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# Essential role of Moringa-albumin (MA) formulation in the maintenance of complement regulatory proteins on erythrocyte (TER-119) cells in diabetic mouse models

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## ABSTRACT

**Aim** To evaluate the essential role of Moringa-albumin (MA) formulation in the maintenance of complement regulatory proteins through CD55 and CD59 on erythrocyte (TER-119).

**Methods** Streptozotocin (145 mg/kg) was used to induce diabetes mice. In diabetes mellitus (DM) model mice were treated with MA formulation for 14 days at a D1 - 500 mg/kg (M) + 620 mg/kg BW (A), D2 - 1000 mg/kg (M) + 420 mg/kg (A), and D3 - 1500 mg/kg (M) + 200 mg/kg (A). On the 15th day, the mice were dissected for the isolation of their bone marrow by the flushing method. The analysis flow cytometry was performed to find out the TER-119, CD55, and CD59 expressions. The data were statistically analysed with one-way ANOVA ($\rho \leq 0.05$) and Tukey test using SPSS version 16 for Windows.

**Results** The effect of MA administration in D3 group have a significant effect on increasing the profile of erythrocytes (TER-119), compared to DM ($\rho \leq 0.05$) group (STZ Injection-no treatment), as well as CD59 expressed by erythrocytes. MA administration in D1 group and D3 group significantly increased the profile of CD55 expressed by erythrocytes compared to DM ($\rho \leq 0.05$).

**Conclusions** MA formulation with D3 group (1500 mg/kg (M) + 200 mg/kg (A)) was able to maintain or control complement regulatory proteins (CD55 and CD59) on red blood cells (erythrocytes/TER-119) in DM.

**Keywords:** albumin, complement regulatory, diabetes mellitus, erythrocytes, *Moringa oleifera*

## INTRODUCTION

Diabetes mellitus (DM) is one of the fastest growing diseases in the world with high prevalence. DM affected 537 million people worldwide in 2021 and is predicted to increase by 46% to 783 million by 2045 (1). This disease can lead to several complications including cardiovascular disease, diabetic retinopathy, neuropathy, and diabet-

ic kidney disease (2). DM is characterized by hyperglycaemia (blood glucose levels ≥ 200 mg/dL) (3,4) and hyperglycaemia is a major factor causing DM through increased reactive oxygen species (ROS). The increased oxidative stress in diabetes occurs through the formation of advanced glycation end products (AGEs), the end products of a molecule formed by the non-enzymatic covalent bonding of glucose residues to free amino groups in proteins, lipids, and nucleic acids (5). Increased accumulation of this product can trigger non-enzymatic glycation reactions in both haemoglobin and glycoproteins in erythrocyte cell membranes, causing the inability of erythrocytes (TER-119) to change their form (deformability) (6).

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Erythrocyte is one of the cells most susceptible to damage caused by oxidative stress, mainly due to the presence of fatty acid content in the membrane, and the high concentration of cellular oxygen and haemoglobin (7). Oxidative damage to the lipids of the erythrocyte membrane and changes in the cytoskeleton protein will cause erythrocyte adhesion and aggregation, decreased ability of deformability, and life span decline. Factors affecting the erythrocyte life are regulators of the complement system existing on the erythrocyte's external membrane (8,9). Several studies showed that in the microvascular complications of diabetes, the complement system was activated and it suppressed the expression of CD55 and CD59, as well as glycation on the CD59 molecule (10–12). Based on the facts, it was clear that the complement system played an important role in the occurrence of microvascular complications in diabetics (13). Previous studies evidenced that CD55 and CD59 levels decreased in diabetic microvascular disease. CD55 and CD59 expressions in peripheral blood leukocytes were decreased in diabetic patients with macrovascular disease. The results suggested that declining levels of complement regulatory proteins might play an important role in the macrovascular disease of diabetes (13).

Based on the above description, to treat diabetes, drugs that can prevent cells from damage and also can repair damaged organs are needed. The use of natural ingredients sourced from plants and animals is believed to be a safe alternative therapeutic agent, safe for consumption. *Moringa oleifera* L. has anti-inflammatory, antimicrobial, antioxidant, antitumor, anticancer, cardiovascular, antihyperglycemic, and diuretic effects (14–16). Snakehead fish (*Channa micropeltes*) is potential medicine due to its high albumin content (17). Albumin has a sulfhydryl (-SH) group functioning as a binding of free radicals thus it has a role in reducing the reactive oxygen species (ROS) in the body (18). The formulation of important ingredients derived from Moringa leaves and snakehead fish was expected to help treat DM.

