

Effects of *Moringa oleifera* aqueous leaf extract in alloxan induced diabetic mice

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Abstract: *Objective:* There is a lack of knowledge regarding the underlying mechanisms of the antidiabetic activity of *Moringa oleifera*. This study investigates the antidiabetic effect of *M. oleifera* and its impact on the immune tolerance. *Methods:* Alloxan-induced diabetes model for mice was used. A dose of 100 mg/kg of Moringa extract was orally administered to diabetic treated mice. Glucose and insulin levels were evaluated to calculate insulin resistance. Total antioxidant capacity (TAC), creatinine, and blood urea nitrogen (BUN) levels were measured. The relative percentage of CD44, CD69, and IFN- γ was investigated by flow cytometry. *Results:* In diabetic mice, insulin resistance by homeostasis model assessment of insulin resistance (HOMA-IR) was increased 4.5-fold than in the control group, and HOMA-IR was decreased 1.3-fold in the Moringa treatment group. The level of TAC was declined 1.94-fold in diabetic mice, and increased 1.67-fold in diabetic treated group. In diabetic mice, creatinine and BUN levels were significantly reduced 1.42- and 1.2-fold, respectively, in Moringa treatment mice. The relative percentage of CD44 was not changed in diabetic mice, but the relative percentage of CD69 was found to be increased. INF- γ was decreased 2.4-fold in diabetic mice and elevated in treated groups. *Conclusion:* Moringa may ameliorate insulin resistance, increase TAC, and improve immune tolerance.

Keywords: blood urea nitrogen, creatinine, insulin resistance, total antioxidant capacity, immune tolerance

Introduction

Diabetes mellitus is one of the most common worldwide diseases. As diabetes is a multifactorial disease, its treatment is complicated and requires multiple therapeutic strategies. Diabetes is associated with the increased level of blood glucose, which causes hyperglycemia. The latter triggers oxidative stress that halts the biological activities, and cause diabetic complications [1, 2]. Hyperglycemia-mediated oxidative stress plays a key role in the pathogenesis of diabetic complications such as nephropathy [3]. So, the optimal antidiabetic drug should combine both hypoglycemic and antioxidant properties. The main drawback of the current drugs available for diabetes is their potential toxicity in the long run and lacking efficiency [4]. Moringa leaves are rich in proteins, calcium, iron, potassium, vitamins (particularly

C and E), β -carotene [5], and in antioxidant and bioactive compounds, such as flavonoids, phenolic acids, glucosinolates and isothiocyanates, tannins, and saponins [6]. Therefore, it seems that Moringa leaves are the first source of the numerous pharmacological properties attributed to *Moringa oleifera* leaves. In this regard, flavonoids and polyphenols are described as natural antioxidants. Since polyphenols and flavonoids can directly react with superoxide anions and lipid peroxyl radical and consequently inhibit or break the chain of lipid peroxidation [7]. This radical scavenging activity of extracts could be related to the antioxidant nature of polyphenols or flavonoids, thus contributing to their electron/hydrogen donating ability. Interestingly, the β -sitosterol isolated from the leaves of *M. oleifera* is a plant sterol with close chemical resemblance to cholesterol, which enables it to block the absorption of

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cholesterol by competitive inhibition [8]. Since *Moringa* has an impact on the immune system, it could stimulate both cellular and humoral immune responses [9, 10]. Oxidative stress has emerged in the pathogenesis of many diseases including diabetes [11, 12]. Individual oxidative stress markers including the measurement of antioxidant enzymes-superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, ceruloplasmin, and proteins such as metallothioneins have been used for decades for mentoring the potency of the antioxidant defense system. Recently, a new test to measure the total antioxidant status was introduced, which has been designated as total antioxidant capacity (TAC) [13]. The major advantage of this test is to measure the TAC of all antioxidants in a biological sample and not just the TAC of a single compound. Since the measure of TAC considers the cumulative action of all the antioxidants present in plasma and body fluids, thus providing an integrated parameter rather than the simple sum of measurable antioxidants [14]. Furthermore, assessment of plasma TAC could provide a clear picture about the physiological status, and may help to identify the state and potential of oxidative stress in the organism. The aim of this study was to investigate the ameliorative effects of low doses of aqueous *Moringa* extract on diabetes and its impact on the TAC and immune tolerance, which have not yet been studied. In another study on the same model with alloxan, I found that *Moringa* promotes the activity of both CD4⁺ and CD8⁺ T cells in diabetic treated mice that may occur through the Sca-1⁺CD117⁺ stem cell factors, which play an important role as hematopoietic regulators (data not shown). Also, administration of *Moringa* leaf extract enhanced the percentage of the endothelial progenitors (CD34⁺CD117⁺) and mature endothelial cells (CD34⁺CD117⁻). *Moringa* also increased the percentage of blood-derived circulating angiogenic cells (Sca-1⁺/CD34⁺). This study investigated the levels of CD44 as a marker for the T-cell activation, the transmembrane CD69 protein, which is supposed to be highly up-regulated in all immune cells, and INF- γ , which is a potent activator of macrophages.

