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# *In vitro* Antisickling and Radical Scavenging Activities of a Poly-herbal Formula (Drepanoalpha®) in Sickle Cell Erythrocyteand Acute Toxicity Study in Wistar Albino Rats

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author KNN designed the study and wrote the protocol, author PTM wrote the first draft of the manuscript. Authors DSTT, PPM and JNK performed the statistical analysis and managed the analyses of the study. Authors BZG, EKA and FMK managed the literature searches. All authors read and approved the final manuscript.*

***Original Research Article***

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## ABSTRACT

**Aims:** To evaluate the antisickling and radical scavenging activities and acute toxicity of indigenous nutritive formula Drepanoalpha®, produced through a bio-guided based plant selection.
**Study Design:** Drepanoalpha® extracts, Antisickling activity by Emmel test, Antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl bleaching methods; acute toxicity on rats, determination of biological and haematological parameters.
**Place and Duration of Study:** Science Faculty University of Kinshasa, between January 2013 and February 2014.
**Methodology:** The antisickling and antioxidant activities of Drepanoalpha® were determined using Emmel and the 1,1-diphenyl-2-picrylhydrazyl bleaching methods

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respectively. Acute oral toxicity test was performed to determine the LD50. Liver and kidney functions, the hematological and histopathological examinations were assessed using standard techniques.
**Results:** Obtained results revealed that Drepanoalpha® possessesinteresting *in vitro* antisickling and antioxidant activities as revealed by the observed normal biconcave form of sickle erythrocyte (normalization rate >80%) and the radical scavenging activity (ED50= 0.604 ± 0.028 µg/mL). Acute toxicity assessment revealed that the medium lethal dose (LD50) is higher than 4000 mg/kg. Drepanoalpha® significantly increases the values of WBC, RBC, Hb, HCT, PLT, IDR-CV and PCT. Furthermore, this polyherbal formula significantly decreases the values of IDR-SD, P-RGC, AST and ALT ($p<0.05$). Both the control and treated groups displayed comparable non altered histological architecture of the liver cells.
**Discussion:** The mean values of biochemical markers and hematological markers of treated rats revealed that Drépanoalpha® is potentially safe indicating non-toxic effect of the phytomedicine on immune cells and blood clotting factors. Moreover, this poly-herbal formulation increases the hemoglobin rate in the all treated rats (500-4000 mg/kg bodyweight) and preserves the histological architecture of the liver cells.
**Conclusion:** Drepanoalpha® may increase weight gain, promote erythropoiesis and thrombopoeisis in sicklers patients. This phytomedicine could be used in the treatment of all form of anemia and may also prevent bile duct obstruction or intra-hepatic cholestasis. The results can form the basis for clinical trials in humans.

*Keywords: Sickle cell disease; Drepanoalpha®; DL50; wistar albino rat; anthocyanins; organic acids.*

## 1. INTRODUCTION

Drepanocytosis or Sickle cell disease (SCD) is known as a life-long blood disorder which is characterized by abnormal, rigid and sickle shape erythrocytes. This disease is a genetically inherited one in which a single base substitution in the gene encoding the human β-globin subunit results in replacement of β6 glutamic acid by valine, This modification leads to the damaging clinical manifestations of SCD [1,2]. The valine substitution causes serious solubility reduction of sickle cell hemoglobin (Hb S) when deoxygenated. In such conditions, the Hb S molecules aggregate and form long crystalline intracellular polymers which induce the deformation of sickle erythrocytes. The clinical manifestations include retarded growth, periodic attacks of pain and progressive organ dysfunction [3]. Epidemiological data indicate that each year around 300,000 children are born with hemoglobinopathy of which 70% are affected by this disease. In Democratic Republic of the Congo (DRC), almost 2% of the population is affected by this chronic disease [3-5].

Medullar transplantation, repeated blood transfusion and the use of chemical agents that can interfere with the sickling process are the first-line clinical management of SCD. However, all proposed therapies are expensive for low income population [6-8]. Therefore, there is a need for more affordable and effective treatments for the disease. Herbal extracts have been used in African traditional medicine for many years in the management of common ailments [9-12].

More recently our research team showed that various plants species traditionally used in DRC for managing SCD had in vitro, antisickling activity which is mainly due to anthocyanins

[13-20]. These secondary metabolites, as several other flavonoids, are natural products with a broad spectrum of pharmacological activities including free radical scavenger activity [21-22]. Our previous studies have also identified organic acids as antisickling agents [15,23]. Although, recent findings have shown that many plants used in traditional medicine are potentially toxic. To determine the safety of herbal remedies for human use, toxicological evaluation is carried out in various experimental animals to predict toxicity and to provide guidelines for selecting a "safe" dose in humans. The highest overall concordances of toxicity in animals with humans are with haematological, gastrointestinal and cardiovascular adverse effects, acute toxicity tests are generally the first test conducted in any toxicity study. They provide data on the relative toxicity likely to arise from a single brief exposure to any substance [24]. Drepanoalpha® is a poly-herbal formula produced by our research group (Faculty of Science, University of Kinshasa) through a bio-guided based plant selection. In human clinical use, this phytomedicine is taken three times daily for the treatment of SCD. The aim of this study was the evaluation of the *in vitro* antisickling and free radical scavenging activities in sickle cell erythrocytes and the acute toxicity study of Drepanoalpha®.

