Moringa oleifera extract regulates the expression of some sickle cell related genes in normal Wistar rats

[Extracto de *Moringa oleifera* regula la expresión de algunos genes relacionados con la anemia falciforme en ratas Wistar normales]

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Abstract

Context: In the current study, we employed gene-regulatory based approach to underscore the putative mechanism of how components of *Moringa oleifera* (MO) ethanol extract exert its antisickling effects.

Aims: To evaluate the relationship between mRNA expression profiles of some sickle cell related genes in normal rats.

Methods: Normal Wistar rats were administered with MO at 50 and 100 mg/kg body weight and the positive control group administered with folic acid for 14 days. The bone marrow and kidneys were harvested, followed by RT-PCR to assess the expression of Gardos channel [kccn1, kccn4], antioxidant [CAT, G6PD], and progression of meiosis [p21, p27] genes. Hematological parameters were also analyzed.

Results: A downregulation of the Gardos channel genes (KCNN-4 and KCNN-1) was observed on MO intervention at 100 mg/kg while folic acid showed no significant expression. Significantly ($p < 0.05$), MO intervention increased antioxidant system in sickle cell anemia scenario due to the expression pattern of antioxidant genes analyzed in this study and caused changes in hematological parameters such as PCV, HBC, MCH relative to the control groups.

Conclusions: The study concluded that the administration of the ethanol extract of *Moringa oleifera* leaf increased blood production, has high antioxidant properties and reduced the expression of the Gardos channel pathway. These are important factors in the management of sickle cell disease and MO could be a candidate of interest in drug development.

Keywords: antioxidant; antisickling; Gardos' channel; gene expression; *Moringa oleifera*.

Resumen

Contexto: En el estudio actual, empleamos un enfoque basado en la regulación de genes para subrayar el mecanismo putativo de cómo los componentes del extracto de etanol de *Moringa oleifera* (MO) ejercen sus efectos antidrepanocíticas.

Objetivos: Evaluar la relación entre los perfiles de expresión de ARNm de algunos genes relacionados con la anemia falciforme en ratas normales.

Métodos: A ratas Wistar normales se les administró extracto de MO a 50 y 100 mg/kg de peso corporal y al grupo control positivo se le administró ácido fólico durante 14 días. Se recolectaron la médula ósea y los riñones, seguidos de RT-PCR para evaluar la expresión de los genes del canal de Gardos [kccn1, kccn4], antioxidantes [CAT, G6PD] y progresión de la meiosis [p21, p27]. También se analizaron parámetros hematológicos.

Resultados: Se observó una regulación a la baja de los genes del canal Gardos (KCNN-4 y KCNN-1) con la intervención de MO a 100 mg/kg, mientras que el ácido fólico no mostró una expresión significativa. Significativamente ($p < 0.05$), la intervención de MO aumentó el sistema antioxidante en el escenario de anemia de células falciformes debido al patrón de expresión de los genes antioxidantes analizados en este estudio y provocó cambios en los parámetros hematológicos como PCV, HBC, MCH en relación con los grupos de control.

Conclusiones: El estudio concluyó que la administración del extracto etanólico de hoja de *Moringa oleifera* incrementó la producción de sangre, tiene altas propiedades antioxidantes y redujo la expresión de la vía del canal de Gardos. Estos son factores importantes en el manejo de la enfermedad de células falciformes y MO podría ser un candidato de interés en el desarrollo de fármacos.

Palabras Clave: antidrepanocítica; antioxidante; canal de Gardos; expresión génica; *Moringa oleifera*.

INTRODUCTION

Sickle cell anemia is an inherited disease characterized by the presence of chronic haemolysis, infections and recurrent occlusion of microcirculation which results in episodes of painful crises and organ damage (Kennedy et al., 2015). Ion channel transport imbalance and drastic reduction in erythropoietin level which truncate erythropoiesis are key factors that fuel the severity of this disease. The erythropoietin (EPO) is a glycoprotein hormone that regulates the production of red blood cell process (erythropoiesis) (Cernaro et al., 2019). It is synthesized by the interstitial fibroblasts (Olmos et al., 2018) in the kidney in close association with peritubular capillary and tubular epithelial tubule. In the absence of erythropoietin, specific erythropoiesis does not occur. However, under hypoxic situations, the kidney will synthesize and secrete erythropoietin to elevate the synthesis of erythrocytes. Scientists have intensively studied the pivotal role of erythropoietin and its cell surface receptor (EPO-R) in the mediation of red blood production. In severe anemia condition like the sickle cell anemia, EPO expression has been reported to be reduced (Nnodim et al., 2015), which may lead to short-circuiting of erythropoiesis. Ion transport pathways play a pivotal role in the transmembrane ionic distribution that allow erythrocyte to maintain various functional characteristics (Maher and Kuchel, 2003). Folate (folic acid) has been reported to have crucial roles in erythropoiesis (Casadevall, 1995).

