The Effect of *Moringa oleifera* Leaves and VipAlbumin® on The Immune System of Diabetes Mellitus Balb/C Mice Model

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Abstract

Diabetes Mellitus is known as a disease grown worldwide rapidly prevalent day by day. This disease caused by a chronic hyperglycemic condition and also glucose intolerance resulting from defects in insulin secretion, insulin action, or both. *Moringa oleifera* is one of the plants used in most developing countries for traditional medicine for treating diabetes. In this research, *Moringa oleifera* mixed with VipAlbumin®, taken from snakehead fish albumin. Albumin found as an antioxidant against ROS. This research aimed to evaluate the effect of *Moringa oleifera* mixed with VipAlbumin® supplement towards the profile changing of T-cell, (CD4+ CD8+) B220+, CD4+ IFN-γ, and TNF-α. The experiments were done by dividing Balb/c mice into five groups and induced with 100 mg.BW^{-1} STZ. Afterward, the mix of Moringa oleifera and VipAlbumin® orally administrated into five different doses. Negative control contains of healthy mice, Positive dose administrated with 145 mg.kg bw^{-1} STZ, Dose 1 of 100 mg.kg bw^{-1} (Mo) + 416.25 mg.kg bw^{-1} (A), Dose 2 of 150 mg.kg bw^{-1} (Mo) + 208.15 mg.kg bw^{-1} (A), Dose 3 of 50 mg.kg bw^{-1} (Mo) + 624.375 mg.kg bw^{-1} (A). One way ANOVA was applied to analyze the data with p-value 0.05% and combined with Tukey test using SPSS version 16 for Windows. The results showed that a relative number of CD4 and CD8 T cells decreased in dose 3 of *Moringa oleifera* and Albumin, as well as B220 in dose 3 gave a significant decreased compared to healthy mice ($p<0.05$). The inflammation showed decreasing after treatment with dose 3 of *Moringa oleifera* and albumin extract. Taken together that proinflammatory cytokines decreased after treatment compared to a positive group.

Keywords: Albumin, Inflammation, *Moringa oleifera*, T cells.

INTRODUCTION

Diabetes Mellitus (DM) is one of the autoimmune disease caused by the destruction and secretion of insulin production in pancreatic β-cells [1]. There are two types of DM. Insulin Dependent DM (IDDM) is known caused by a lack of insulin secretion by β-cells of the pancreas. While on the other hand, Non-Insulin Dependent DM (NIDDM) caused by decreased sensitivity to insulin of target tissues or cells, which often called insulin resistance (IR).

Inflammation was first linked to insulin resistance and diabetes in the early 1990s and could increase TNF-α in adipose tissue [2]. Inflammatory cytokines such as IL-1β and IFN-γ, which increased in obesity, also modulate insulin signaling. Furthermore, previous research asserts macrophages are the primary inflammatory cell type in the glucose utilizing tissues such as adipose tissue and liver [3].

Obesity and systemic inflammation are known to induce insulin resistance. The sign could be seen mostly in the adipose tissue, which releases fatty acids, adipokines, and other cytokines with the capability to downstream effects on the muscle and liver [4].

Another feature of insulin resistance is an increased release of free fatty acid (FFA) [5]. Obesity, characterized as a state of chronic low-grade inflammation caused by overnutrition, is a major cause of decreased insulin sensitivity, which makes obesity a major risk factor for IR.

Tumor Necroses Factor-α (TNF-α) reported by as a proinflammatory cytokine that induces insulin resistance [4,6]. Proinflammatory molecules such as leptin, monocyte, resistin, visfatin, interleukin-6 (IL-6), chemoattractant protein-1 (MCP-1), and (TNF-α), are expressed at high levels in macrophages and other cells when activated [7].

Nowadays, it is estimated that more than 80% of people in developing countries use medicinal herbs to maintain their health [8]. They use of plants in the diabetic management provides better alternatives as they are less toxic, readily available, and affordable. Since there is the fact that oxidant stress involved in diabetes, this research used herbals, which comprise anti-diabetic and also antioxidant.

