# The Role of *Moringa oleifera*- Ifalmin® Formulation in Regulation of $B220^+IgM^+$ and $B220^+IgG^+$ in Diabetic Mice

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**Abstract**

It has been known that the immunoglobulin levels were altered in diabetes mellitus (DM) conditions. This study aimed to evaluate the levels of immunoglobulins in DM mice after the administration of *Moringa oleifera*-Ifalmin® formulation (MI). Streptozotocin, at a dose of 145 $mg.kg^{-1}$, was injected intraperitoneally to experimental mice to obtain diabetic mice. The groups were divided into normal mice, diabetic mice without treatment, diabetic mice with metformin treatment (307.5 $mg.kg^{-1}$ BW), and diabetic mice with MI treatment at dose 1 (M:I= 800 $mg.kg^{-1}$ BW: 800 $mg.kg^{-1}$ BW), dose 2 (M:I= 615 $mg.kg^{-1}$ BW: 615 $mg.kg^{-1}$ BW), and dose 3 (M:I= 800 $mg.kg^{-1}$ BW: 615 $mg.kg^{-1}$ BW). Mice were orally treated by MI for 14 days. Subsequently, the levels of immunoglobulin IgM and IgG were evaluated using flow cytometry analysis. IgM and IgG levels were significantly lower in the DM group than the normal group. These results indicated that DM altered immunoglobulin levels. MI treatment for 14 days significantly increased the number of IgM and IgG at the level equivalent to the normal group and significantly different as compared to the DM group. Based on the results, MI can be used as an immunomodulatory agent in humoral immunity through the precise regulation of IgM and IgG.

**Keywords:** Diabetes mellitus, Ifalmin®, Immunoglobulin, immunomodulator, *Moringa oleifera*.

## INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease characterized by high levels of blood glucose (hyperglycemia) [1]. In 2019, the prevalence of DM globally increased to 463 million people, and predictably raised to 700 million in 2045. DM caused 4.2 million deaths in 2019 [2] and was the seventh leading cause of death, according to the World Health Organization (WHO) [3]. In DM, hyperglycemia resulted from a defect in insulin secretion or action [4].

Some studies demonstrated that there was a tight correlation between the immune system and DM pathogenesis [5,6]. The immunoglobulins are essential components of humoral immunity that have an important role in effector function. The immunoglobulin levels are often determined to represent the status of humoral response in clinical practice [7]. The previous study demonstrated that the levels of immunoglobulin changed in the mice model of diabetes [8]. DM is also associated with the alteration of immunoglobulin [7,9].

The use of synthetic hypoglycemic drugs and insulin can cause side effects in DM patients. Furthermore, both drugs and insulin usually target a single pathway of metabolic, which only focuses on hyperglycemia regulation [10]. Therefore, another alternative treatment is needed to minimalize these side effects. Nowadays, herbal medicine becomes a great prospect for alternative treatment in some diseases, including DM.

*Moringa oleifera* (MO) and Ifalmin® are good sources of nutrition. MO leaves are rich in polyphenols such as flavonoids, phenolic acid, tannins, etc. MO also contains vitamins including vitamin A, E, and C [11] and minerals such as calcium (Ca), zinc (Zn), iron (Fe), potassium (K), manganese (Mn), copper (Cu), and magnesium (Mg) [12]. Ifalmin® is a food supplement from *Channa micropeltes* extract that contains high levels of albumin. Previous studies demonstrated that both MO leaves [13] and albumin [14] have anti-diabetes activities. However, the efficacy of MO and Ifalmin® formulation in the regulation of IgM and IgG in diabetic mice never been studied.

In this study, we used the formulation of MO and Ifalmin® (MI). We assumed that MI, based on their nutritional contents, could be used as an alternative treatment in DM. This formulation was expected to target some mechanisms in DM conditions, including the regulation of altered immunoglobulins. Therefore, this study aimed to evaluate the levels of IgM and IgG in mice models of DM.

