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Research Article
Preventive and Curative Effect of *Morinda lucida* Extract on the Anemia and its Toxicological Evaluation

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Abstract

Background and Objective: Chronic anemia is the main cause of death of sickle cell patients. The way to manage their anemia is the stimulating of erythropoiesis by daily folate supplementation with its side effects. This study was aimed to evaluate *in vitro* antihemolytic and *in vivo* erythropoietic properties of *Morinda lucida* leaves hydroalcoholic extract (MLHE). **Materials and Methods:** The hemolysis was induced by a range of hypotonic solutions of NaCl in the absence and presence of 1.5 and 3 g L^{-1} of MLHE. The toxicity of 28 days was also evaluated for MLHE at the doses of 250, 500 and 1000 mg kg^{-1}. **Results:** MLHE at 1.5 and 3 mg mL^{-1} reduced, respectively 14.7% ($p<0.01$) and 23.2% ($p<0.0001$) this hemolysis. On other hand, three days after the anemia induction by phenylhydrazine administration, the reticulocyte rate had increased from 4.6% of red blood cells to 17.6% for the control versus 23.1% ($p<0.02$) in rats treated with 500 mg kg^{-1} of MLHE. After 28 days of MLHE oral administration (250, 500 and 1000 mg kg^{-1}), hematology, nephrology and hepatic parameters showed no significant difference between the control groups and the treated rats. Only the platelets and white blood cells showed significant increasing but this is in the rat standard blood cell counts. **Conclusion:** The MLHE have anti-anemia properties by increasing the erythropoiesis and osmotic resistance of the red blood cells. Indeed, the results showed that it can admit that MLHE is not toxic.

Key words: *Morinda lucida*, sickle cell anemia, erythropoiesis, antihemolytic, toxicity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sickle cell disease is a qualitative abnormality of blood inducing chronic sickle cell anemia. It is hemolytic anemia characterized by a very short life of blood cells. This erythrocyte has a life span of 120 days in healthy subjects (AA) to 10 days in the sickle cell subject (SS, S/β° thalassemia)[1]. This basic chronic anemia is aggravated by hemolysis of sickled erythrocytes trapped in the spleen during the crisis[2-4]. This increased hemolysis in sickle cell patients may be followed by the suppression of erythropoiesis which can lead to acute chronic anemia[2,5-7]. Treatment and prevention of this anemia are the stimulation of erythropoiesis by acid folic taking by sickle cell patient during all their life[8,9].

In earlier work, the anti-sickling properties of *M. lucida* was screened by Joppa *et al.*[10] and Avaligbe *et al.*[11]. Avaligbe *et al.*[12] had also demonstrated antihemolytic properties of aqueous extract of *M. lucida* in sickle red blood cell. Moreover, sickle cell disease is intimately linked to oxidative stress[13]. Then, others researchers work on sickle cell disease was targeting antioxidant property of plants[14]. So, there are evidence that *M. lucida* leaves extract protect red blood cells against oxidative stress[15,16]. Indeed, previous study showed that *M. lucida* inhibited deoxyhemoglobin polymerization *in vitro*[15]. All these findings reveal anti-drepanocytary properties of *M. lucida*.

Besides, involvement of sickling, oxidation process and deoxyhemoglobin polymerization[1,17,18], hemolysis is involved in chronic and acute anemia of sickle cell patients. That anemia is a prime cause of sickle cell patients' death[19,20]. So, this study was aimed to evaluate the effect of the extract of *M. lucida* on the resistance of the hemolysis induced by osmotic pressure *in vitro* and on the erythropoiesis *in vivo*.

In addition, *M. lucida* being used to treat this chronic disease all the life, sickle cell anemia, a toxicological study of this plant was done by exploring its effect on the organs of detoxification and some hematological and biochemical parameters.

MATERIALS AND METHODS

Research duration: These studies took place in the Animal Physiology and Pharmacology Laboratory of the University of Lomé from September, 2013 to March, 2014 for osmotic resistance and erythropoiesis and from April-May, 2016 for toxicological studies.

Material

Biological material: The leaves of *Morinda lucida* were harvested in the rainy season at Adétikopé. A tropical area situated at 20 km North of Lomé, Togo. The plant extract is prepared from the maceration of dried leaves under cooling in an ethanol-water mixture at 80% (80:20). The filtrate of this maceration was evaporated under vacuum in a bath of 45°C using a Buchi Rotavapor R-210.