One of the plants used for traditional medicine for treating diabetes in developing countries is *Moringa oleifera.* The efficacy of *Moringa oleifera* has been reported by many researchers. The root of *M. oleifera* was able to reduce stone forming constituents in the kidneys and also reduce the elevated urinary oxalate of calculogenic rats as a result of ethylene glycol treatment [9]. The positive effect of ethanolic leaf extract to insulin resistance and beta-cell

function in hyperglycemia HFD/STZ which induced to diabetic mice has also reported [10].

Reactive Oxygen Species (ROS) will increase in the individual suffering from hyperglycemia and lead to oxidative stress [11]. However, an exogenous antioxidant from *M. oleifera* can overcome oxidative stress. Another strong antioxidant can be obtained from snakehead fish *(Channa striata*) [11]. This fish albumin found to act as an antioxidant and also will overflow group (-SH), which serves as a binder radical that plays a role in the arrest of ROS.

This research used VipAlbumin® as a supplement from snakehead fish *(Ophiocephalus stiatus)* with a high content of albumin compared to the other kinds of fish. One of the albumin's benefits is as antiinflammation and antioxidant. Therefore, this study conducted to determine the effect of VipAlbumin® in change quantitative T cell lymphocytes and a decrease in blood glucose levels [12]. This research was aimed to analyze the influence *M. oleifera* and Albumin extract towards the pro-inflammation cytokine such as TNF-α, IFN-γ, and IL-6 in diabetics Balb/c mice.

MATERIALS AND METHODS

STZ Induction

Female Balb/c mice (n=25, 6-7 weeks old, body weight ±25-30 g) were acclimatized for seven days and fed with standard feed *ad libitum*. The mice assigned into five groups. There are negative control, positive DM, Dose 1, Dose 2, and Dose 3. Each group will have five times of repeated treatment. The mice in the DM group and D1, D2, and D3 intraperitoneally injected with STZ at a dose of 145 $mg.kg^{-1}$ of body weight. Afterward, mice were kept for 14 days. In the incubation period, blood glucose was checked every three days after STZ induced. Mice were reported to suffer from type 2 diabetes when its blood glucose levels exceed 200 $mg.dL^{-1}$.

Oral Administration of *M. oleifera* and VipAlbumin®

Moringa oleifera samples taken from leaves (100 g) were dried and powdered using a blender then mix with 100 mL of distilled water for 24 hours and then stored at 4°C. Afterward, the mixture was filtered twice through a 2-µm pore filter paper, then stored at 4°C for five days. Albumin extract using VipAlbumin®, which contains 500 mg of Snakehead fish extract and 30,20% albumin, and also vitamin A, vitamin D, and calcium.

The female balb/c mice divided into five different groups following the oral administration of *M. oleifera* and VipAlbumin®. The five groups are as follows (Table 1).

Table 1. Five Groups of Balb/c Mice

Group	($mg.kg^{-1}$ Body Weight)		
	STZ injection	*M. oleifera* extract	VipAlbumin®
Normal	-	-	-
DM	145	-	-
Dose 1 (D1)	145	100	416.25
Dose 2 (D2)	145	150	208.15
Dose 3 (D3)	145	50	624.375

Notes: Normal = healthy mice (negative control); DM= mice with diabetes mellitus by STZ Injection (positive control).

Spleen Cells Isolation

The mice were killed by dislocation in their neck. The spleen was separated from the mice's body and washed with sterile PBS solution and laying down on the sterile Petri dish containing 2 mL of sterile PBS. Afterward, the puree spleens were taken with the spuit in one way. The homogenates were strained and put it in the 15 mL of sterile propylene tubes, and centrifuged at 2500 rpm for 5 minutes at 4°C. Finally, the pellet resuspended 1 mL of sterile PBS.