Materials and Methods

Moringa oleifera aqueous extract preparation

Moringa aqueous extract was prepared by mixing 10 g of dried and powdered *M. oleifera* leaves with 100 mL of distilled water for 24 h and then stored at 4 °C. Afterward, the mixture was filtered twice through a 2- μ m pore filter paper. The aqueous extract stock solution (100 mg/mL) was stored at 4 °C for up to 5 days, or freshly prepared for each set of experiment.

Animals care and treatments

Forty albino mice (20 \pm 5 g) were adapted in the laboratory for 2 weeks under the same natural environmental condition of temperature and photoperiod and with free access of food and water. All the procedures were in accordance with the protocol of National Animal Care and Use Committee and Guidelines for the Care and Use of Experimental Animals. Mice were randomly divided into four groups (10 mice each) as follows: control group, diabetic untreated group, mice received oral administration of *M. oleifera* aqueous extract (100 mg/kg), and diabetic treated groups with 100 mg/kg of *M. oleifera* aqueous extract given by an oral gavage for 14 days after diabetes induction (mice lived for 21 days). Diabetes was induced with two intraperitoneal injections of alloxan (Sigma-Aldrich), previously dissolved in ice-cold phosphate-buffered saline, pH 6.8 (Merck Millipore). The first dose was 150 mg/kg as recommended by Bromme et al. [15], and the second dose was 100 mg/kg given 2 days after the first dose to ensure the induction of diabetes throughout the experimental duration. Diabetes threshold was plasma glucose level >250 mg/dL (Diagnostics, Indianapolis, IN, USA).

Plasma Measurements

Insulin resistance by homeostasis model assessment of insulin resistance (HOMA-IR)

Blood was taken from the tail vein of the fasting mice (12 h); the glucose level was estimated by LifeScan OneTouch® UltraEasy meter. Insulin level was determined according to the instructions of kit's manufacturer (Merckodia-10-1251-01). HOMA-IR was determined using the following formula: HOMA-IR = fasting glucose value (mg/dL) \times fasting insulin value (μ U/mL)/405 [16].

Biochemical analysis

The TAC was measured in the plasma according to a previously described method [17], and its activity was expressed in mM/mg protein. The total protein was determined using Bio-diagnostic kit according to a previously described method [18]. The levels of creatinine and blood urea nitrogen were determined by the commercially available kits (Bio-Diagnostic Co., Egypt).

Flow cytometry analysis

Peripheral blood mononuclear cells and splenocytes from mice were incubated with primary antibody, including

CD44 (156-3C11) mouse mAb, CD69 (Clone H1.2F3), and IFN- γ (bs-0480R). Stained cells were resuspended in Flow Cytometry Staining Buffer and analyzed by flow cytometry. FACSCanto II (BD Biosciences, SanJose, CA, USA) was used for acquisition. CellQuest (BD Biosciences, SanJose, CA, USA) and FlowJo software were used for data analysis. The absolute numbers of cells were calculated using the following formula: the percent of cells \times the total number of white blood cells/100.

Statistical and data analysis

The data were analyzed with Sigma Plot 10 software (Systat Software Inc., San Jose, CA, USA), and Prism 3.0 package (GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) Newman-Keuls multiple test was used as a *post-hoc* comparison test. The significant difference was set at $P < 0.05$.

Results

As shown in *Fig. 1A*, a significant increase was noted in the level of glucose in diabetic mice (321.2 ± 33.93 mg/dL) compared with the control group (140.8 ± 13.61 mg/dL). The level of glucose was significantly higher in diabetic mice treated with Moringa by 1.7-fold when compared with the control group. However, due to treatment with Moringa, the level of glucose was decreased by 1.28-fold compared with the diabetic group. Therefore, treating diabetic mice with Moringa significantly reduced hyperglycemia, maintaining mean glucose levels at 249.2 ± 11.77 mg/dL. The level of insulin in plasma reflects the function of the pancreatic beta cells and the sensitivity of tissues to insulin through glucose uptake. As shown in

Fig. 1B, the insulin level was significantly declined from 14.35 ± 1.35 mg/dL in the control group to 5.35 ± 0.84 mg/dL in the diabetic group. The level of insulin was significantly increased to 9.800 ± 1.530 mg/dL in the diabetic group because of treatment with Moringa. Surprisingly, an increase occurred in mice treated with Moringa alone (20.00 ± 1.673), and that was statistically significant compared with the control group.