The SS blood samples used for osmotic resistance test were collected from consenting volunteer donors in vacutainers with anticoagulants.

For toxicological and anti-anemic studies, male as well as female Sprague-Dawley rats have been used. Two groups of 6 males for anti-anemic study and 4 groups of 8 (4 males and 4 females) were used for the toxicity studies. These rats are reared in standard conditions (12 h light/dark, food and water *ad libitum*) at the animal facility of the Laboratory of Animal Physiology and Pharmacology at the University of Lomé.

Chemicals: For induction of anemia, phenylhydrazine ($C_6H_5NHNH_2$) was used and cresyl blue was used for the enumeration of erythrocytes. These solutions are purchased by Sigma-Aldrich.

Methods

Effect on the osmotic resistance of SS sickle cell patient red blood cells: Osmotic resistance was investigated by induction of hemolysis by a NaCl hypotonic solution gradient (0, 1.25, 2.5, 3, 4, 5, 7 and 10 g L^{-1}) either in presence or absence of plant extract by modifying Parpart *et al.*[21] methods.

50 µL of sickle cell patient blood were added to 4.5 mL NaCl solution in the presence of 0.5 mL of *M. lucida* extract at 5 mg mL^{-1}. In the control group, the extract was replaced with the same volume of NaCl solution at 0.9%. The mixture was homogenized and incubated at the room temperature for 30 min. The tubes are centrifuged at 2500 rpm for 5 min. The supernatants were collected and their absorbance was read with Vernier spectrophotometer at 540 nm. The calibration of the spectrophotometer was carried out with supernatant from 10 g L^{-1} of NaCl solution defined as the concentration did not induce hemolysis.

Then, the effect of extract concentration at 1.5 and 3 mg mL^{-1} was studied on the osmotic resistance of the red blood cells at the sodium chloride concentration that induced 95% of hemolysis.

Effect on erythropoiesis: The method used is that described by Diallo *et al.*[22]. On the 1st day (d_0), reticulocytes rate, were evaluated by collecting by retro-orbital sinus 0.5 mL of blood. Then, anemia was induced by intraperitoneal injection of 2 doses of phenylhydrazine at 40 mg kg^{-1} a day 0 and 1 (d_0 and d_1).

On the 3rd day, rats were divided in two groups of 6 male rats each. A treated group received per os alcoholic extract of *M. lucida* (500 mg kg^{-1}) every day from d_2 to d_{15} while control group received distilled water by the same way. Blood samples were taken at d_2, d_7, d_{10} and d_{15} according to Diallo *et al.*[22] methods and the reticulocytes were counted and other hematological parameters were determined.

Subchronic toxicity study: Subchronic test is performed according to Adeneye and Agbaje[23] method by slight modification. Four groups of 8 rats (4 females and 4 males) of 178±3 g were used. The control group received distilled water at a dose of 10 mL kg^{-1} and the other groups received respectively by oral rout *M. lucida* extract at doses of 250, 500 and 1000 mg kg^{-1}.

The weight of the rats was measured every 4 days (d_1, d_5, d_9, d_{13}, d_{17}, d_{20}, d_{24} and d_{28}). On the 28th day, rats are fasted for 18 hours. On day29, they are weighed and anesthetized under ether and 2 mL of blood was taken from each rat of them for biochemical parameters analysis on serum and hematological parameters on whole blood respectively by hemogram analyzer BC-2800 and Biochemistry Analyzer SINNOWA BS-300M. Then their abdomen was opened and the vital organs were removed and weighed.

Statistical analysis: Statistical analysis (ANOVA and student test) were performed with Systat 11 and differences are significant if $p<0.05$. The results are presented as Mean±SEM (standard error of the mean). The graphics are performed with GraphPad 5.0.

RESULTS

Preventive effect on the hemolytic anemia erythrocyte osmotic resistance: Figure 1 shows the absorbance curve of hemolysate either in absence or in presence of *M. lucida* extract at 0.55 mg mL^{-1}. At all hemolyzing concentrations of NaCl, absorbance in presence of 0.55 mg mL^{-1} of *M. lucida* extract were lower than the control. That inhibition of hemolysis in the presence of the extract of *M. lucida* is concentration-dependent. Hemolysis induced by 2.5 g L^{-1} of NaCl solution was inhibited at 14.7% ($p<0.01$) and 23.2% ($p<0.0001$), respectively by 1.5 and 3 mg mL^{-1} of *M. lucida* extract (Fig. 2).