The aim of this study was to evaluate the essential role of Moringa-albumin (MA) formulation in the maintenance of complement regulatory proteins through CD55 and CD59 on erythrocyte (TER-119).

## MATERIALS AND METHODS

### Animals and research design
Male mice (8-10 weeks) used in this study were obtained from Malang Murine Farm, Singosari, Malang, East Java, Indonesia, in good health condition based on inspection number 524/306/35.73.319/2019 by the Department of Agriculture and Food Security, Malang City, Indonesia. The mice were placed in a chamber and acclimatized for about 1 week to adjust to the new environment to avoid stress. This study was conducted at animal room, biology molecular building, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University; Malang, East Java, Indonesia in August 2019. Mice were placed in a chamber with controlled environmental conditions. Food in the form of standard pellets used in animal centres and mineral water were given daily. Having been acclimatized, the mice began to be treated. The mice were divided into 5 groups: healthy controls-untreated, DM group - mice with streptozotocin (STZ) injection, and three different groups with various dose administered to the DM group. The dosage used consists of a combination of Moringa (M) and Albumin (A). Three of these doses are: dose 1 (D1 group) with 500 mg/kg (M)+ 620 mg/kg (A), dose 2 (D2 group) with 1000 mg/kg (M) + 420 mg/kg (A)), and dose 3 (D3 group) with 1500 mg/kg (M) + 200 mg/kg (A)).

Experimental research using these animals was carried out under an ethical license approved by the National Ethics Committee of Brawijaya University with the registration number 1068-KEP-UB / 2019.

### Methods
**Preparation of M. *oleifera* (MO) extract** *M. oleifera* was obtained from Materia Medica Batu, East Java, Indonesia in the form of dried simplicia. The simplicia is used in the form of dry leaf powder packed in sterile containers and stored at room temperature. Moringa leaf powder was extracted with sterile aquadest in the ratio of 1:10. The dried Moringa leaves were weighed with an analytical balance of 5 g, and the simplicia was then boiled in 50 mL boiling distilled water for approximately 5 minutes before being filtered using filter paper to separate the pulp and substrate. The extract obtained was then evaporated with a freeze dryer for about 3 days to get pellets ready to be used for the treatment.

**Diabetes mellitus induction using streptozotocin** Induction of diabetes mellitus using streptozotocin (STZ) was done based on DiaComp Protocols with modification to get type 1 diabetes model mice. STZ (145 mg/kg of body weight - BW) injection was applicated on mice intraperitoneally. The preparation of STZ was done by dissolving STZ in a citrate buffer with a pH of 4.5. STZ dissolved in a citrate buffer solution (pH=4.5) must be injected immediately. Before STZ injection, the mice fasted for 4-6 hours. To discover the success of diabetes mellitus induction using STZ, blood glucose levels were measured using a glucometer on the 4th day after STZ injection. Blood samples were taken from the tails of mice by cutting the tip of the mice's tails. Blood sugar was then measured using a glucometer. Mice with blood glucose levels ≥200 mg/dL were considered positive for DM (3).

**Treatment of Moringa-albumin (MA) formulation** The extract of MO formulated with albumin from Ifalmin product was obtained from PT. Ismut Fitomedika (Ma-

kassar, Indonesia). Ifalmin is a product derived from Toman fish (*Channa micropeltes*). MA was mixed and dissolved in sterile aquadest with a specified dose. The combined dose of MA used was following the Food Drug Administration (FDA) (4). The treatment of MA was given orally for 14 days, followed by measurement of glucose in the blood every 3 days. This regular measurement was conducted to determine the effect of the MA formulation extract in reducing glucose level in blood plasma. The treatment dosages included D1, D2, and D3 (Table 1).