Considerable evidence exists that red blood cells (RBC) are oxidatively damaged *in vivo* and red cell lipids and proteins may be the target of these oxidants (Prasartkaew et al., 1988). The cells have limited ability to repair such damage, the cumulative injury resulting from oxidation can contribute to cell death. In red cells characterized by congenital or acquired structural defects, susceptibility to oxidant injury is often increased (Stocks et al., 1972). The stress placed on the membrane during shape change, particularly in pathologic red cells, may alter membrane organization and increase susceptibility of lipid and protein domains to oxidant injury (Weiss, 1980). Repeated polymerization leads to a cyclic cascade inciting blood cell adhesion, vaso-occlusion, and ischemia- reperfusion injury (Belcher et al., 2010). Studies have shown relationships between markers of oxidative stress and common secondary diseases in SCD, such as Acute Chest Syndrome and Pulmonary Hypertension (Morris et al., 2003). The antioxidant activity of natural compounds is now intensively studied due to the current growing demand from the pharmaceutical and food industries, which are interested in natural bioactive compounds that possess health benefits.

Red cell sickling can be measured by the amount of potassium efflux from deoxygenated, haemoglobin S-containing red cells *in vitro*, particularly during tests with antisickling agents (Roth et al., 1981). This is critical for erythrocytes from patients with sickle cell disease (SCD), where decreased cell volume and hence increased haemoglobin concentration promotes HbS polymerization under deoxygenated conditions, leading eventually to irreversible sickling (Eaton and Hofrichter, 1990). In Hb S RBCs, one pathway of solute loss is mediated via the K^+–Cl^- cotransporters (KCC, probably KCC3 and KCC1; Gamba, 2005), which are abnormally active in red cells from SCD patients (Brugnara et al., 1986; Crable et al., 2005). Another involves a non-selective ion and small solute permeability pathway, often termed P_{sickle}, which acts as a direct efflux pathway for K^+ loss but also promotes Ca^{2+} entry and hence activation of the Gardos (Ca^{2+}-activated K^+) channel pathway (Hoffman et al., 2003; Joiner, 1993; Lew et al., 1997). The consequence is rapid K^+ loss with Cl^- following via a separate conductive pathway. Blockade of potassium loss from the erythrocyte should, therefore, prevent the increase in HbS concentration and reduce erythrocyte sickling. Two ion transport pathways, the K-Cl cotransport and the Ca^{2+}-activated K^+ channel play prominent roles in the dehydration of sickle erythrocytes. Possible therapeutic strategies include inhibition of K-Cl cotransport by increasing red cell Mg^{2+}content and inhibition of the Ca^{2+}-activated K channel by oral administration of clotrimazole.

Blood parameters are key indicators in diagnosing the actual physiological status of an organism (Pankaj and Varma, 2013). The assessment of hematological parameters can be diagnostic of adverse effects of foreign compounds on the blood constituents of an animal (Ashafa et al., 2012). Administration of the chemical compounds at toxic doses often results in changes in blood parameters that are indicative of hematological disorders such as anemia which is characterized by low hemoglobin content (Price and Schrier, 2008), neutropenia, which occurs in cases of reduced production of white blood cells or increased utilization and destruction, or both.

Medicinal plant is an important element of indigenous medical systems all over the world. The ethnomedicine provides a rich resource for natural drug research and development (Aziz et al., 2018; Ghafari et al., 2018). Experimental evidence has revealed the extract of *Moringa oleifera* (Adejumo et al., 2012; Cyril-Olutayo et al., 2018) to be quite potent and ameliorates the adverse effect of sickle cell anemia, but the mechanism is still unclear. In the current study, we

employed gene-regulatory based approach to underscore the putative mechanism of how components of *Moringa oleifera* exert its antisickling effect. The erythropoiesis index, the antioxidant properties, Gardos channel index, and hematological parameters were evaluated.

MATERIAL AND METHODS

Plant material

Moringa oleifera Lam. (MO) (family *Moringaceae*) leaves were collected at the Department of Civil Engineering, OAU Ile-Ife, Nigeria (latitude 7°31'14.7612" N and longitude 4°31'49.1340" E). It was identified, and authenticated by B.E Omomoh at the IFE Herbarium, OAU, Ile-Ife and a voucher specimen was deposited with voucher number IFE 17255. MO leaves were oven dried at 40°C, and extracted by macerating in absolute ethanol for 72 h after which the extract was filtered and evaporated to dryness *in vacuo* at 40°C.

Animal handling

Laboratory animals (Wistar rats) were divided into four groups of six rats each. Groups A and B received *Moringa oleifera* extracts at 50 mg/kg and 100 mg/kg body weight, respectively, while groups C and D represented the positive control and placebo groups administered with folic acid (50 mg/kg) and distilled water, respectively. The samples were administered for 14 days, after which the animals were humanly sacrificed. Blood samples from the heart were collected for hematological analysis, while tissues of the bone marrow, liver, and kidney were collected for RNA isolation.