Flowcytometry Analysis

To analyze the mixture of *M. oleifera* and Albumin extract against pro-inflammatory cytokines, the isolated cells were taken for 200 µL and placed on the sterile microtubes and centrifugated at 2500 rpm for 5 minutes at 4°C. Subsequently, the supernatant was separated and added with 40 µL of antibody staining and incubated for 15-20 minutes in the icebox with the dark condition. The cells then were added with 300 µL of PBS sterile and placed into flow cytometry for analysis.

Data Analyis

The data were analyzed using one-way ANOVA with significance level $p < 0.05$ using SPSS to determine the relationship between variables, and then continued with a Tukey test to find the most involving variable.

RESULTS AND DISCUSSION

Moringa oleifera and Albumin Decreased the Relative Number of T Cells

The administration of *M. oleifera* and Albumin against DM on the Balb/c mice showed no significant result. The relative number of CD4+ CD8+ increased compared to DM positive control. This research tested three groups of different doses of *M. oleifera* and VipAlbumin® and compared them to negative control and DM

positive control. We found that Dose 1 contains 100 mg.kg^{-1} body weight *M. oleifera* + 416.25 mg.kg^{-1} bodyweight VipAlbumin® gave a result ($p<0.05$), but it is not significant compared to DM positive control (Fig. 1).

The number of CD4+CD8+ cells on the positive control number showed an increasing number ($p<0.05$) compared to healthy mice from 1.84% to 2.52%. Relatively, which given *M. oleifera* and VipAlbumin® orally showed no significant decrease. Dose 1 became 1.43%, Dose 2 became 1.87%, and Dose 3 became 1.56% (Fig. 1). Based on the result shown by flowcytometry analysis, the conclusion is, *M. oleifera* and VipAlbumin® did not give a specific result to reduce the relative number of CD4+ CD8+.

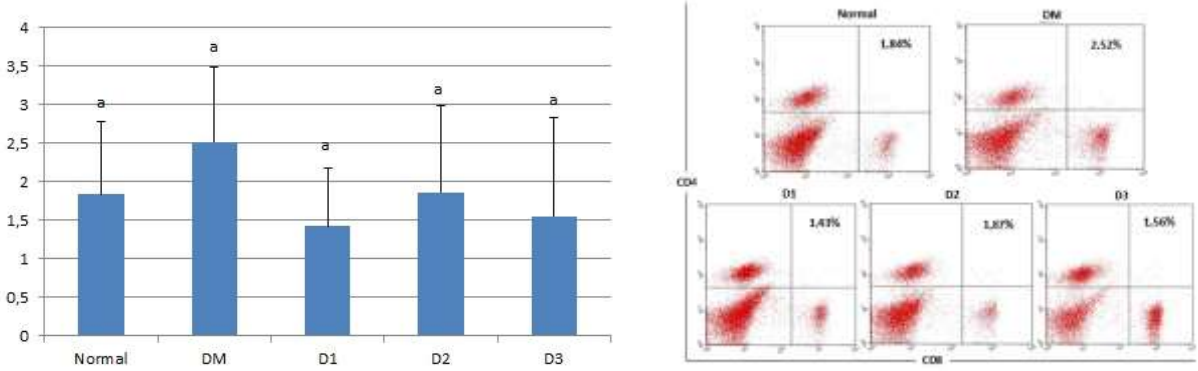

Figure 1. The Administration of *Moringa oleifera* and Albumin increased a relatively number but not significant of T cells. **Normal** : a healthy group, **DM** is positive control induced with 145 mg.kg^{-1} body weight STZ, **D1** : dose one with STZ injection and 100 mg.kg^{-1} *Moringa oleifera* + 416.25 mg.kg^{-1} VipAlbumin®, **D2** : dose 2 with STZ injection and 150 mg.kg^{-1} *Moringa oleifera* + 208.15 mg/kg VipAlbumin®, **D3** : dose 3 with STZ injection and 50 mg.kg^{-1} *Moringa oleifera* + 624.375 mg.kg^{-1} VipAlbumin®.