## MATERIAL AND METHOD

### Experimental Design

A total of 30 male mice (*Mus musculus*), strain BALB/c, with the ages between 8-10 weeks were obtained from Singosari Breeder (Malang, Indonesia) and used in this study. The mice

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divided into six groups including the normal group (N) or non-diabetic mice, diabetic mice with metformin administration (DM-M), diabetic mice without MI administration (DM), and diabetic mice with administration of MI dose 1 (DM-D1), dose 2 (DM-D2), and dose 3 (DM-D3). The formulation of used doses were D1 (MO 800 $mg.kg^{-1}$ BW: I 800 $mg.kg^{-1}$ BW), D2 (MO 615 $mg.kg^{-1}$ BW: I 615 $mg.kg^{-1}$ BW) and D3 (MO 800 $mg.kg^{-1}$ BW: I 615 $mg.kg^{-1}$ BW). Each group consisted of five replications. The experiment using the animal model in this study conducted according to ethical clearance approved by The Ethical Committee of Brawijaya University (Reg. No. 1180-KEP-UB).

**Induction of Diabetic Mice**

A single high dose of Streptozotocin (STZ) used to induce the DM in the mice [15]. The mice fasted for four hours then they were injected by STZ at a dose of 145 $mg.kg^{-1}$ BW mice. The levels of blood glucose were measured from the mice tail using Easy Touch glucometer (Bioptik Technology Inc., Taiwan) after four days of STZ injection. Mice considered diabetic when the levels of blood glucose were higher than 200 $mg.dL^{-1}$ [16].

**Oral Administration of MI and Metformin**

Five grams of MO leaves powder (Materia Medica Batu, Malang) dissolved in 50 mL of boiled water. Then, it was filtered using Whatman no. 1 paper and subjected to freeze-drying. The extract of MO formulated with Ifalmin®. Ifalmin® obtained from PT. Ismut Fitomedika (Makassar, Indonesia). MI was administrated in diabetic mice (DM-D1, DM-D2, and DM-D3) by oral for 14 days.

Metformin, as a standard drug in this study, was obtained from PT. Hexparm Jaya (Bekasi). Metformin (307.5 $mg.kg^{-1}$ BW mice) dissolved in water. The metformin was administrated in the DM-M group by oral for 14 days. The conversion of human dose to mice equivalent dose in this research is determined based on the Food and Drug Administration (FDA) [17].

**Isolation of Spleen**

After 14 days of MI treatment, the mice sacrificed by the cervical dislocation technique. Then, the spleen organ was isolated from the mice to obtain the immunoglobulin-expressing lymphocyte B cell. The spleen homogenized in phosphate buffer saline (PBS), and the homogenate moved into microtube, then centrifuged at 2500 rpm, 10°C for 5 minutes. The supernatant removed, and the pellet added by PBS. A total of 50 µL sample moved into a microtube for antibody staining.

**Antibody Staining and Flow Cytometry Analysis**

The antibodies applied at a concentration of 0.005 $mg.100\ \mu L^{-1}$ based on the company's protocol. The sample containing lymphocyte cells was stained with extracellular antibody before intracellular antibody staining. Staining of extracellular antibody conducted by adding 50 µL of FITC-conjugated rat anti-mouse B220 (Biolegend®, San Diego) into the cells, then incubated for 20 minutes in the icebox (4°C).

Next, the cells were added by 50 µL of cytofix (BD-Biosciences Pharmingen), incubated for 20 minutes in the icebox, and added by wash perm solution (WPS) (BioLegend®, USA), and centrifuged at 2.500 rpm, 10°C for 10 minutes. Then, the supernatant removed from the sample, and each pellet stained with 50 µL of PE/Cy5-conjugated rat anti-mouse IgG (Biolegend, San Diego) and PE/Cy5-conjugated rat anti-mouse IgM (Biolegend, San Diego). The sample was moved into a flow cytometry tube and added with 400 µL of PBS. Flow cytometry tube was put into the flow cytometer (BD FACSCalibur, USA) to run the flow cytometry. The flow cytometry data were analyzed using BD Cellquest Pro™ software (BD Biosciences, San Jose, CA, USA) [18].

**Data Analysis**

The data were statically analyzed using the SPSS program with one way of variance analysis (ANOVA) and were continued to the Post Hoc test with the p-value <0.05.