Animal beddings were changed three times per week and the animals fed with standard diet and water *ad libitum*. All experiments were carried out according to the guidelines for care and use of experimental animals and approved by Institutional Animal Ethical Committee of Afe Babalola University, Ado-Ekiti (ABUADREC 887359-DR12020/214).

RNA isolation and cDNA preparation

The tissues (bone marrow of tissues femoral shaft, liver and kidney) for RNA isolation were homogenized in 100 µL RNAse cocktail (4 M guanidinium isothiocyanate, 20 mM sodium acetate, 0.1 mM dithiothreitol, 0.5% N-luroylsarcosine, pH 5.5), 200 µL phenol: chloroform: isopropanol (125:49:1 v/v/v) solution was added to the homogenate and incubated at 4°C for 20 min. RNA was pelleted (10,000g, 20 min) from the supernatant (100 µL) after incubating at 4°C for 1 hr in ammonium acetate (5 µL, 0.2 M) and isopropanol (100 µL). RNA samples were washed twice

in 75% ethanol and dissolved in 25 µL nuclease-free water. The purity (O.D 260/180 >1.8) and concentration of the RNA were determined spectrophotometrically. DNA contaminant was removed following DNAse I treatment (ThermoFisher Scientific) following manufacturer's protocol. 100 ng DNA-free RNA was converted to cDNA immediately using M-MLV reverse transcriptase (GibcoBRL, Carlsbad, CA) in a 20 µL of reaction buffer containing 3 µL of random primers, and 1 mM dNTPs, following incubation at 42°C for 52 min. The reaction was terminated at 65°C (5 min). All experiments were done in triplicates (n = 3).

PCR and gel electrophoresis

PCR was performed in a final reaction volume of 50 µL containing 25 µL mater mix (Thermo Scientific), 2 µL of cDNA, 1 µL (10 mM) each of forward and reverse primers (Table 1) and 21 µL nuclease-free water. The PCR was programmed as follows: 95°C/5 min, 30 cycles of (95°C/ 30 sec, (Tm-4)°C/45 sec, 72°C/1 min) and 72°C for 5 min. All experiments were done in triplicates (n = 3).

Quantification of potassium efflux protocol

Preparation of plant extracts

Moringa oleifera extract was first dissolved completely with DMSO (0.1%) before final dissolution in distilled water to make a stock concentration of 100 mg/mL.

Preparation of red blood cells

Erythrocyte isolation from sickle-cell patient whole blood samples were performed based on the protocols developed by Evans et al. (2013), and the experiments were performed in triplicates (n = 3).

Quantitation of potassium (K^+) leakage from RBC

Aliquot containing 20 µL RBC was mixed with 2 µL phosphate buffer (pH 7.4) containing 0 (control), 3, 10, 30 or 100 M mannitol to stimulate K^+ leakage from the RBC. Two additional experiments were performed to test the effects of MO extract or folic acid (FO) on mannitol-induced K^+ leakage from the RBC. In both test experiments, the reaction buffer cocktail contained 3 mg/L (final concentration) of MO or FO. In all experiments, incubation was performed at room temperature for 3600 seconds, followed by centrifugation at 2500 rpm for 5 min. Supernatants were collected for K^+ quantification using potassium reagent kit (TECO® Diagnostic, Lakeview, CA) following manufacturer's recommendations. All experiments were done in triplicates (n = 3).

Table 1. Forward and reverse primer set.

Primer name	5'-Forward sequence -3'	5'-Reverse sequence -3'
Erythropoietin	AGGCGCGGAGATGGGGGTGC	ATCGGATGTGGGTGGTCATAGG
Erythropoietin receptor	TTGAGAGCAAAGCGGCCCTGCT	ATCGGATGTGGGTGGTCATAGG
rKCCN4	CACCTTGCCCTGGAGAAGA	GTCCTGAGTAGAATGTAGATCGA
rKCCN1	CCTGGAGTTGGGTTGTCTAAGA	CATCAGCCCTCACCAGTCATCTC
G6pDD	AGCCTGGCGTATCTTCACAC	GCTCAGAGCTTGTGAGGGTT
Catalase	GTGCATGCATGACAACCAGG	GAATGTCCGCACCTGAGTGA
p21	GACATCTCAGGGCCGAAAAC	CGGCGCTTGGAGTGATAGAA
p27	CCTTCGACGCCAGACGTAAA	AGCAGTGATGTATCTAATAAACAAGGAATT
β-actin	GTCGAGTCCGCGTCCAC	AAACATGATCTGGGTCATCTTTTCAC

All oligonucleotides were products of Inqaba Biotec (South Africa). rKCCN4= Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4, rKCCN1= Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1, G6pDD= Glucose-6-Phosphate Dehydrogenase