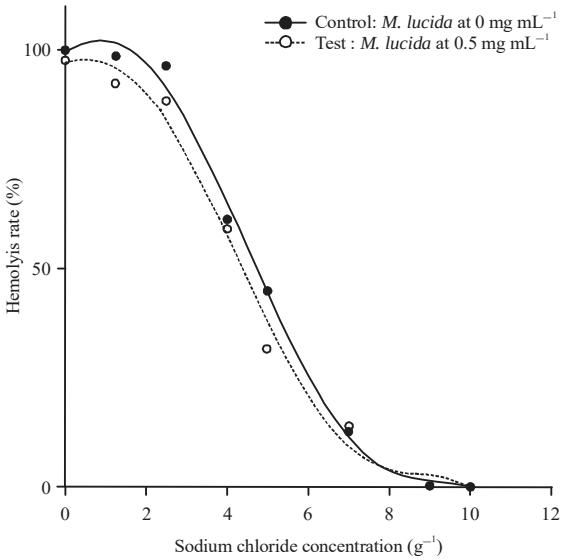

Fig. 1: Hemolysis in presence and absence of *M. lucida* extract
Hemolysis have been induced with sodium chloride solutions at different concentrations from 0-10 g dm^{-3} (n = 3), the absorbance indicates the hemolysis rate corresponding to each sodium chloride concentration with or without *M. lucida*, at 2.5 g L^{-1}, sodium chloride induces 95% of hemolysis

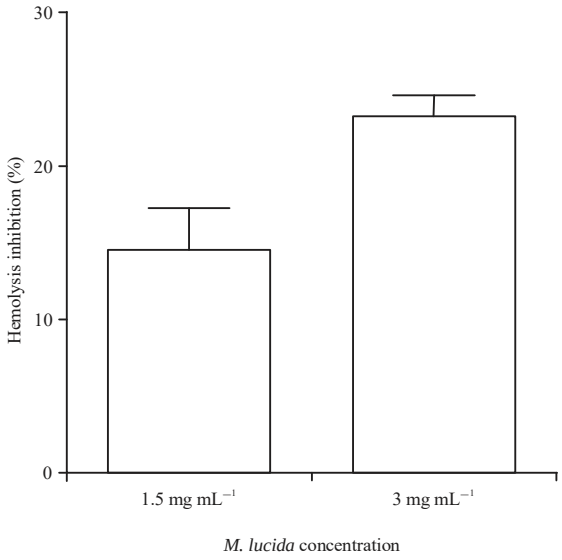

Fig. 2: Hemolysis inhibition rate at two concentrations of *M. lucida* at the sodium chloride concentration inducing a 95% of hemolysis in the control tubes (n = 5)
Inhibition rate (IR) at 3.0 mg mL^{-1} is significantly higher than the inhibition at 1.5 mg mL^{-1} ($p<0.05$), $IR = 100\times(DO_{témoin} - DO_{test})/DO_{témoin}$

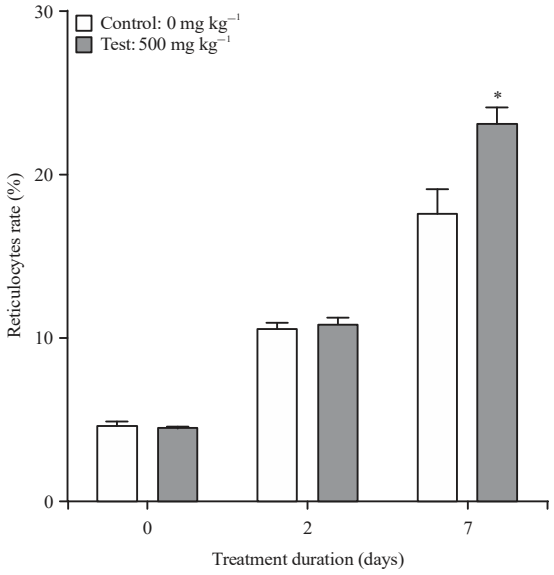

Fig. 3: Reticulocytes production after anemia induced by phenylhydrazine

Day 2 correspond to the real anemia induced, any difference is not observed in the reticulocytes rate of the both lots, At this day begun the treatment with *M. lucida* at 500 mg kg^{-1}, the control group received a distilled water, After 5 days of treatment, a significant difference is observed in the reticulocyte rate of the treated and controlled groups (*p<0.01)