Moringa oleifera and Albumin Suppressed the Pro-Inflammatory Cytokines

As a pro-inflammatory role, TNF-α is a contributor to β-cell destruction. The result showed a decrease in inflammation of TNF-α produced by CD4 in healthy mice was 3,76%. After being injected by STZ, the number of inflammation of TNF-α produced by CD4 increased became 4.21% but not significant ($p > 0.05$) (Fig. 2b).

The administration of *Moringa oleifera* and VipAlbumin® in D1, D2, and D3 groups decreased the inflammation number significantly ($p < 0.05$). The result showed that the inflammation number of TNF-α produced by CD4 T cells for Dose 1 became 1.95%, Dose 2 became 3.47%, and Dose 3 became 0.96% compared to DM positive, which 4.21%.

IFN-γ produced by CD4 T cells seems important in susceptibility and progression of DM. This study result showed a decrease in the inflamation number of IFN-γ in control positive DM compared to healthy mice (normal). The inflammation number of healthy mice was 2.43% became 2.05% but not significant ($p>0.05$).

The inflammation number of changed following the administration of *M. oleifera* and VipAlbumin® showed both a decrease and an increase. A significant decrease of inflammation number of IFN-γ produced by CD4 T cells was observed in D1 ($p<0.05$) compared to DM positive control was 1.39%. While in D2, it has an increased inflammation number but not significant ($p>0.05$) DM positive control was 2.3%. For D3, it has a decrease inflammation number significantly ($p<0.05$) compared to DM positive control was 1.56%.

Moringa oleifera and Albumin Decreased the Relative Number of T Cells

Flowcytometry analysis results for B220 cells showed both a significant increase and a decrease (Fig. 3). The result found that the relative number of B220 cells of the positive control DM was 8.6% compared to healthy mice 12.82%. The relative number of B220 cells showed significant ($p<0.05$) difference on both decrease and increase result after given *M. oleifera* and VipAlbumin® orally. A significant increase observed on D1 ($p>0.05$) compared to DM positive control was 10.03%. While on D2, a significant increase observed and compared to DM positive control, was 23.67%. On D3, a different result showed. A significant decrease observed on D3 ($p<0.05$) compared to DM positive control was 4.2%.

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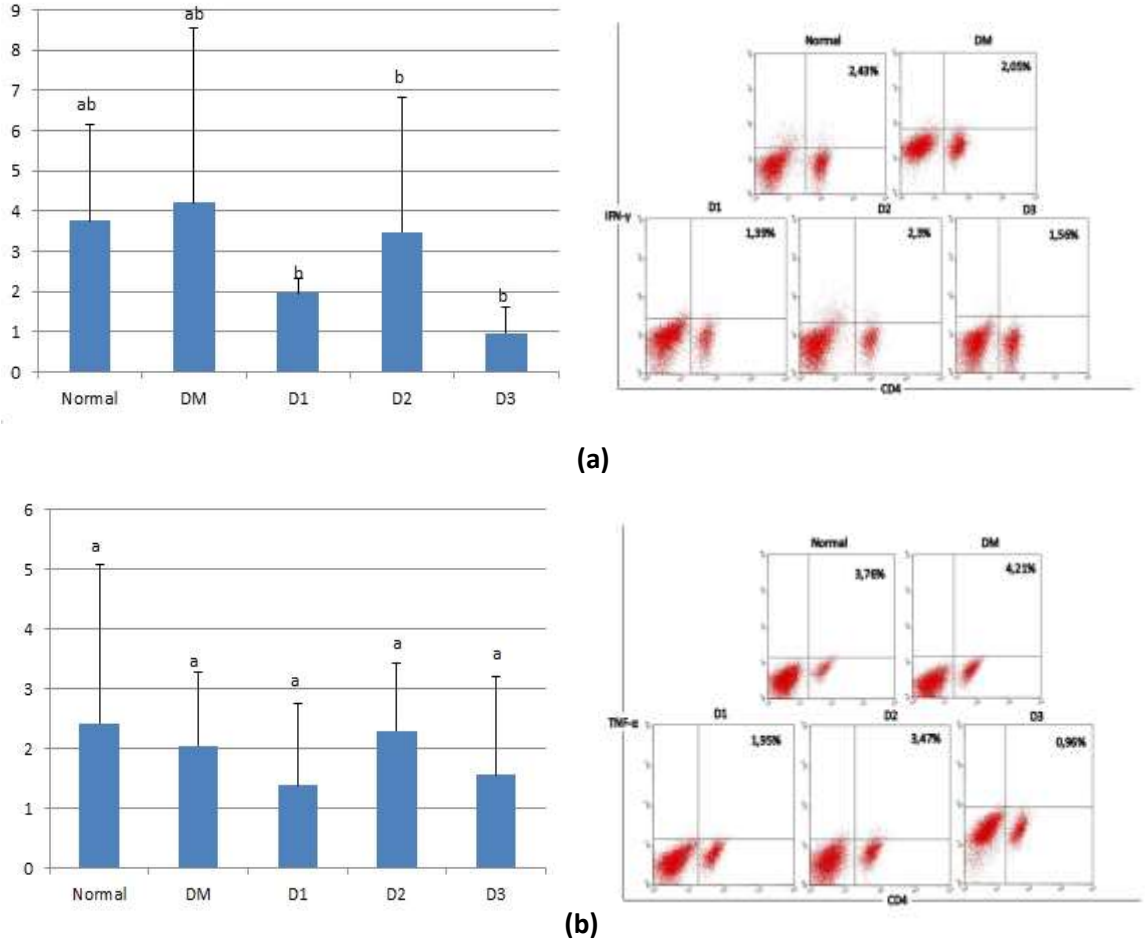