Quantitation of leaked hemoglobin from RBC

Aliquot containing 200 µL RBC was mixed with 10 µL buffer containing 0 (control), 3, 10, 30 or 100 mM hydrogen peroxide (H_2O_2) to stimulate hemoglobin (Hb) leakage resulting from RBC membrane lipoperoxidation. Two additional experiments were performed to test the ability of MO extract or folic acid (FO) to reverse H_2O_2-induced Hb leakage from the RBC. In both test experiments, the reaction buffer cocktail contained 30 mg/L (final concentration) of MO or FO. In all experiments, incubation was performed at 37°C for 180 min, followed by centrifugation at 2500 rpm for 5 min. Supernatants were collected for Hb quantification using Drabkins solution kit (Fortress Diagnostics, Lot#: 482-01-038/2) following manufacturer's recommendations. All experiments were done in triplicates (n = 3).

Statistical analysis

Unless otherwise stated, all experiments were done in triplicates and all absorbance values were read off modulus microplate reader (Turner, Biosystems). All Statistical analyses were performed on Graph pad Prism (ver. 7.0). For dose-response curves, EC_{50} values were calculated using ordinary fit of log of agonist vs. normalized response (three parameters) and $p \leq 0.05$ was considered significant. The data was represented as sample mean of a triplicate experiment and expressed as means ± SEM.
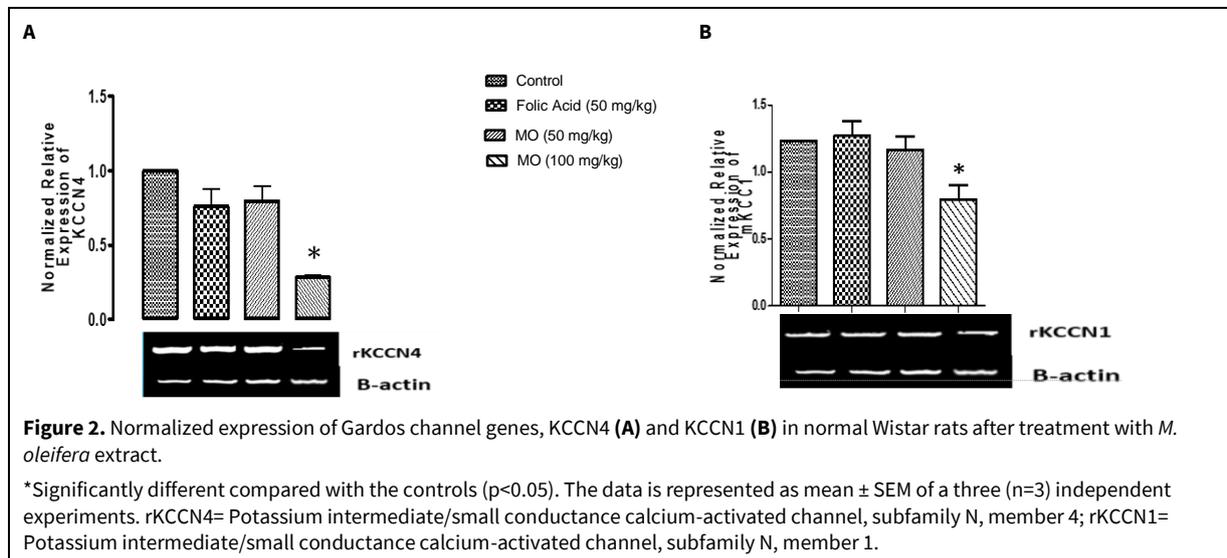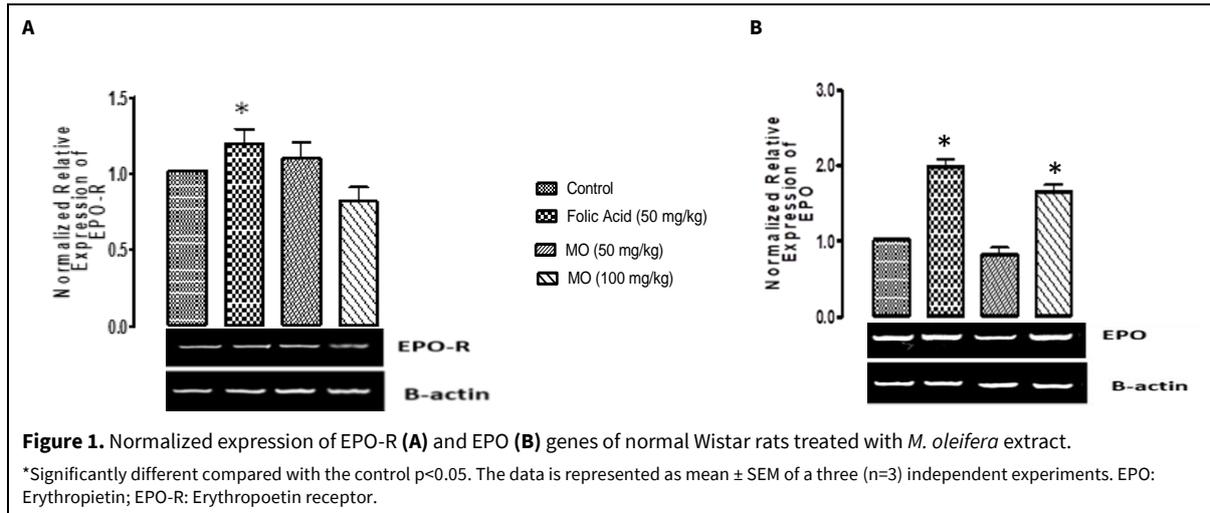
RESULTS

