diabetic Mouse after Administration of *S. grandiflora* Seeds Decoction for 18 days

Effect of Hyperglycemia and Administration of *S. grandiflora* Seed Decoction in Pro-inflammatory Responses

CD62L (L-selectin) is a molecule expressed on most circulating leucocytes and mediates leucocyte rolling on endothellium at sites of inflammation.[20] Naive T-cells express high surface levels of L-selectin. After naive T-cells become activated by exposure to an antigen, they develop an immune response. Activated cells rapidly divide and differentiate into L-selectin low effector cells, which home to inflammatory sites and induce pathogen clearance. After successful elimination of the antigen, most T-cells undergo apoptosis, but some develop into memory cells.[21]

Activated T-cells in this experiment were marked by $CD62L^-$ which, when in negative uppercase, means that a T-cell has lost CD62L (L-selectin) from its surface. Indeed, the more T-$cell^+CD62L^-$ content, the more activated T-cells found relative to the total number of splenocytes. As shown in Fig. 3a and Fig. 3b, relative amounts of either $CD4^+CD62L^-$ or $CD8^+CD62L^-$ were not significantly different between normal mice and diabetic mice. This result indicated that hyperglycemia did not induce activation of either $CD4^+$ or $CD8^+$ in STZ-induced diabetic mice. Moreover, decoction administration did not alter the percentage of activated T-cells.

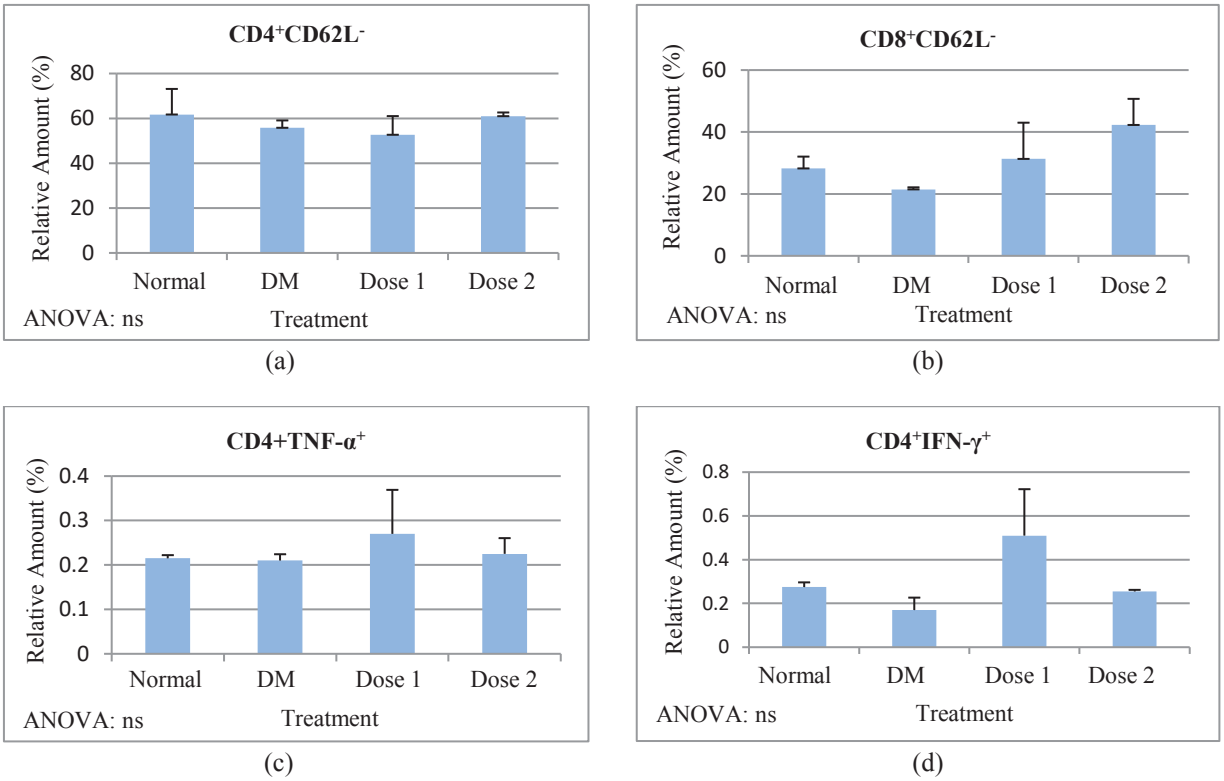

FIGURE 3. Effect of Administration of *S. grandiflora* Seeds Decoction on T-cells Activation and Relative Amount of Cytokines TNF-α and IFN-γ in STZ-induced Diabetic Mouse.
The data were analyzed by Analysis of Variance (ANOVA) test.

This finding contradicted previous research that found an increase of activated CD8 in diabetic mice compared to normal mice.[18] Possibly, our results showed a different tendency because we used an STZ injection method to induce the type 1 diabetic mouse model, while Rifa'i *et al.*[18] used S961 peptide administration which resulted in type 2 diabetes. Furthermore, there is no explanation for the mechanism of how hyperglycemia could induce T-cell activation. Thus, a direct link between hyperglycemia and activation of T-cells remains speculative and unclear.