HOMA-IR analysis

HOMA-IR was calculated from the values of serum glucose (mg/dL) and serum insulin (μ U/mL) in mice fasted over night (*Fig. 2*). The level of insulin resistance was significantly increased from 7.172 ± 0.815 (mg/dL \times mU/mL) in the control group to 13.09 ± 0.965 (mg/dL \times mU/mL) in the diabetic untreated group. As a result of Moringa treatment, insulin resistance was significantly decreased to 10.31 ± 0.466 (mg/dL \times mU/mL) compared with the diabetic untreated group, although it was still significantly higher compared with the control group.

Total antioxidant capacity

The TAC was prominently declined to 0.27 ± 0.057 mM/mg protein in the diabetic untreated mice when compared with the control group, which recorded 0.526 ± 0.026 mM/mg protein (*Fig. 3*). There was no significant difference between the mice received Moringa, which found to be 0.463 ± 0.02 mM/mg protein, compared with the control group. Treatment of diabetic mice with Moringa significantly enhanced and restored the TAC to 0.453 ± 0.029 mM/mg protein.

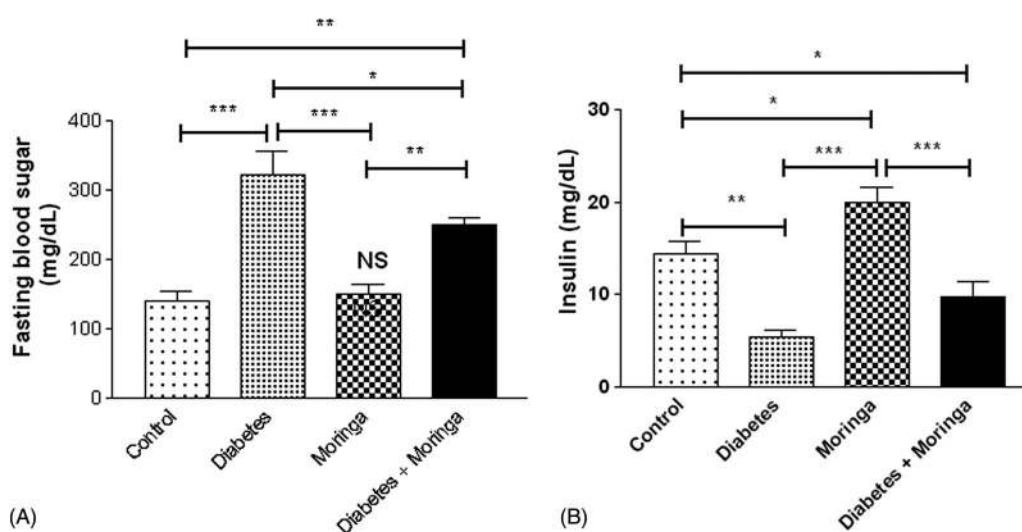


Fig. 1. Fasting glucose (A) and insulin (B) levels in different mice involved in this study. Data were expressed as mean \pm SE of 10 mice in each group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, NS: statistically non-significant

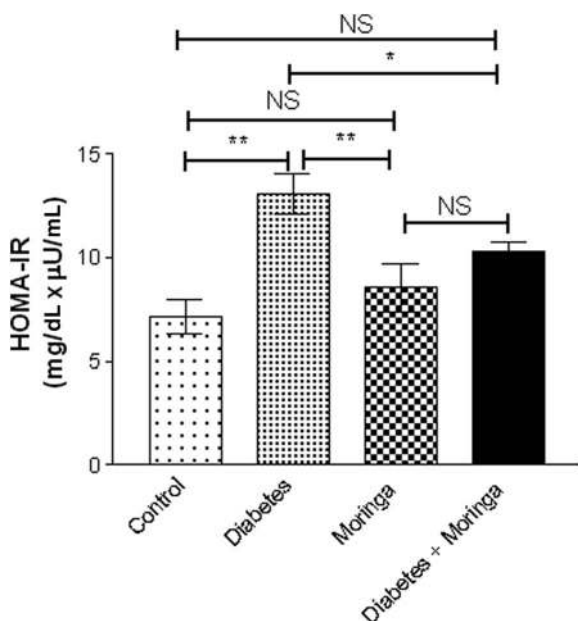


Fig. 2. HOMA-IR analysis in different mice involved in this study. HOMA-IR was calculated from glucose (mg/dL) and insulin (μU/mL) levels using the following formula: HOMA = fasting glucose value (mg/dL) × fasting insulin value (μU/mL)/405. Data were expressed as mean ± SE of 10 mice in each group. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, NS: statistically non-significant