**Table 1. The comparison of the Moringa (M) and albumin (A) extracts given to each mouse***

| Mouse group | Comparison of MA formulation (%) | Comparison of MA formulation (mg/kg BW) |
|---|---|---|
| D1 | 25 M: 75 A | 500 (M): 620 (A) |
| D2 | 50 M: 50 A | 1000 (M): 420 (A) |
| D3 | 75 M: 25 A | 1500 (M): 200 (A) |

*The data presented as mean the doses for treatment MA formulation; BW, body weight; D1, Dose 1: 500 mg/kg BW (M): 620 mg/kg BW (A); D2, Dose 2: 1000 mg/kg BW (M): 420 mg/kg BW (A); D3, Dose 3: 1500 mg/kg BW (M): 200 mg/kg BW (A)

**Bone marrow cell isolation** The treated mice were dislocated by the neck and dissected. Bone marrow (BM) cells were isolated from the femur and tibia. The cell isolation was carried out by flushing with phosphate buffer saline (PBS). The resulting suspension was transferred to a 15 mL propylene tube. After all cell suspensions had been removed, the propylene tube was centrifuged at 2500 rpm, at 10 °C temperature for 5 minutes. The pellet was resuspended with 1 mL PBS before being homogenized. A sample of 50 µL was transferred to 1 mL Eppendorf containing 500 mL PBS and centrifuged at 2500 rpm, at 10 °C temperature for 5 minutes. The supernatant was aspirated and pellets were stained with extracellular and intracellular antibodies.

**Antibody staining and flow cytometry analysis** The centrifugation pellets were added with 1 µL of antibodies that had been diluted with 50 µl PBS and 10% fetal bovine serum (FBS) and incubated in a 4 °C icebox for 20 minutes. The antibody concentration was 0.005 mg/100 µL. Extracellular staining had been performed before intracellular staining. Intracellular antibody staining was performed on cell suspensions that had been fixed with 100 µL cytofix fixative solution and incubated for 20 minutes in an icebox. Then the cells were given 500 µL washperm according to the kit's use protocol. The suspension was again centrifuged at 2500 rpm, at 10 °C temperature for 5 minutes. The pellet was stained with intracellular antibodies and incubated for 20 minutes in an icebox. The cells that had been stained with extracellular and intracellular antibodies were added with PBS of 300-500 µL before being transferred to cuvettes and analysed with flow cytometry (FCM BD FACSCalibur, BD Biosciences, San Jose, CA).

**Statistical analysis**
The data from the flow cytometry were analyzed using BD Cellquest ProTM software. The analysed data were then statistically analysed by one-way parametric ANOVA (p≤0.05), followed by the Tukey test when treatments were significant to the parameters.

## RESULTS

**MA administration promotes the development of TER-119**
The profile of erythrocytes (TER-119) after the treatment for 14 days was analysed using flow cytometry. Based on the results of the analysis, it was stated that the relative amount of TER-119 in normal mice differed significantly compared with DM mice (p≥0.05). The relative amount of TER-119 in normal mice was 39.73%, while in DM mice it was 19.03%. Based on these results, it is clear that in DM mouse models there was a decline of relative amount TER-119 cells. Furthermore, the treatments were performed on mice that indicated DM using MA with a variety of different doses. The D3 administration gave a more significant effect than other doses in increasing TER-119 when compared to DM mice without treatment. The administration of D1 and D2 had no significant effect in either decreasing or increasing the relative amount of TER-119 compared to DM mice. The relative amount of TER-119 at D1 was 27.24%, D2 was 24.88%, and D3 was 41.35%, respectively (Figure 1).

**MA administration promotes the development of TER-119$^+$CD55$^+$**
Flow cytometry analysis on MA formulation treatment on the development of TER-119$^+$CD55$^+$ had been carried out. The obtained results showed that the MA formulation treatment was able to improve the development of TER-119$^+$CD55$^+$. The profile of TER-119$^+$CD55$^+$ in normal mice and DM mice had a significant difference. Normal mice expressed TER-119$^+$CD55$^+$ higher (68.17%) than DM mice (25.86%). It proved that DM sufferers had low TER-119$^+$CD55$^+$ expression levels. The provision of MA with D1 and D3 could significantly increase the TER-119$^+$CD55$^+$ level compared to D2. In addition, the administration of these doses was able to significantly increase the development of TER-119$^+$CD55$^+$ cells compared to the DM group. The expression of TER-119$^+$CD55$^+$ in D1, D2, and D3 treatments were 69.47%, 51.61%, and 67.45%, respectively (Figure 2).