Figure 2. (a) Production profile of TNF-α Cytokines by CD4 cells with T cell CD4 TNF-α total percentage. (b) Production profile of IFN-γ Cytokines by CD4 cells with CD4 IFN-γ total percentage. (**Normal** : a healthy group, **DM** is positive control induced with 145 mg.kg^{-1} body weight STZ, **D1** : dose one with STZ injection and 100 mg.kg^{-1} *Moringa oleifera* + 416.25 mg.kg^{-1} VipAlbumin®, **D2** : dose 2 with STZ injection and 150 mg.kg^{-1} *Moringa oleifera* + 208.15 mg/kg VipAlbumin®, **D3** : dose 3 with STZ injection and 50 mg.kg^{-1} *Moringa oleifera* + 624.375 mg.kg^{-1} VipAlbumin®)

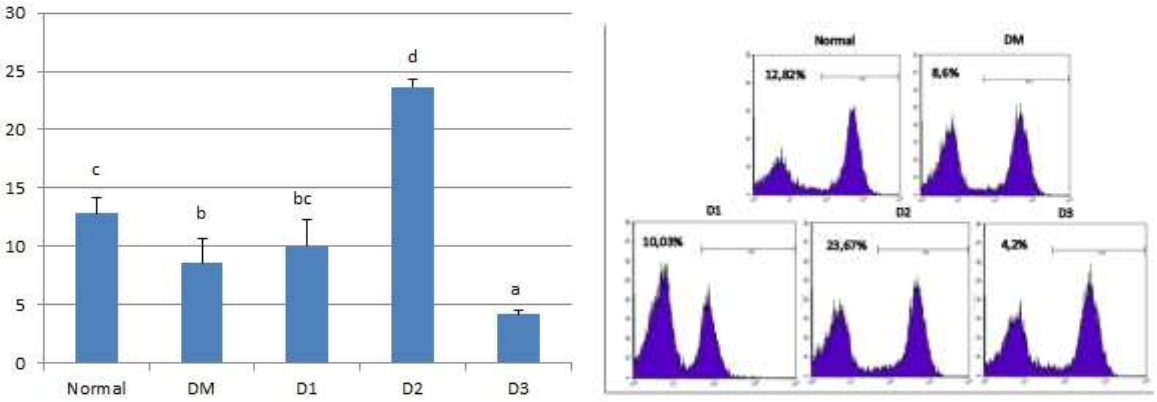

Figure 3. Result graphic calculation for B220. (**Normal** : a healthy group, **DM** is positive control induced with 145 mg.kg^{-1} body weight STZ, **D1** : dose one with STZ injection and 100 mg.kg^{-1} *Moringa oleifera* + 416.25 mg.kg^{-1} VipAlbumin®, **D2** : dose 2 with STZ injection and 150 mg.kg^{-1} *Moringa oleifera* + 208.15 mg/kg VipAlbumin®, **D3** : dose 3 with STZ injection and 50 mg.kg^{-1} *Moringa oleifera* + 624.375 mg.kg^{-1} VipAlbumin®)