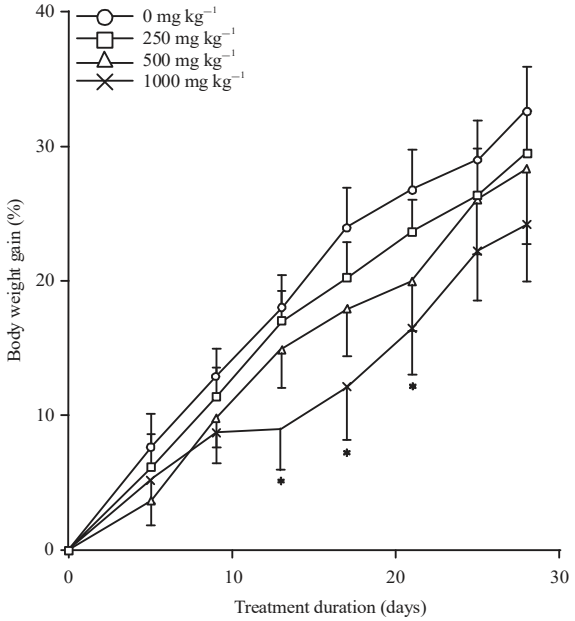

Fig. 4: Evolution of rat's body weight gain during the 28 days of daily administration of different doses of *M. lucida*

Rats treated daily with *M. lucida* have dose-dependent low body weight gain compared to the control group, this decreasing of the body weight is significant (*p<0.05) only for the lot treated daily with *M. lucida* at 1000 mg kg^{-1} from 13-21 day

Curative effect on chronic anemia of sickle cell disease: 48 h after intraperitoneal injection of phenylhydrazine, the hemoglobin rate of the rats which initially was 15.8 g dm^{-3} dropped to 8.2 g dm^{-3} about 47% reduction of original hemoglobin level (Table 1). Seven days after the induction of anemia, the reticulocytes rate has increased from 4.6% of red blood cells to 17.6% for the control versus 4.5-23.1% ($p<0.02$) in rats treated with 500 mg kg^{-1} of *M. lucida* extract (Fig. 3).

Toxicological study of alcoholic *M. lucida* extract

Body weight of rats: After 28 days of daily gavage with different *M. lucida* doses 32.6, 29.5, 28.4 and 24.2% increase in body weight was recorded respectively for rats of the lot 0, 250, 500 and 1000 mg kg^{-1} (Fig. 4). These decreases in body weight gain were only significant for 1000 mg kg^{-1} 13-21day.

Effect on detoxification organs and heart biochemical markers and their relative weights: After 28 days treatment, the relative weights of the heart, the liver, the spleen and the kidney of the rats treated with different doses of hydroalcoholic extract of *M. lucida* didn't have any significant changes compared to the rats in the control group (Table 2). There is no correlation between their values and the extract doses ($p>0.05$ and $R<0.45$).

At the doses of 250 and 1000 mg kg^{-1}, the ASAT concentrations were 147 and 107 UI dm^{-3}, respectively versus 119 UI dm^{-3} in the control group. Their effects were not significant on ASAT concentration ($p>0.05$). The same results were obtained for ALAT (Table 3).

As kidney function indicators investigated through plasma creatinine and urea concentration was not affected by the plant extract at all doses (Table 3). Total proteins were not also presented any variation after 28 days treatment at all doses. Table 3 shows their values which are almost identical to those of the control ($p>0.1$).

Effect on hematological parameters: After 28 daily oral administration of *M. lucida* extract, white blood cells count had increased from 6.1×10^3 µL^{-1} for the control rats to 6.9×10^3 µL^{-1} ($p<0.05$) and 10.4×10^3 µL^{-1} ($p<0.001$), respectively for the doses of 500 and 1000 mg kg^{-1}. Platelets rate was also significantly increased ($p<0.05$) for all doses. Its concentration was 297×10^3 µL^{-1} for the control 451, 457 and 545×10^3µL^{-1}, respectively for 250, 500 and 1000 mg kg^{-1} (Table 4). These increases were dose-dependent. Unlike platelets and white blood cells, these doses had no effect on red blood cells and hemoglobin rate (Table 4).