**MA administration promotes the development of TER-119$^+$CD59$^+$**
Based on the analysis of the development of TER-119$^+$CD59$^+$ in healthy mice and DM mice showed a significant difference (p≥0.05). DM mice expressed TER-
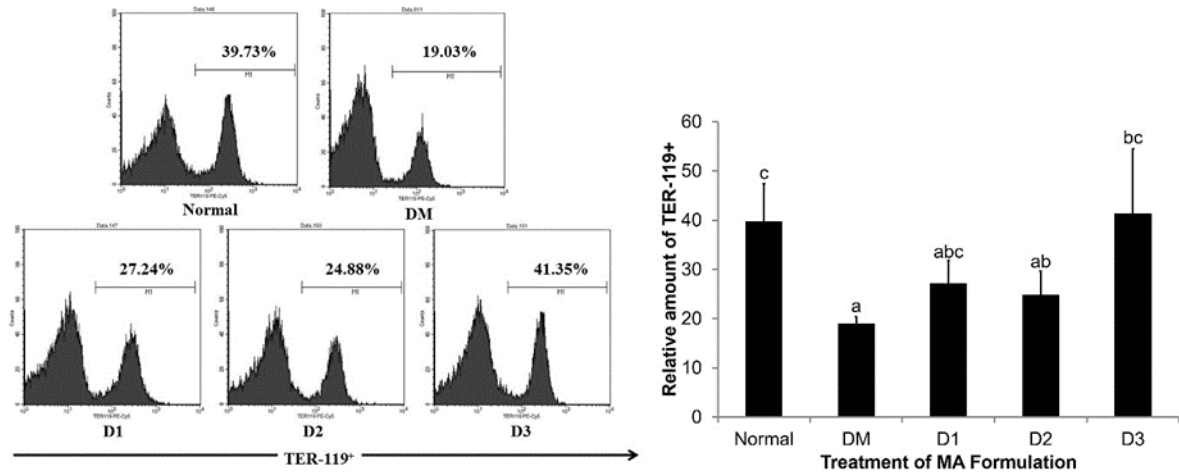
**Figure 1. The administration of Moringa-albumin (MA) triggers the development of erythrocytes (TER-119+) in different treatments. N, normal (healthy mice); DM, DM mice without treatment; D1 (500 mg/kg - M: 620 mg/kg - A); D2 (1000 mg/kg - M: 420 mg/kg - A); D3 (1500 mg/kg - M: 200 mg/kg - A)**

The data were obtained from the mean±SD in each treatment with a p≤0.05

M, Moringa; A, albumin

$119^+CD59^+$ lower than healthy mice. The administration of D1 and D2 did not significantly influence the development of TER-$119^+CD59^+$. However, the relative amount of TER-$119^+CD59^+$ in DM mice was lower than in normal mice and DM mice receiving MA treatment. D3 significantly influences the expression of TER-$119^+CD59^+$. The relative amount of TER-$119^+CD59^+$ on the normal, DM, D1, D2, and D3 are 25.43%, 4.71%, 9.03%, 13.25%, 23.81%, respectively (Figure 3).

## DISCUSSION

The focus of this research was to find herbal medicines coming from nature for treating DM effectively. We used natural ingredients derived from plants namely *Moringa oleifera* and animals namely *Channa micropeltes* believed to be able to treat DM sufferers. Based on the results of this study we presumed that the formulation of