From the mRNA profiling of erythropoietin receptor and its cognate ligand (erythropoietin), folic acid at 50 mg/kg showed the upregulation of both EPO and EPO-R, thus claim its reported ability in erythro-poiesis mechanism. On MO exposure to the treatment groups at 50 mg/kg and 100 mg/kg, there was no significant mRNA expression of EPO-R when compared to the basal control and folic acid group. For EPO mRNA profiling however, MO treated groups especially at 100 mg/kg showed more expression compared to the control group, but no significant expression was observed when compared to folic acid group (Fig. 1A-B).

As sickle cell dehydration is thought to result from a complex interplay of Hb S polymerization and several cation transport systems in sickle cells. A transport pathway that normally regulates volume in reticulocytes, the potassium-chloride cotransporter (KCC) appears to function pathologically in sickle cells, overshooting its target hemoglobin concentration (Brugnara, 2018) and priming the reticulocyte to sickle. Hb S polymerization activates a nonselective cation leak pathway in a fraction of sickle cells upon deoxygenation. Calcium entry via this sickling-induced pathway triggers activation of the Gardos channel, which mediates rapid KCl and water loss (Lew et al., 2002). Abnormal KCC activity in the sickle reticulocyte may thus facilitate a vicious spiral in which sickling and Gardos channel activation reinforce each other to dehydrate the cell. This makes it a therapeutic target for sickle cell disease (Brugnara et al., 1993). From the result of mRNA profiling of the gene that encode Gardos channel (KCNN-4), the folic acid at 50 mg/kg showed no significant expression when compared to the control. But downregulation was observed on MO intervention at 100 mg/kg (Fig. 2A).

The observed downregulation may alter the protein structure function that dictate its inhibition. The result here showed significant correlation with work

Figure 1. Normalized expression of EPO-R **(A)** and EPO **(B)** genes of normal Wistar rats treated with *M. oleifera* extract.

*Significantly different compared with the control p<0.05. The data is represented as mean ± SEM of a three (n=3) independent experiments. EPO: Erythropietin; EPO-R: Erythropoetin receptor.

Figure 2. Normalized expression of Gardos channel genes, KCCN4 **(A)** and KCCN1 **(B)** in normal Wistar rats after treatment with *M. oleifera* extract.

*Significantly different compared with the controls (p<0.05). The data is represented as mean ± SEM of a three (n=3) independent experiments. rKCCN4= Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4; rKCCN1= Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1.

by Brugnara et al. (1993). The maintenance of intracellular Cl^- concentration is critical for the normal function of many physiologic processes, including regulation of red cell volume (Gamba, 2005). Intracellular Cl^- concentration is controlled in part by the K-Cl cotransporters, which pump Cl^- and K^+ ions with the obligatory movement of water out of the cell. Brown et al. (2015) reported the important new insights into the regulation of K-Cl cotransporters and provides *in vivo* evidence that increased KCC activity worsened end-organ damage and diminished survival in sickle cell. This drives the current study to profile mKCC-1 gene. From the mRNA expression result, folic acid group showed no significant expression when compared to the control. Interestingly, upon MO intervention there is observed downregulation of the gene (Fig. 2B), which correlate with the work by Brown et al. (2015), this suggests that MO may be a good regulator of ion channel that fuel this genetic disorder.

SCD is triggered by a mutation in a gene called β-globin, which switches one amino acid called β6 glutamic acid with another named valine (Townes and McCune, 1999), leading to increased levels of plasma cell-free hemoglobin, as well as reactive oxygen species (ROS), which is accountable for oxidative stress (Olsson et al., 2010). The generation of ROS may cause damage in the system and amplify various conditions like inflammation and immunologic disorders (Ricklin et al., 2010). Sickle cell disease patients has been reported to produce higher levels of ROS, which make them subject to increased oxidative stress (Amer et al., 2006). The best defense method against ROS and oxidative stress are antioxidants, including molecules like glutathione, vitamins C and E, and the enzyme superoxide dismutase, catalase, GSR (Iuchi, 2012). Recently, several reports have suggested that oxidative stress is a complex mechanism rather than a simple imbalance between the production and elimi-