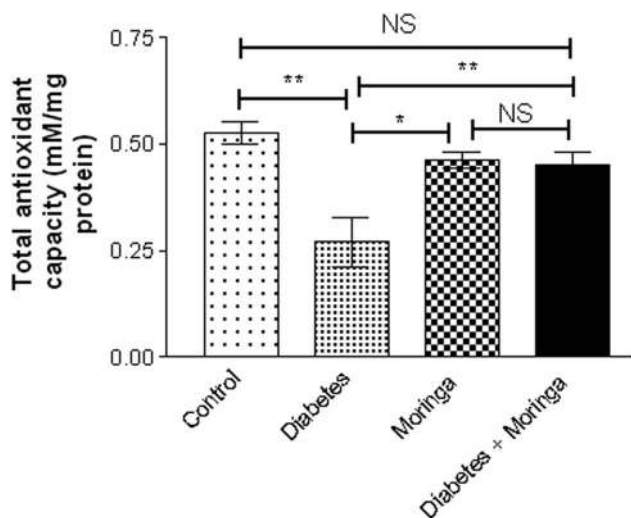


Fig. 3. Total antioxidant capacity in different mice involved in this study. Data were expressed as mean ± SE of 10 mice in each group. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, NS: statistically non-significant

Creatinine and urea levels

As shown in *Fig. 4*, the level of plasma creatinine was significantly increased in diabetic untreated mice (0.49 ± 0.066 mg/dL) compared with the control group (0.11 ± 0.0089 mg/dL). There was a non-significant difference in the mice received Moringa (0.49 ± 0.066 mg/dL)

when compared with the control group. Due to treatment of diabetic mice with Moringa, the level of creatinine was significantly reduced to 0.344 ± 0.078 mg/dL. On the other hand, the level of urea was significantly enhanced from 6.050 ± 0.27 mg/dL in the control group to 11.12 ± 1.24 mg/dL in the diabetic untreated group. The level of urea in the mice received Moringa was recorded 7.02 ± 0.511 mg/dL, reflecting a non-significant difference when compared with the control group. The level of urea was significantly declined to 9.16 ± 0.96 mg/dL when compared with the diabetic untreated group.

Flow cytometry analysis

The Fluorescence Minus One gating boundaries for CD44, IFN-γ, and CD69 molecules are shown in *Fig. 5*. As shown in *Fig. 6*, there was no significant difference in the percent of CD44 molecules when the diabetic untreated group was compared with the control group (73.74 ± 0.98 and 70.30 ± 1.70, respectively). Compared with all groups, the maximum percent of CD44 molecules was recorded in the diabetic treated group with Moringa (87.20 ± 2.048).

Although there was an increase in the percent of the expression of CD69 PE.Cy7 in the diabetic untreated group, which recorded 4.138 ± 0.512, when compared with the control group 1.43 ± 0.092, but that increase was not significant (*Fig. 7*). When compared with the control group, Moringa promotes the activity of CD69 PE.Cy7 expression as indicated in the mice received Moringa and in the diabetic mice treated with Moringa, which were recorded 5.49 ± 0.87% and 8.33 ± 1.30%, respectively.

INF-γ production was significantly decreased in diabetic mice, which recorded 2.059 ± 0.417%, compared with the control group that recorded 5.03 ± 0.80%. Moringa significantly enhanced the production of INF-γ in mice to 9.70 ± 0.57% in normal mice and 12.24 ± 1.34% in diabetic treated mice (*Fig. 8*).

Discussion

Insulin is a critical hormone for the process of cellular glucose uptake, and thus mainstreaming the normal levels of blood glucose. Diabetes mellitus is a multifactorial disease marked by hyperglycemia due to the impairment in insulin secretion and/or peripheral insulin resistance [19]. Insulin resistance is defined as a reduced responsiveness of insulin on a target cell or a whole organ, which results in reducing insulin-mediated glucose utilization in peripheral tissues, accompanying glucose intolerance and insulin intolerance [20]. Although HOMA-IR is a marker usually used in human type 2 diabetes, not routinely