MA was able to work synergistically in regulating free radicals produced by DM sufferers. The use of MA with D1, D2, and D3 was able to control or improve the condition of erythrocytes (TER-119) and complement regulatory proteins (CD55 and CD59). It is known that D3 can increase the relative amount of TER-119 significantly compared to other doses. This was presumably due to the active compound content in the formulation of MA able to control the life span of erythrocytes in people with DM. MA contains iron (Fe), magnesium (Mg), zinc (Zn), and copper (Cu) where these substances are needed as catalysts in the steps of haemoglobin synthesis [19]. Iron (Fe) is also able to stimulate the synthesis of the erythropoietin hormone [20]. In addition, Fe is known as an erythropoietic agent because it can stimulate the production of the EPO (erythropoietin) hormone in the kidneys, which will be transmitted to the bone marrow to stimulate erythrocyte production [21].
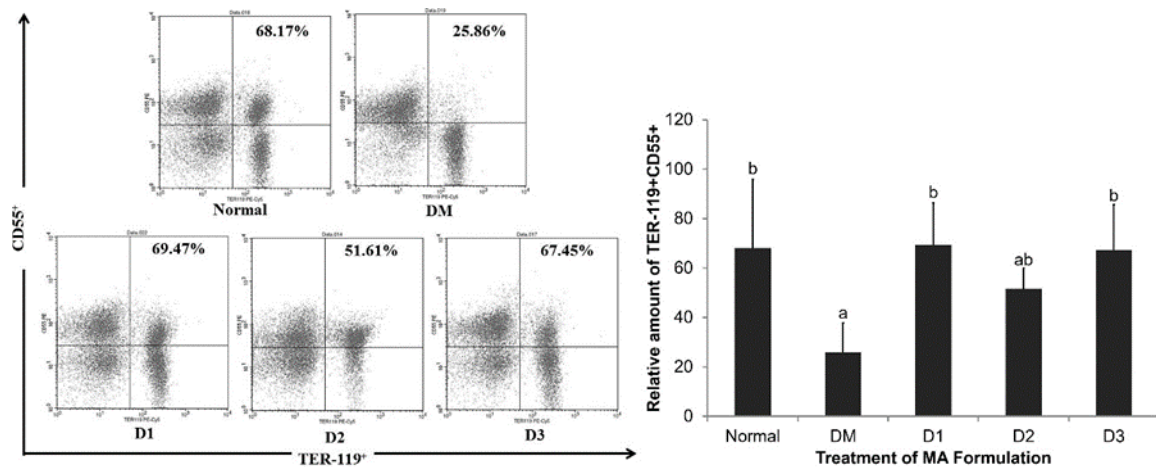


**Figure 2. The administration of Moringa-albumin (MA) can increase the expression of CD55+ molecules in erythrocytes. N = normal (healthy mice); DM, DM mice without treatment; D1 (500 mg/kg - M: 620 mg/kg - A); D2 (1000 mg/kg - M: 420 mg/kg - A); D3 (1500 mg/kg - M: 200 mg/kg - A)**

The data were obtained from the mean of ±SD in each treatment with a p≤0.05
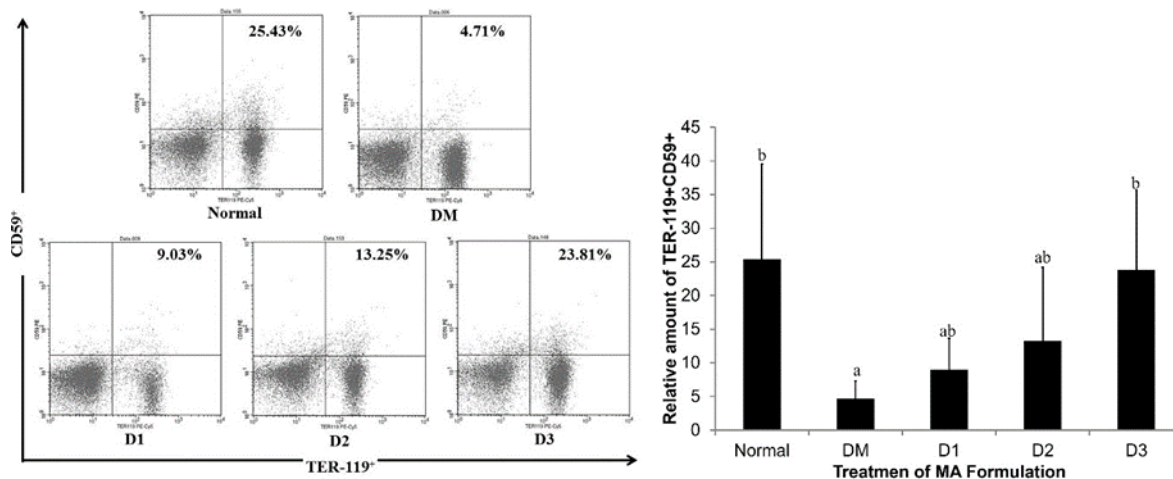
M, Moringa; A, albumin

**Figure 3. The administration of Moringa-albumin (MA) increases the expression of CD59+ molecules in erythrocytes. N = normal (healthy mice); DM, DM mice without treatment; D1 (500 mg/kg - M: 620 mg/kg - A); D2 (1000 mg/kg - M: 420 mg/kg - A); D3 (1500 mg/kg - M: 200 mg/kg - A)**
The data were obtained from the mean of ±SD in each treatment with a p≤0.05
M, Moringa; A, albumin

The EPO hormone stimulates erythropoiesis by increasing the number of erythrocyte progenitor cells to proliferate and differentiate to form haemoglobin (22).