nation of ROS. Oxidants and free radicals are continuously produced in living organisms with endogenous and external sources such as oxygen and nitric oxide [reactive nitrogen species (RNS). Chronic redox imbalance in erythrocytes of individuals with sickle cell disease (SCD) contributes to oxidative stress and likely underlies common etiologies of hemolysis (Cho et al., 2010). Considering the several molecular mechanisms that have been proposed to contribute towards a high oxidative burden in sickle cell patients. Some of the mechanisms that disturb the redox state include, the excessive levels of free hemoglobin that catalyze the Fenton reaction (Chirico and Pialoux, 2012). It has been reported that SCD patients will require an increased antioxidant capacity to combat oxidative stress (Kimmons et al., 2009) due to increased ROS generation in the disease scenario. Here, we profiled battery of anti-oxidant system gene in response to MO. Understanding the erythrocyte protective role of G6PD against oxidative stress, it is the key regulatory enzyme of the pentose phosphate pathway, which is essential for the supply of reduced NADPH. NADPH enables cells to counterbalance oxidative stress that can be triggered by several oxidant agents, and to preserve the reduced form of glutathione (Manganelli et al., 2010). Since erythrocytes lack mitochondria, the pentose phosphate pathway is the only source of NADPH; therefore, defense against oxidative damage is dependent on G6PD. From the mRNA expression profiling of the gene (G6PDD), when the folic acid at 50 mg/kg group was compared to control group, there is observable significance in the expression pattern. Upon MO intervention at 50 and 100 mg/kg, there was also significant up regulation when compared to the control group (Fig. 3). GPx-1 as one of the batteries of the antioxidant system showed slight downregulation when folic acid was compared to the control group. Interestingly, MO group at both doses (50 and 100 mg/kg) showed no significant difference in the expression when compared to the control. In expression pattern of catalase gene (CAT) in response to MO, when folic acid group was compared to the control group, there is no observable difference, Upon MO intervention, there is spike in the expression of catalase compared to the control group (Fig. 3). Here, we infer that MO may increase antioxidant system in sickle cell anemia sce-

nario via the spike in the expression pattern of antioxidant gene underscored in this study.

Cell cycle mechanism is one of the daily erythropoietic output that requires enormous progenitor cell expansion to generate massive numbers of erythroblasts (EBs), followed by cell cycle withdrawal at the final stages of differentiation, during the occurrence of hemoglobinization and cellular remodeling (Randle et al., 2015). Careful regulation of the G1 phase is crucial for erythropoiesis, and both pro-proliferative and anti-proliferative activities are essential. Here, we underscore some key genes that are involved in governing G-phase during cell cycle progression. Cyclin D is a member of the cyclin protein family that is involved in regulating cell cycle progression. The synthesis of cyclin D is initiated during G1 and drives the G1/S phase transition (Sherr and Sicinski, 2018).