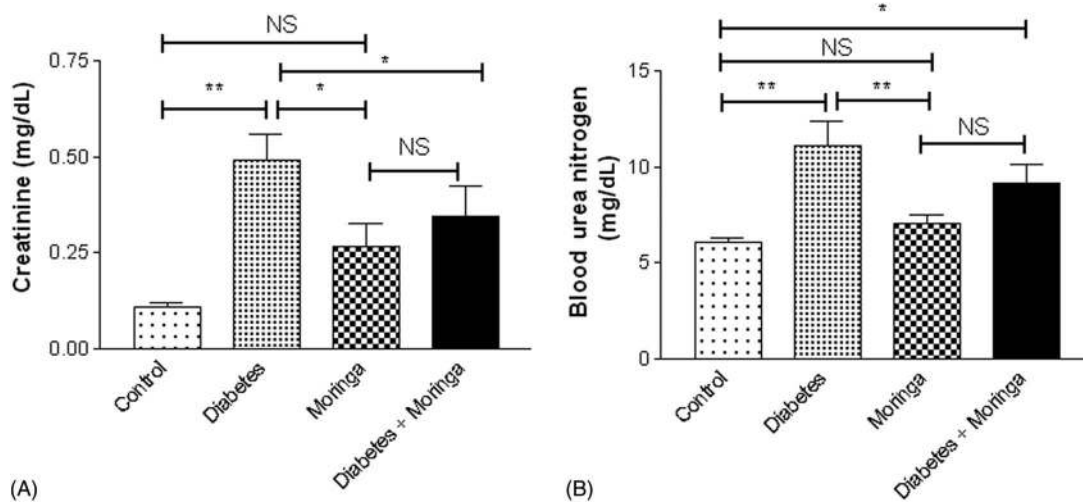


Fig. 4. The level of creatinine (A) and blood urea nitrogen (B) levels in different mice involved in this study. Data were expressed as mean \pm SE of 10 mice in each group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, NS: statistically non-significant

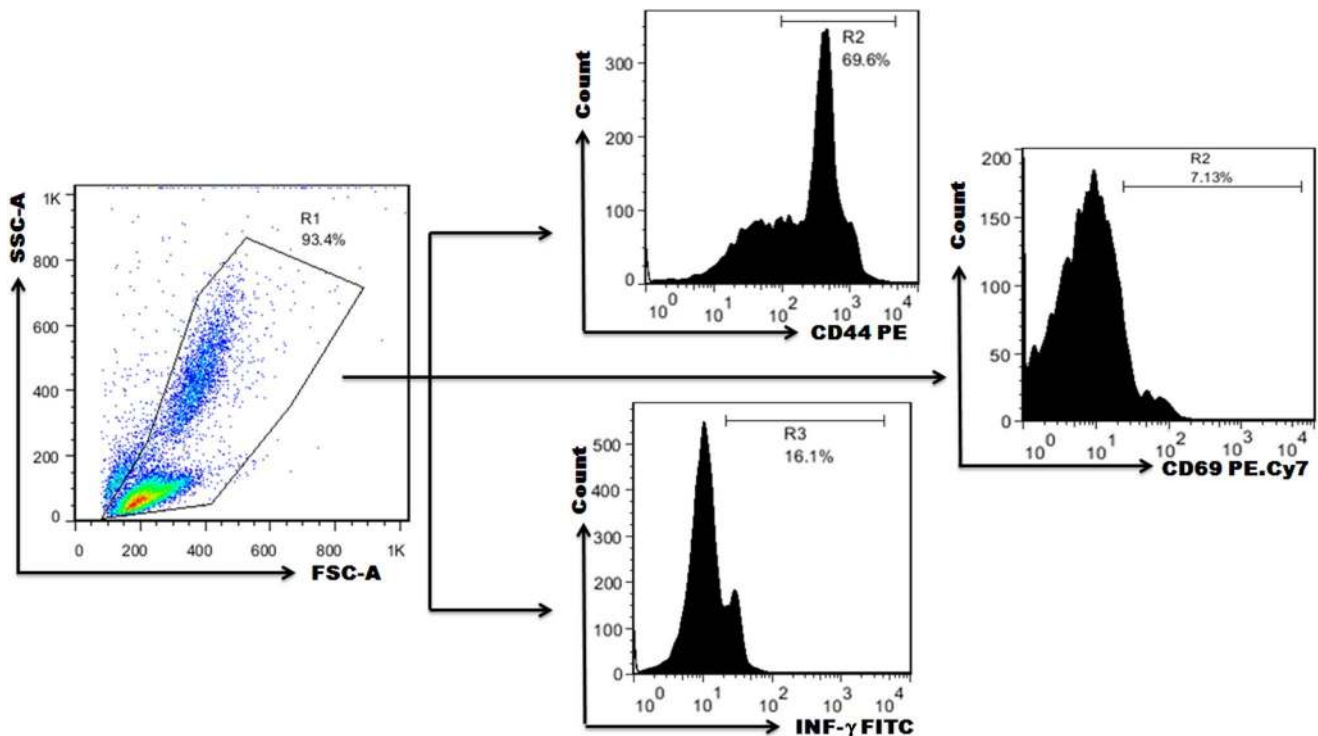
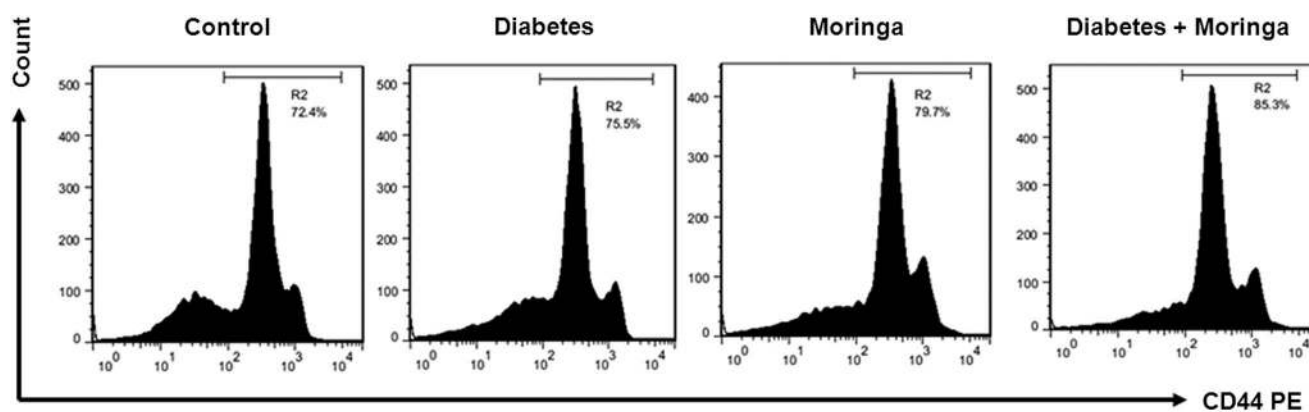