**RESULT AND DISCUSSION**

The results showed that the levels of $B220^{+}IgM^{+}$ (Fig.1) and $B220^{+}IgG^{+}$ (Fig. 2) were significantly lower in the DM group as compared to the normal group ($p<0.05$). The levels of IgM reduced from 14.6% (N) to 9.43% (DM), while the IgG levels decreased from 8.05% (N) to 5.03% (DM). The decreasing levels of IgM and IgG in diabetic mice caused by hyperglycemia conditions, but the mechanism remains unclear [7]. Hyperglycemia in this study results from the cell death mechanism of β-pancreatic after injection of STZ. STZ enters the β-pancreatic cells through glucose transporter 2 (GLUT-2) and induces its toxicity. The toxic effect of STZ is due to the DNA alkylating agent, methyl-nitrosourea (MNU), which causes DNA damage of β-pancreatic cells and eventually induces hyperactivation of poly (ADP-ribose) (PARP) enzyme [19,20].

Hyperactivation of PARP can suppress cell energy reserves ($NAD^+$) and reduce ATP, thereby inducing necrosis.

Necrosis in β-pancreatic cells will reduce the secretion of insulin, a hormone that plays an important role in glucose metabolism. Thus, insulin lack results in hyperglycemia. Hyperglycemia can prevent the production of IgM in splenocytes [21] and decrease the production of IgM in B lymphocytes cells [8]. Moreover, hyperglycemia can increase hydroxyl radical (OH.) [22,23], one of the reactive oxygen species (ROS) with high reactivity and is known able to damage IgG structure [24] so that it decreases the levels of IgG. The decline of IgM and IgG levels also seen in type 1 and 2 DM [9,25].

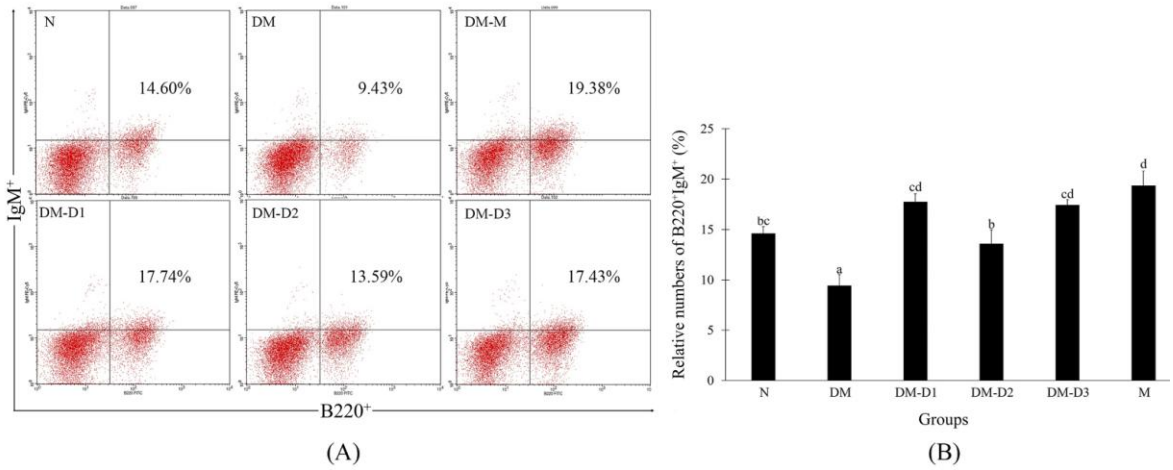


**Figure 1.** MI administration increased the levels of IgM expressed by $B220^+$-expressing cells in diabetic mice. (A) Spleen cells ($2x10^6$) were obtained from all mice, then subjected to extracellular staining cells with anti-B220 antibody, intracellular staining cells with anti-IgM antibody, and analyzed by flow cytometry. N: non-diabetic mice without STZ induction; DM: diabetic mice with STZ induction without MI treatment; DM-D1: diabetic mice with MI treatment dose 1 (MO 800 $mg.kg^{-1}$ BW: I 800 $mg.kg^{-1}$ BW); DM-D2: diabetic mice with MI treatment dose 2 (MO 615 $mg.kg^{-1}$ BW: I 615 $mg.kg^{-1}$ BW); DM-D3: diabetic mice with MI treatment dose 3 (MO 800 $mg.kg^{-1}$ BW: I 615 $mg.kg^{-1}$ BW); DM-M: diabetic mice with metformin treatment. (B) The bars are a calculation of $B220^+IgM^+$ in splenic cells. The data are mean value ± SD of five mice in each group with a significant value<0.05 (n = 30).