Understanding the continuous self-renewal process of erythroid progenitors in erythrocyte formation. It has been documented that in the first 72 h the cells undergo five cell divisions. Thereafter, they arrest in G1 and complete their maturation into red blood cell (RBC) without further divisions. There is reduction in the length after the induction of differentiation which bring about the drastic reduction of G1 while S-phase length is intact. At the same time, the differentiating cells underwent an extensive and concerted switch in their gene expression pattern. Interestingly, the shortening of G1 was accompanied by a rapid down-regulation of D-type cyclins and their partner, cyclin-dependent kinase type 4 (cdk4), while expression of S- and G2-M-associated cell cycle regulators (cyclin A and cdk1/cdc2) remained high until the cells arrested in G1 72-96 h after differentiation induction (Dolznig et al., 1995). In this study MO extract down regulated the expression of both the p21 and p27 genes at 100 mg/kg (Fig. 4) and this expression is evident in the hematological parameters such as the RBC count, PCV, and Hb concentration. Administration of MO increased hematological parameters significantly when compared to folic acid and the basal control (Table 2). There was evidence that MO stimulated RBC production and new blood cells were being produced. In general, MO caused significant (p<0.05) change in hematological parameters relative to control group.
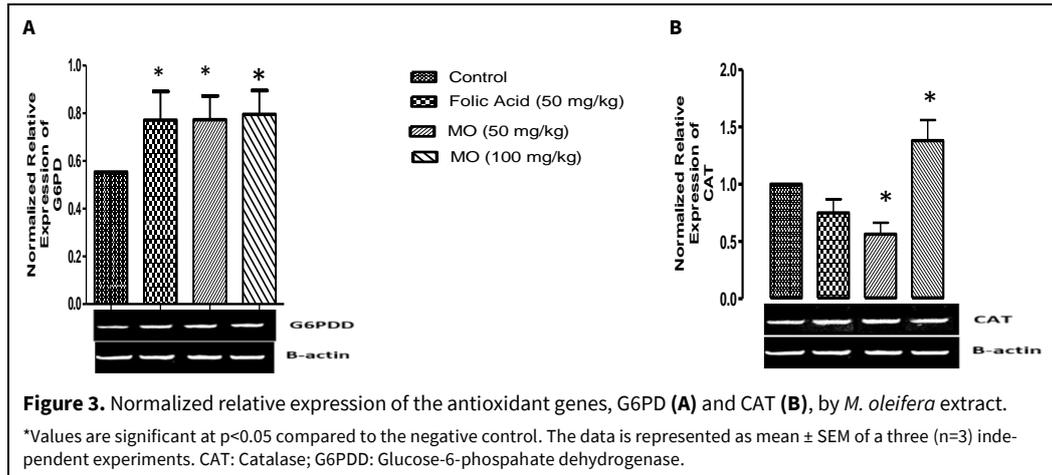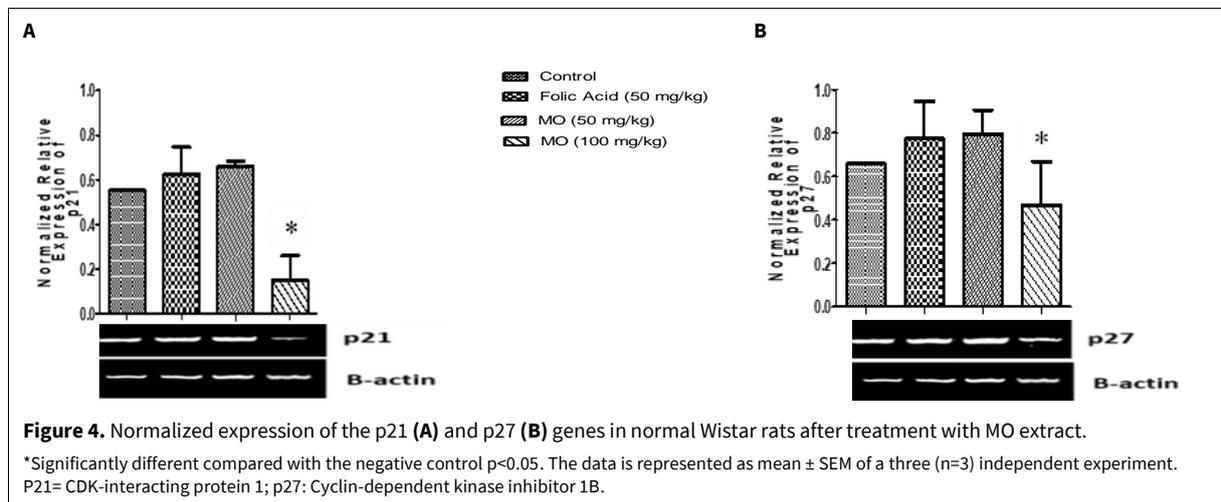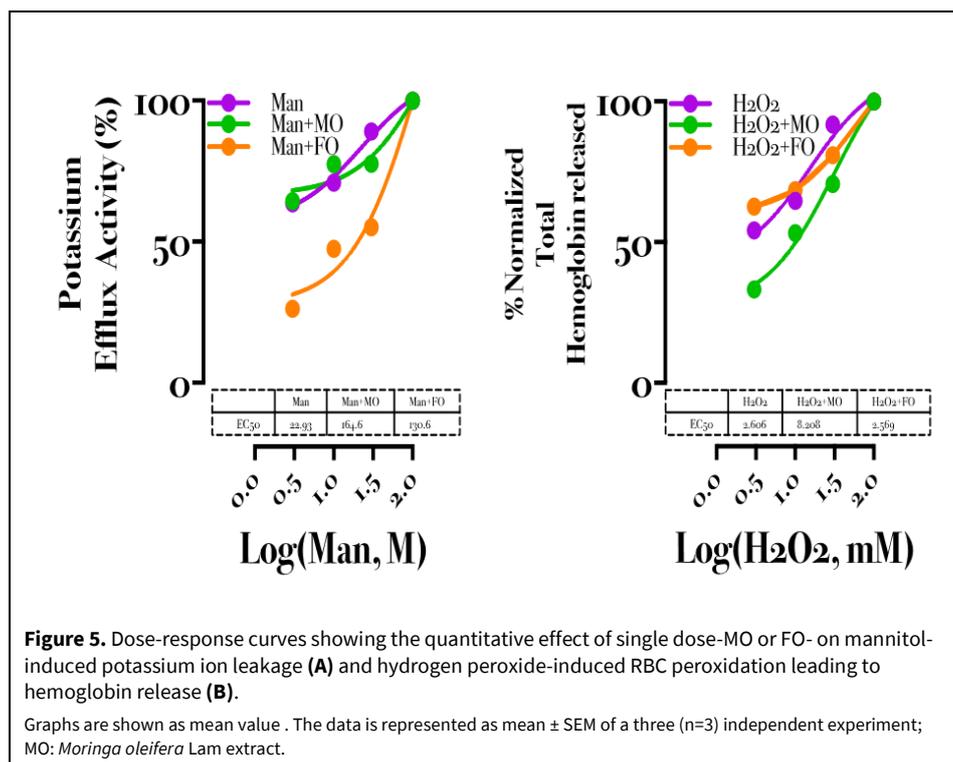
Figure 3. Normalized relative expression of the antioxidant genes, G6PD **(A)** and CAT **(B)**, by *M. oleifera* extract.