Fig. 5. The Fluorescence Minus One gating boundaries for CD44, IFN- γ , and CD69 molecules

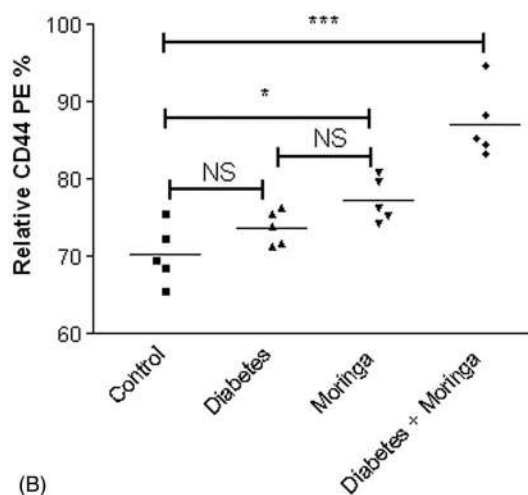
evaluated in mice, this study determined it in order to investigate whether treatment with *Moringa* could aid us to overcome the insulin resistance. HOMA-IR in diabetic mice was increased about 4.5-fold than that in the control group. As a result of treatment of diabetic mice with *Moringa*, HOMA-IR was decreased 1.3-fold compared with the diabetic untreated mice. The antihyperglycemic and antioxidant abilities of *Moringa* leaves extract may be

associated with its ability to improve insulin sensitivity in diabetic mice.

On the other hand, oxidative stress has recently emerged and involved in the etiology of many diseases including diabetes. Oxidative stress causes membrane damage leading finally to membrane rupture in different cellular types. Cells possess an efficient antioxidant defense system against destructive damage induced by oxidative



(A)



(B)

Fig. 6. Representative histograms showing the expression of the CD44 molecules in the peripheral blood of different mice involved in this study (A). A representative histogram showing the relative percentage of CD44 molecules (B). Data were expressed as mean \pm SE of five mice in each group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, NS: statistically non-significant

stress. An elevation of blood glucose induces oxidative stress resulting in an increased production of oxygenated free radicals and decreased antioxidant enzyme activities [21, 22]. This may result in intracellular structure modification and ultimately affect the normal cellular function, leading to pathogenesis and the development of diabetic complications [22, 23]. In 2006, Shin et al. [24] reported an inverse association between the insulin resistance and plasma levels of TAC in non-diabetic hypercholesterolemic patients. Furthermore, TAC was inversely associated with fasting plasma glucose [25]. In this study, the TAC was significantly declined 1.94-fold in diabetic mice compared with the control group. Due to treatment with Moringa, the level of TAC was increased 1.67-fold compared with the diabetic untreated mice.

Free radical generation-mediated stress in diabetes is one of the leading reasons for renal dysfunction associated with the elevation of urea and creatinine levels due to the persistent hyperglycemia, and hemodynamic changes within the kidney tissue [26, 27]. Since, creatinine is also a protein breakdown product and urea is a protein metabolism product, thus the elevation of the blood urea

and creatinine levels can be used as indicators for the excessive breakdown of protein. In this study, blood urea and creatinine levels were significantly increased 1.83- and 4.45-fold, respectively, in diabetic mice compared with normal mice due to excessive breakdown of protein. Administration of Moringa to the diabetic mice significantly reduced the creatinine 1.42-fold and urea 1.2-fold compared with the diabetic untreated mice. This reflects the preventive action of Moringa supplementation on kidney damages in diabetic condition perhaps due to the antioxidant properties. We have previously indicated that hyperglycemia causes osmotic diuresis and depletion of extra-cellular fluid volume, which could explain the elevated plasma creatinine in diabetic untreated group [28]. It is very important to mention that creatinine does not bind to plasma proteins, and is freely filtered by the glomerulus of the kidney, which has an important clinical implication for overestimating creatinine clearance of kidney function [29, 30].