Discussion

This study used *M. oleifera* and Albumin as anti-diabetics to reduce inflammation and blood glucose levels on a diabetic Balb/c mice model. *Moringa oleifera* contains many important biological substances such as flavonoid pigments like kaempferol, rhamnetin, isoquercitrin, and kaempferitrin.

Several phytochemicals become a particular interest because of their medicinal function [13]. Phytochemical investigations of *M. oleifera* have revealed the presence of 4-(4'-o-acetyl-α-L-rhamnopyranosyloxy) benzyl isothiocyanate, niazimicin, pterygospermin, benzyl isothio-cyanate, and 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolates [14].

Furthermore, the previous study also reported that flavonoids act as insulin mimetic or insulin secretagogues by influencing the pleiotropic [14]. They also proposed that Albumin is necessary for the proper distribution of body fluids between intravascular compartments and body tissues and functions as a plasma carrier by binding several hydrophobic hormones. Albumin can maintain the blood from leaking out from blood capillaries, after being treated by STZ.

Lymphocyte cells, such as CD4+CD8+, has been known to play an essential role in obesity and obesity-induced insulin resistance [3]. Furthermore, this research explained that $CD4^+$ effector T cells could be further divided into pro-inflammatory Th17, Th1, Th2, and regulatory T cells ($CD4^+CD25^+Foxp3^+$). This division is closely related to its functionality and the production of cytokine. Th1 cells could produce interleukin-2 (IL-2) and tumor necrosis factor-beta (TNF-β), interferon-gamma (IFN-γ), triggering phagocyte-dependent inflammation and cell-mediated immunity. CD4 T cells produce TNF-α molecule as potent inflammatory mediator. TNF-α also produced by monocytes, macrophages, $CD8^+$ T cells, B cells, endothelial cells, NK cells, and lymphokine-activated killer (LAK) cells [15].

Interferon-γ produced by lymphocytes as a result of the activation by specific antigens or mitogens. INF-γ is a potent activator of macrophages, and thus, it has important immunoregulatory functions [2].

The result of this study showed that the relative number of T cells decreased significantly ($p<0.05$) after the administration of *M. oleifera* and VipAlbumin® compared to DM positive control from 2.52% to 1.43% for Dose 1. The administration of *M. oleifera* and VipAlbumin® also decreased pro-inflammatory cytokines significantly ($p<0.05$). This study showed that TNF-α produced by CD4 T cells decreased compared to DM positive control in Dose 3, from 4.22% to 0.96%.

Meanwhile, IFN-γ produced by CD4 T cells gave decrease results compared to DM positive control in Dose 1, which contains 100 $mg.kg^{-1}$ body weight *Moringa oleifera* + 416.25 $mg.kg^{-1}$ body weight Albumin, from 2.05% to 1.39%. On the other hand, the IL-6 which is produced by isolated mouse islets after being exposed to interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) gave a significant result after the administration of *M. oleifera* and Albumin, on Dose 3 from 8,6% to 4,2%.

CONCLUSION

In this research, *Moringa oleifera,* combined with Albumin, gave a result that could not be used as an anti-inflammatory. This result happened because the dose used here was low. *Moringa oleifera* and VipAlbumin® can be used to cure the inflammation in DM, but with another dose, which is not part of this research. The activity of *M. oleifera* and VipAlbumin® with the right dose was able to decrease the relative number of a proinflammatory cytokine such as TNF-α, IFN-γ, produced by T lymphocytes or macrophages.

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