*Values are significant at p<0.05 compared to the negative control. The data is represented as mean ± SEM of a three (n=3) independent experiments. CAT: Catalase; G6PDD: Glucose-6-phospahate dehydrogenase.

Table 2. Effect of *M. oleifera* (MO) extracts on hematological parameters of rats.

Samples (mg)	PCV	RBC	HB	MCHC	MCH	MCV
Control	39.00 ± 2.89^a	6.21 ± 0.35^a	13.50 ± 0.93^a	33.00 ± 0.12^a	19.33 ± 0.33^a	59.00 ± 1.16^a
Folic acid	43.67 ± 1.73^b	7.10 ± 0.06^b	14.27 ± 0.52^a	34.00 ± 0.10^b	20.63 ± 0.33^a	60.64 ± 1.09^b
MO (50)	41.67 ± 1.86^a	6.89 ± 0.42^b	14.09 ± 0.61^a	33.00 ± 0.88^a	19.00 ± 1.00^a	58.00 ± 2.00^a
MO (100)	45.00 ± 0.58^b	7.55 ± 0.73^c	15.00 ± 0.17^b	35.00 ± 0.35^c	21.10 ± 0.10^b	63.38 ± 0.29^c

Values with different alphabets in superscripts are significantly different (p<0.05), values with same alphabet in superscript are comparable (p>0.05). The data is represented as mean ± SEM of a six (n=6) independent experiment. PCV: Packed cell volumes; RBC: Red blood cell; HB: Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; MHC: Mean Corpuscular Hemoglobin; MCV: Mean Corpuscular Volume.

Figure 4. Normalized expression of the p21 **(A)** and p27 **(B)** genes in normal Wistar rats after treatment with MO extract.

*Significantly different compared with the negative control p<0.05. The data is represented as mean ± SEM of a three (n=3) independent experiment. P21= CDK-interacting protein 1; p27: Cyclin-dependent kinase inhibitor 1B.

Figure 5. Dose-response curves showing the quantitative effect of single dose-MO or FO- on mannitol-induced potassium ion leakage **(A)** and hydrogen peroxide-induced RBC peroxidation leading to hemoglobin release **(B)**.

Graphs are shown as mean value . The data is represented as mean ± SEM of a three (n=3) independent experiment; MO: *Moringa oleifera* Lam extract.

Hematological parameters

Loss of erythrocyte K+ has been identified as a key driver of cellular integrity via dehydration (Agoreyo and Nwanze, 2010). Incubation of SS-RBC with MO increased the EC50 to 164.6 ± 12.60 M while incubation with FO increased the EC50 value to 130.6 ± 22.10 M (Fig. 5).

Potassium ion efflux

This data strongly indicates that in the presence of either MO or FO, there is an apparent arrest of RBC K^+ leakage; which in turn prevents dehydration (Glader and Sullivan, 1979; Gallagher, 2017; Glogowska and Gallagher, 2015) of SS-RBC. Furthermore, when RBC is exposed to oxidants including hydrogen peroxide, loss of membrane integrity is often associated with loss of intracellular hemoglobin (Peralta et al., 2013) extracts from plants have been shown to block the process. The EC_{50} for hemoglobin leakage from SS-RBC in the presence of MO extract was estimated to be 8.208 ± 2.10 mM. These results provide evidence that in addition to the ability of MO and FO to alter erythropoiesis, they exhibit divergent mechanisms for SS-RBC protection. While FO preferentially inhibits transmembrane K^+ leakage, MO both protects K^+ leakage and membrane integrity; allowing cellular maintenance of K^+ and hemoglobin levels. These effects are consistent with antisickling potencies as observed in some compounds such as BT1118, which is under clinical study for the management is sickle cell disease (Al Balushi et al., 2019).

CONCLUSION

It can be inferred from our study that *Moringa oleifera* ethanol extract expressed strong antioxidant properties, increased red blood cell production and can be used in the development of antisickling remedy.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTION:

Contribution	Cyril-Olutayo MC	Omotuyi IO
Concepts or ideas	x	x
Design	x	x
Definition of intellectual content	x	x
Literature search	x	
Experimental studies		x
Data acquisition	x	x
Data analysis	x	
Statistical analysis	x	
Manuscript preparation	x	
Manuscript editing		x
Manuscript review	x	x