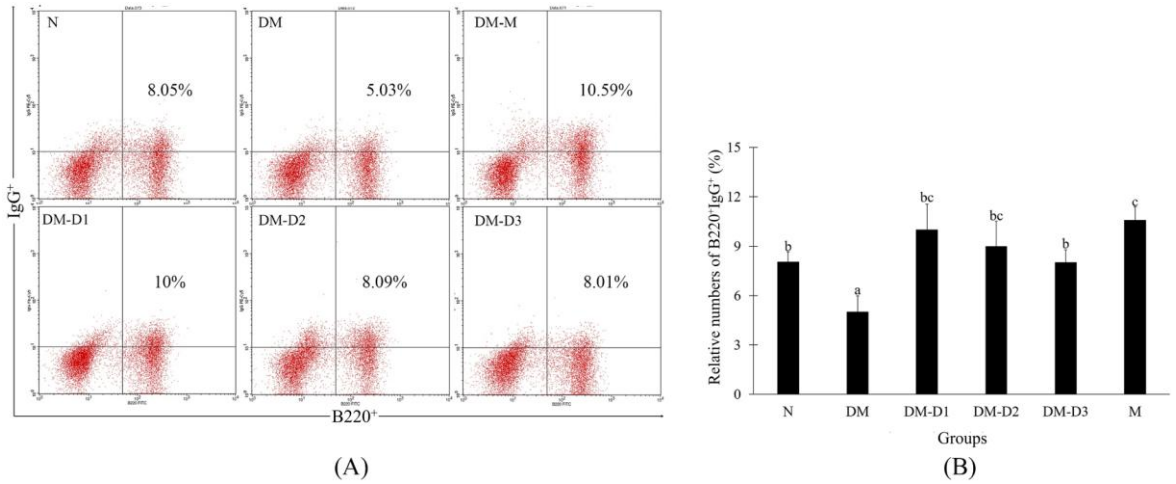


**Figure 2.** MI administration increased the levels of IgG expressed by $B220^+$-expressing cells in diabetic mice. (A) Spleen cells ($2x10^6$) were obtained from all mice, then subjected to extracellular staining cells with anti-B220 antibody, intracellular staining cells with anti-IgG antibody, and analyzed by flow cytometry. N: non-diabetic mice without STZ induction; DM: diabetic mice with STZ induction without MI treatment; DM-D1: diabetic mice with MI treatment dose 1 (MO 800 $mg.kg^{-1}$ BW: I 800 $mg.kg^{-1}$ BW); DM-D2: diabetic mice with MI treatment dose 2 (MO 615 $mg.kg^{-1}$ BW: I 615 $mg.kg^{-1}$ BW); DM-D3: diabetic mice with MI treatment dose 3 (MO 800 $mg.kg^{-1}$ BW: I 615 $mg.kg^{-1}$ BW); DM-M: diabetic mice with metformin treatment. (B) The bars are a calculation of $B220^+IgG^+$ in splenic cells. The data are mean value ± SD of five mice in each group with a significant value<0.05 (n = 30).


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IgM and IgG have the immune-regulatory activities in controlling of auto-antibody response during autoimmune diseases, including type 1 DM. These immune-regulatory activities are possibly performed by suppressing the activity of phagocytosis through Fc receptor interaction, thereby preventing the binding of auto-antibody to its cell target [26,27]. The inability of B lymphocyte cells to secrete IgM correlated to impaired B lymphocyte development, which can ultimately lead to the development of autoreactive B cells in mice model of type 1 DM [26]. Furthermore, low levels of IgG and IgM were associated with the prevalence of type 2 DM [7]. Therefore, the decreasing levels of IgM and IgG in this study might indicate the progression of DM.