Table 1: Induction of anemia and changing of hemoglobin rate in presence and absence of 500 mg kg^{-1} of *M. lucida*

	Hemoglobin rate (g dL^{-1})				
Groups	D_0	D_2	D_7	D_{10}	D_{15}
Control (0 mg kg^{-1})	15.8±0.2	8.2±0.3	13.0±0.4	14.3±1.5	16.0±0.3
Test (500 mg kg^{-1})	15.5±0.6	8.2±0.4	12.4±0.6	15.4±0.3	16.1±0.5

Anemia was induced by intraperitoneal administration of phenylhydrazine during D_0 and D_2, From D_2-D_{15} control and test group received, respectively distilled water and 500 mg kg^{-1} of hydroalcoholic extract of *M. lucida* by oral route

Table 2: Relative weight of detoxification organs and the heart in (%)

Doses (mg kg^{-1})	Heart (%)	Liver (%)	Spleen (%)	Kidney (%)
0	0.35±0.01	3.10±0.13	0.21±0.01	0.52±0.02
250	0.34±0.01	3.21±0.15	0.22±0.01	0.53±0.01
500	0.35±0.01	3.09±0.11	0.21±0.01	0.51±0.02
1000	0.35±0.02	3.21±0.15	0.22±0.01	0.56±0.01

Values in this table represent mean of relative weight (RW) of each organs, It was calculated with the formula RW = 100×OW/BW, OW: Organ weight, BW: Body weight

Table 3: Biochemical serum parameter measurements

Doses (mg kg^{-1})	ASAT (UI dm^{-3})	ALAT (UI dm^{-3})	Creatinine (UI dm^{-3})	Urea (UI dm^{-3})	Total proteins (UI dm^{-3})
0	119±10	61±2	7.5±0.2	64±1	63.5±1.3
250	147±14	68±5	7.7±0.2	65±2	64.6±2.1
500	97±03	63±5	7.3±0.3	64±2	64.2±1.9
1000	107±11	67±6	7.8±0.2	64±3	64.4±2.5

For all doses and parameters, any significant difference is not observed

Table 4: Hematological parameters in rats after 28 days of *M. lucida* leaves extract

Extract doses (mg kg^{-1})	WBC (×10^3 µL^{-1})	RBC (×106 µL^{-1})	Hemoglobin (g dL^{-1})	Hematocrit (%)	Platelet (×10^3 µL^{-1})	MCHC (g dL^{-1})	VGM (fL)
0	6.1±0.7	8.4±0.2	15.8±0.4	47.1±1.1	297±54	33.4±0.2	56.0±0.4
250	6.9±0.5	8.4±0.3	15.7±0.4	46.7±1.2	451±48*	33.5±0.2	56.1±0.4
500	8.5±0.7*	8.5±0.1	16.1±0.3	47.7±0.8	457±31*	33.6±0.2	56.0±0.5
1000	10.4±0.6**	8.5±0.2	15.9±0.3	48.0±1.0	545±66*	33.1±0.2	56.4±0.7

Blood was collected by retro orbital sinus, the increasing of white blood cell (WBC) and platelet are significant (*p<0.05, **p<0.001) but their values for all the doses are in standard hemogram of Sprague-Dawley rat, different doses had no effect on red blood cell (RBC)

DISCUSSION

Results showed that the *M. lucida* extract increases resistance to hemolysis of sickle red blood cells. Then, it can been affirmed that *M. lucida* has *in vitro* antihemolytic properties that enable this to prevent hemolytic anemia in sickle cell patients. Avaligbe *et al.*[12] study highlighted this antihemolytic effect of aqueous extract of *M. lucida* leaves on SS sickle red blood cell. Unlike the results of Avaligbe *et al.*[12], the hydroalcoholic extract of *M. lucida* leaves showed concentration-dependent antihemolytic effect (Fig. 2).

In the other hand, the severe anemia was induced by decreasing of 47% of hemoglobin rate like Diallo *et al.*[22] and Gbenou *et al.*[24] who obtained respectively 45 and 47%.In control and test groups, the hemoglobin rate was increasing consistently till it reached the basic rate. Contrary to these previous studies[22,23] that obtained significant effect respectively for *Tectonia grandis* and *Justica secunda Vahl* at 1000, 2000 and 500 mg kg^{-1}*M. lucida* didn't show significant increasing of hemoglobin rate. At 300 mg kg^{-1} 4 plants anti-anemic plants studied by Lee *et al.*[25] didn't demonstrate significant increasing of hemoglobin rate compared to the control group during the correction of hemoglobin rate after anemia induced by phenylhydrazine.