In addition, the omega-3 fatty acid content in MA was presumed to play an important role in modulating the development of erythrocyte counts. Omega-3 also had a role to reduce the amount of malondialdehyde (MDA) significantly. The accumulation of free radicals occurring in diabetes increases lipid peroxide known to be able to trigger rigidity of the erythrocyte membrane. Malondialdehyde (MDA) produced through lipid peroxide can affect the intrinsic mechanical properties of the erythrocyte membrane which can cause deformability in erythrocytes or the ability for decline of erythrocytes to change its form (23). In addition, MA also has antioxidant properties that are able to regulate ROS activity occurring due to β cell damage caused by the STZ exposure (24). STZ causes the deposition reaction of ATP and helps xanthine oxidase in the formation of superoxide and reactive oxygen species (ROS) in β cells (25). A person suffers from hyperglycaemia because the β cells are damaged. High glucose levels will enter the mitochondria and activate the mechanism of ROS release. Damage and apoptotic mechanisms occurring in beta cells are triggered by low antioxidants (26). Antioxidants present in MA were hypothesized to be able to work to control ROS released from mitochondria, thus protecting beta cells and then controlling hyperglycaemia (27,28).

Our results showed reduced erythrocytes (19.03 %) in DM presuming to be due to the loss or decrease in expression of CD55 and CD59 complement regulatory proteins. The presence of CD55 and CD59 bound by glycosylphosphatidylinositol (GPI) is very important for the survival of the erythrocyte. According to our results, the expression of CD55 and CD59 in erythrocyte DM

mice was less than normal mice. It was presumed that in hyperglycaemic conditions the synthesis of CD55 and CD59 molecules was reduced or even lost at the beginning of the development of erythrocytes. In addition, according to Nie et al. (29), STZ in normal mice caused a significant expression decline of CD55 in spinal dorsal 3 days after the STZ injection which continued for 28 days. The expression decline of CD55 has an important role in the occurrence and development of diabetic nephropathy (DNP), which may be the initiator of abnormal complement activation involved in the occurrence and development of neuropathic pain (NPP) (30).

The decreased expression of CD55 and CD59 molecules was presumed to be due to the mutation of the PIG-A gene triggering the emergence of paroxysmal nocturnal haemoglobinuria (PNH) disease. PNH is a kind of disorder associated with abnormalities in hematopoietic stem cells caused by the mutation of the PIG-A gene. The PIG-A gene is an enzyme-coding gene that is needed in the early stage of the biosynthesis of glycosylphosphatidylinositol (GPI). PIG-A mutations trigger the decline or loss of protein expressed on cell surfaces classified as GPI-anchored protein including complement regulatory proteins such as (CD55 and CD59) (31,32).

The effect of MA on D1 (500 mg/kg BW (M) + 620 mg/kg BW (A)) and D3 (1500 mg/kg BW (M) + 200 mg/kg BW (A)) can significantly increase or maintain CD55 expression in erythrocytes (TER-119$^+$CD55$^+$). However, D2 (1000 mg/kg BW (M) + 420 mg/kg BW (A)) did not significantly influence the relative amount (TER-119$^+$CD55$^+$) compared to DM (mice STZ injection, blood glucose ≤ 200 mg/dl). At D2 a transduction signal was presumed to appear suppressing the synthesis of the CD55 molecule.

However, the limitation of this study is an inability to explain how the mechanism of suppression of CD55 molecular synthesis. It presumably occurred because MA was immunomodulatory, which can potentially be an immunostimulant, on the other hand, MA was also an immunosuppressor depending on the amount of dose given.

In conclusion, administration of MA (a combination of *Moringa oleifera* and *Channa micropeltes*) had a potential of being an alternative treatment for DM by maintaining the expression of complement regulatory proteins (CD55 and CD59) bound by GPI to erythrocytes. Overall, administration of MA D3 group with 1500 mg/kg (M) + 200 mg/kg (A) affected the increase in TER-119, CD55 and CD59 compared to DM without treatment.

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## TRANSPARENCY DECLARATION

Conflict of interests: None to declare.

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