Activated T cells exhibit surface expression of molecules such as CD69, CD44, and INF- γ . The effect of cell activation is a cascade of molecular events leading to

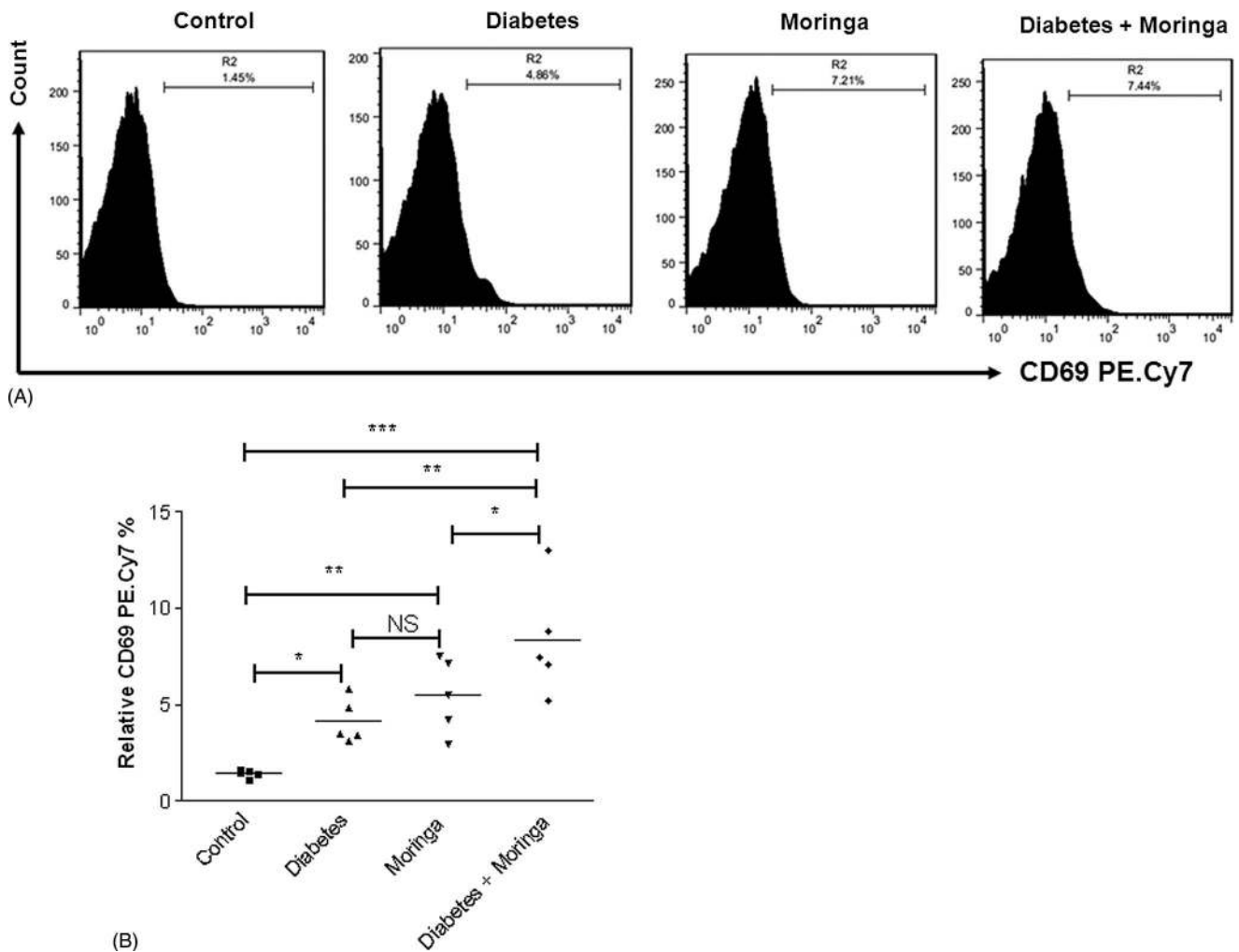


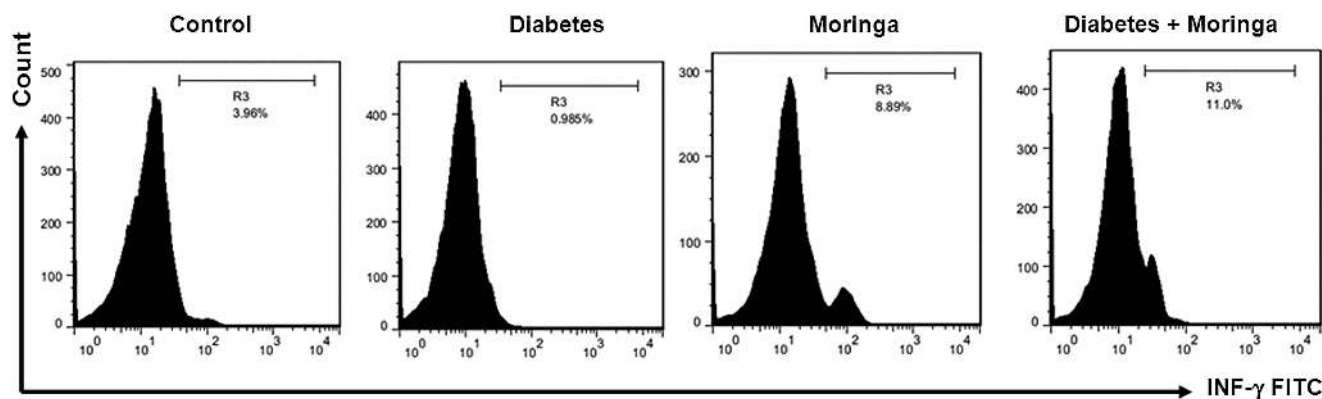
Fig. 7. Representative histograms showing the expression of the CD69 molecules in the peripheral blood of different mice involved in this study (A). A representative histogram showing the relative percentage of CD69 molecules (B). Data were expressed as mean \pm SE of five mice in each group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, NS: statistically non-significant

proliferation and clonal expansion of antigen-specific T cells. Increased surface levels of CD44 are the characteristic of T-cell activation. In this study, the expression of CD44 was increased 1.2-fold as a result of treatment of diabetic mice with Moringa. These findings along with the findings in this study are consistent with previous study in non-obese diabetic mice showed that the transfer of diabetes by spleen cells from diabetic donors into immunocompromised recipients required the presence of both CD4⁺ and CD8⁺ T cells [31].

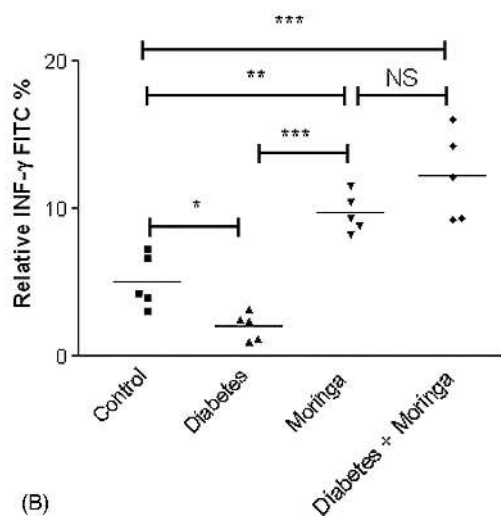
The relative percentage of CD69 PE.Cy7 was significantly increased 2.8-fold in diabetic mice compared with the control mice. These results are in line with the previous investigation that diabetes mediates high levels of co-stimulatory molecules CD69 [32, 33]. In mice received Moringa, the transmembrane CD69 protein is highly up-regulated in all immune cells. Since, the expression of CD69 was significantly higher in each of mice received Moringa (Positive control group) and diabetic

treated mice with Moringa compared with the control and diabetic untreated groups. Compared with the control group, the relative percentage of CD69 PE.Cy7 was significantly increased 3.8- and 5.8-fold in mice received Moringa and in diabetic treated group, respectively. These results point to up-regulation of CD69 molecule may be a target of therapy to enhance immune potency against diabetes and its complications.