Moreover, at the opposite of Hussain *et al.*[26] who obtained significant hemoglobin rate higher than the standard value (D_0) with 200 mg kg^{-1} of *Trigonella foenum-graecum*, there was no significant increasing of the hemoglobin rate when it reached the basic level with *M. lucida*. An increase more than normal would be detrimental to sickle cell disease patients because high concentration of hemoglobin S is a sufficient condition to trigger sickling[18,27].

The hemolytic anemia induced by phenylhydrazine stimulated erythropoiesis leading to increased production of reticulocytes in the control and test groups. After 5 days, there was 283% increasing of reticulocytes production in controls group compared to the basic reticulocyte of rats. Exacerbation of this natural correction of anemia in the presence of hydroalcoholic extract was noted. In effect, *M. lucida* increased very significantly the rate of production of reticulocyte to 402%.This increase was 40% higher than the control rats (p<0.01) after five days of treatment. Seen the

value of the probability p, this significant increasing of reticulocyte rate can be observed earlier at 3 or 4 days of treatment but blood cannot be sampled at these days because it can affect the rats' survival[13]. Like highlighted by Lee *et al.*[25] works from four plants at 300 mg kg^{-1}, at the dose of 500 mg kg^{-1} *M. lucida* demonstrated significant stimulation of erythropoiesis levels despite having no significant increase in hemoglobin rate.

Oral administration of 250 and 500 mg kg^{-1} doses of *M. lucida* to rats for 4 weeks at one daily dose had no effect on the body weight gain compared to the control rats. However, decreasing in body weight gain was significant for 1000 mg kg^{-1}group during the third week but it was corrected in the 4th week. Even though it was not significant, leaves of *M. Lucida* as its stem bark[28] reduced body weight gain.

Likewise, the *M. lucida* leaves extract has no effect on transaminases, creatinine and urea. The same results were obtained by Oduola *et al.*[29] with the root extract of *M. lucida* on the Wistar rats. Overall, there were no indicators of impairment of body detoxification showed by the oral administration of the extract at the different doses for 28 days. Indeed, previous studies had placed its limit toxicity [11,29], LD50 beyond 5 g kg^{-1} or even 50 g kg^{-1} [29]. So, it can be affirmed that, the hydroalcoholic extract of *M. lucida* leaves is not toxic at the pharmacological doses. These results are confirmed by those of Lee *et al.*[25] whose works had showed that the extract of *M. lucida* is stripped of any toxicity in the liver and kidney[29]. Unlike the root[30] and the stem bark[29], extract of *M. lucida* leaves can be used for clinical trials[29].

Unlike biochemical parameters, some hematological parameters including white blood cells and platelets increased significantly after 28 days of treatment with the three doses. Seen the dose-dependent effect of the extract and its effect on erythropoiesis (Fig. 3), it can be deduced that the increases of the platelet rate is due to a medullary stimulation[31]. This thrombocythemia is, therefore, reactional and induced by treatment with the plant extract for 28 days. Although this increasing of 84% at the higher dose, the platelet rate is in Sprague-Dawley standard blood cell counts[32]. This increasing must be consider abnormal if it was over 200%[31,33]. Since the increasing of the platelet rate and the white blood cell are in the standard blood cell counts[32], they are not toxic effect of *M. lucida*. The thrombocytosis was a reactive one in response to acute anemia induced[34-37].

Overall, it can be resumed that *M. lucida* have no toxicological effect on the hematological parameters. On the other hand, increasing white blood cell it suggests an immunostimulant effect of the extract which involves further investigation. Seen the effectiveness of *M. lucida* on anemia parameters, it can be recommended for management of acute and chronic anemia of sickle cell patients. But seen the chronic character of sickle cell disease, it necessary to confirm this non-toxicity of *M. lucida* leaves by chronic toxicity and its anti-anemic effect by clinical study.

CONCLUSION

The *M. lucida* extract increases resistance to the hemolysis of red blood cells. Moreover, *M. lucida* increases the red blood cells production rate needed to correct the anemia by enhancing the erythropoiesis which is inhibited in the sickle cell patients. Therefore, *M. lucida* can be used in a preventive and curative ways to solve the problem of sickle cell disease related to anemia as it leaves extract is not toxic. Hematological results also suggested that *M. lucida* leaves extract possess immunostimulant effect which require further investigations.

SIGNIFICANCE STATEMENT

This study discovers the erythropoietic property of *M. lucida* leaves that can be beneficial for preventive and curative management of acute and chronic anemia of sickle cell patients. This study will help the researcher to uncover the critical areas of new therapeutic approach of sickle cell disease anemia that researchers were not yet explore.

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