This study proved that oral administration of MI for 14 days in diabetic mice significantly increased the levels of IgM and IgG. MI administration in diabetic mice increased the levels of IgM from 9.43% to 17.74% (D1), 13.59% (D2), and 17.43% (D3), respectively (Fig. 1). Furthermore, the administration of MI in diabetic mice increased IgG levels from 5.03% to 10% (D1), 8.99% (D2), and 8.01% (D3) (Fig. 2). The administration of MI could increase the levels of IgM and IgG at the levels equal to the normal group and significantly different from the DM group. These results indicated that MI could restore the decreased levels of IgM and IgG to normal levels in diabetic mice.

The abilities of MI to increase the levels of immunoglobulin in diabetic mice represent its immunomodulatory activities in humoral immunity. These immunomodulatory activities of MI might occur through hypoglycemic activity and antioxidant mechanisms as the decreasing levels of IgM and IgG affected by hyperglycemia and stress oxidative. STZ can increase the levels of ROS [28] which also have a destructive effect on β-pancreatic cells. Furthermore, hyperglycemia as well increases the production of ROS and decreases antioxidant enzyme, thereby inducing oxidative stress [29].

MI was assumed to have antioxidant activity due to high flavonoids in MO leaves and abundant albumins in Ifalmin®. Flavonoids exert its antioxidant activity by donating its electron and hydrogen atom to some radicals, including hydroxyl radicals, superoxide, alkoxyl, and peroxyl, thereby stabilizing them [30]. Quercetin in MO leaves can increase the endogenous antioxidant enzymes via the nuclear-related factor 2 (Nrf2) pathway [31]. Another plant with antioxidant properties reported having an immunostimulant effect in IgM and IgG [32]. Furthermore, flavonoid and phenolic acid in MO leaves have hypoglycemic activity through inhibition of amylase enzyme [33]. This enzyme functions to convert the starch into glucose, decrease in intestinal absorption of glucose, and eventually reduce the glucose levels.

Albumin in Ifalmin® is known to activate Nrf2 signaling for gene expression of endogenous antioxidant enzymes, including superoxide dismutase (SOD), heme oxygenase-1 (HO-1), glutathione S-transferase, and glutathione peroxidase [34]. Some studies demonstrated that antioxidant enzymes were decreased in DM conditions [35,36]. Albumin contains high sulfhydryl (-SH) groups that have acted as a radical scavenger to bind ROS, thereby reducing the levels of ROS and preventing stress oxidative. Moreover, anti-hyperglycemic of albumin has been demonstrated by Dwijayanti et al. [14] in mice model of type 2 DM. The efficacy of albumin in decreasing the glucose levels occurred due to its anti-inflammatory activity as the inflammatory mediators in DM cause the dysfunction of β-pancreatic cells.

In this study, we also compared the efficacy of MI with a standard drug, metformin, in the regulation of IgM and IgG. The results showed that the administration of metformin in diabetic mice significantly increased the levels of $B220^{+}IgM^{+}$ (Fig. 1) and $B220^{+}IgG^{+}$ (Fig. 2) as compared to the DM group ($p<0.05$). The increasing levels of IgM and IgG after metformin administration possibly occurs through its anti-hyperglycemia effect. Metformin reduces glucose levels by suppressing the production of hepatic glucose and inhibiting intestinal glucose transport [37]. However, the IgM and IgG levels in the DM-M group were significantly higher than the normal group.

**CONCLUSION**

Based on the results, we concluded that the formulation of *Moringa oleifera* – Ifalmin® (MI) could be used as an immunomodulator in diabetic mice through precise regulation of IgM and IgG. The oral administration of MI for 14 days in diabetic mice significantly increased the levels of IgM from 9.43% to 17.74% (D1), 13.59% (D2), and 17.43% (D3), respectively. Meanwhile, the administration of MI in diabetic mice increased IgG levels from 5.03% to 10% (D1), 8.99% (D2), and 8.01% (D3).

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