INF- γ is produced by lymphocytes due to the activation by specific antigens or mitogens [34]. INF- γ is a potent activator of macrophages, and thus it has important immunoregulatory functions [35]. In this study, the production of INF- γ was significantly decreased 2.4-fold in the diabetic group compared with the control group. These findings are in agreement with the previous study demonstrated that the level of plasma INF- γ was decreased in mice with alloxan-induced type 1 diabetes mellitus [36]. The maximum INF- γ production was recorded in diabetic treated group compared with all



(A)



(B)

Fig. 8. Representative histograms showing the expression of IFN- γ molecules in the peripheral blood of different mice involved in this study (A). A representative histogram showing the relative percentage CD69 molecules (B). Data were expressed as mean \pm SE of five mice in each group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, NS: statistically non-significant

groups. Moringa treatment promotes the restoration of IFN- γ production in diabetic treated mice 5.9-fold increase greater than those untreated diabetic mice.

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Authors' contribution: MJT designed the study, performed the experiments, the sequence alignment of biochemical and flow cytometric analysis, the statistical analysis, wrote and revised the manuscript.

Conflict of interest: The author declares no conflict of interest.

Ethics: This article does not contain any studies with human subjects performed by the author. For animal subjects, all the procedures were in accordance with the protocol of National Animal Care and Use Committee and Guidelines for the Care and Use of Experimental Animals.

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