Activation of T-cells by a high blood glucose content could be mediated by a ROS-induced pro-inflammatory response. Due to increased delivery of glucose to adipose tissue, endothelial cells in the fat may take up increasing amounts of glucose molecules, which may lead to increased glucose metabolism. A high rate of glucose metabolism leads to excess ROS production in adipocytes, which then leads to increased production of pro-inflammatory cytokines.[8] Recent studies indicate that pro-inflammatory cytokines were a known third signal for T-cell

activation.[22] However, this mechanism is more common in over-nutrition cases, rather than diabetic hyperglycemia cases. In STZ-induced diabetic mice, hyperglycemia may not increase intra-cell glucose metabolism because glucose molecules are not able to enter cells in the absence of insulin. As a result, both ROS accumulation and inflammation responses would not occur.

In parallel with T-cell activation, we clearly found that pro-inflammatory cytokines (TNF-α dan IFN-γ) were not significantly different between normal and diabetic mice (Fig. 3c and Fig. 3d). This finding supported our speculation that hyperglycemia did not induce either T-cell activation or inflammatory responses in STZ-induced diabetic mice.

While it was clear that inflammation could induce hyperglycemia via insulin resistance[9], the contrary link between hyperglycemia and inflammation is still in debate. Esposito *et al.*[11] found that pro-inflammatory cytokines were higher in hyperglycemic patients than in normal people, even though there was no explanation about the mechanism. Conversely, Wellen and Hotamisligil[8] stated that hyperglycemia could not induce inflammation. Indeed, they argued that the available evidence strongly suggested that type 2 diabetes is an inflammatory disease, and that inflammation is a primary cause of obesity-linked insulin resistance, hyperglycemia, and hyperlipidemia rather than merely a consequence.

Administration of the decoction did not alter the relative amount of TNF-α or IFN-γ compared to normal mice (Fig. 3c and Fig. 3d). This evidence showed that the *Sesbania grandiflora* seed decoction did not disturb immune cell homeostasis. On the other hand, this also indicated that the decrease of blood glucose in STZ-induced diabetic mice did not involve regulation of either T-cell activation or TNF-α and IFN-γ secretion.

Effect of *S. grandiflora* Seed Decoction on Relative Amount of Interleukin-10

Administration of the *S. grandiflora* seed decoction to diabetic mice significantly increased the relative amount of IL-10 (Fig. 4). This evidence indicated that IL-10 was possibly involved in the process of lowering the blood glucose in diabetic mice. This finding was supported by other researchers who have also reported the role of IL-10 in maintaining blood glucose homeostasis.[23-24] In particular, Goudy *et al.*[14] and Goudy *et al.*[15] found that IL-10 could be used to prevent type 1 diabetes in mice. The role of IL-10 in protection from type 2 diabetes was reported by Bary *et al.*[16]

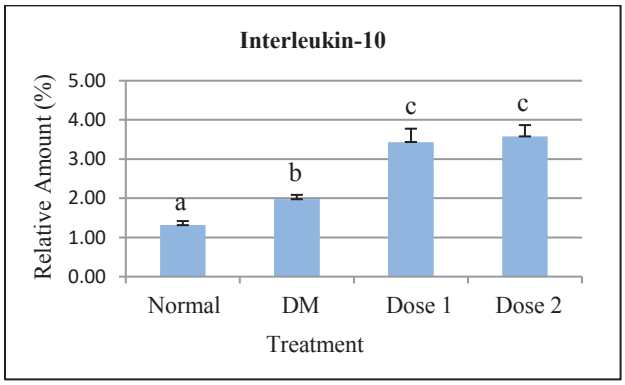

FIGURE 4. Effect of *S. Grandiflora* Seeds Decoction in Relative Amount of Interleukin-10 in STZ-induced Diabetic Mouse. The data were analized by Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT).

The mode of action of IL-10 in preventing diabetes was mostly linked to its properties as an anti-inflammatory cytokine [14, 15, 16, 23, 24]. However, the decrease of blood glucose in this research was not likely related to anti-inflammatory activity of IL-10, since no inflammation occurred in any of the experimental groups. Hence, IL-10 was involved in lowering blood glucose through other mechanisms.

We suggest that IL-10 may help the process of pancreatic beta-cell regeneration. Previous work has also shown that IL-10 was involved in the process of cell regeneration, such as in lung repair during influenza infection[25] or regenerative wound healing in the fetal skin.[26] King *et al.*[27] explained that the ability of IL-10 to facilitate regenerative healing is likely a result of pleiotropic effects, through regulation of the inflammatory response, as well as novel roles as a regulator of the extracellular matrix, fibroblast cellular function, and endothelial progenitor cells.

However, there is not enough data to conclude that IL-10 plays a role in pancreatic beta-cell regeneration. Hence, further investigations are needed to prove this speculation.

CONCLUSION

In summary, we found that the *S. grandiflora* seed decoction (2.5 g/kg BW) could lower blood glucose of STZ-induced diabetic mice. There were no pro-inflammatory responses involved either in the hyperglycemic state or in the blood glucose lowering process, as indicated by no significant difference in the relative amounts of $CD4^+CD62L^-$, $CD8^+CD62L^-$, TNF-α and IFN-γ between experimental groups. On the other hand, relative amounts of interleukin-10 were increased significantly, indicating the role of IL-10 in maintaining blood glucose homeostasis. However, there is not enough data to explain how IL-10 was related to lowering blood glucose; thus, further investigations are needed to clarify the mechanism.

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