## 2. MATERIALS AND METHODS

### 2.1 Extraction and Chemical Screening of Drepanoalpha®

Aliquots of 10g of powder from Drépanoalpha® were repeatedly extracted by cold percolation with 95% ethanol (EtOH) and water (100 mL x 3) for 48 hours. Chemical screening was done in aqueous and organic extract according to a well-known protocol as previously reported [3,6,7]. Fractions were filtered and concentrated to dryness under reduced pressure using a rotary evaporator. Extraction of anthocyanins was then done using 100 g of dried powdered material with acidified methanol (1% HCl) following an established protocol [13-20]. Anthocyanins extract was then defatted by n-hexane. Organic acids were extracted according to the protocol of Ouattara et al. [25] with slight modification. Briefly, the powdered material (50 g) were macerated with 100 mL of methanol-$H_2O$ (50/50) and then percolated with 400 mL of the same solvent at room temperature. The extract was concentrated under reduced pressure until 100 mL. The aqueous solution was basified to pH 9 with $Na_2CO_3$ and repeatedly extracted with ether. The aqueous solution was then acidified with 4% acetic acid. The resulting acidic (pH 3) solution was repeatedly extracted by ethyl acetate. The solution was dried over $Na_2SO_4$ and concentrated to give organic acids crude extract. All extracts were stored at +4ºC.

Chemical screening to detect the present groups of secondary metabolites was done in aqueous and alcoholic according to a protocol previously reported [3,6,7].

### 2.2 Biological Testing

#### 2.2.1 *In vitro* antisickling biossay

Blood samples used to evaluate the antisickling activity of the plant extracts in this study were taken from known drepanocitary adolescent patients attending the "Centre de Médecine Mixteetd'Anémie SS" and "Centre Hospitalier Monkole", both located in Kinshasa area, DRC. None of the patients had been transfused recently with Hb AA blood. All antisickling experiments were carried out with freshly collected blood. In order to confirm their SS nature, the above-mentioned blood samples were first characterized by

Haemoglobin electrophoresis on cellulose acetate gel, as previously reported [5]. They were found to be SS blood and were then stored at +4 °C in a refrigerator. An informed consent was obtained from all the patients participating in the study. All the research procedures have received the approval of Department of Biology Ethics Committee.

An aliquot of Hb S-blood was diluted with 150 mM phosphate buffered saline ($NaH_2PO_4$ 30 mM, $Na_2HPO_4$ 120 mM, NaCl 150 mM) and mixed with an equivalent volume of 2% sodium metabisulfite. A drop from the mixture was spotted on a microscope slide in the presence or absence of lyophilized aqueous, anthocyanins or organic acids extracts and covered with a cover slip. Paraffin was applied to seal the edges of the cover completely to exclude air (Hypoxia). Duplicate analyses were run for each extract. The red blood cells (RBCs) were analyzed by measuring various parameters including the area, perimeter and the radius of each RBC using a computer assisted image analysis system (Motic Images 2000, version 1.3; Motic Chine Group Co LTD) and statistical data analysis were processed using Microcal Origin 7.1 package software.

### 2.2.2 Free radical scavenging assay

The DPPH free radical (1,1-diphenyl-2- picrylhydrazyl ) scavenging assay was carried out as previously reported [6,7,26]. The radical scavenging activity of extracts for DPPH free radical was measured on the principle that antioxidants reduce the DPPH radical to a yellow-coloured compound (diphenylpicrylhydrazin) and the extent of the reaction will depend on the hydrogen donating ability of the antioxidant. Briefly, a 100 µM solution of DPPH radical in methanol was prepared. 3.5 mL of this solution added to 0.5 mL solution of each extract in methanol at concentrations ranging from 0.1 to 1.0 mg/mL, thus obtaining the desired final concentrations in the reaction mixture. The mixture was shaken vigorously and incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using a spectrophotometer SP- 1105 Brand model. Methanol was used as a blank. The control solution consists of 0.5 mL of methanol and 3.5 mL of DPPH radical solution. The antiradical activity of a sample (calculated by the following formula) is given as percentage of reduced DPPH free radical: %I = [(OD control - OD sample)/OD control] ×100. The IC50 value (µg/mL) is the effective concentration at which DPPH radicals were scavenged by 50%. L-ascorbic acid was used as positive control. Duplicate analyses were run for each extract [6,7].

### 2.2.3 Toxicity study (LD50) of Drepanoalpha®

All the experimental animals used were obtained from the animal house of the “Institut National de Recherche Biomédicale”. Acute toxicity study of Drepanoalpha® was carried out using 15 female rats of average weight between 85-190 g (8 weeks age), that were dosed orally with different gradual doses (500-4000 mg/kg body weight). Wistar albino rats of average weight between 95 g to 140 g were selected by stratified randomization and then divided into five groups of three rats. Groups II, III, IV and V were given 500, 1000, 2000 and 4000 mg/kg body weight respectively of Drepanoalpha® orally in a single dose. NaCl 0.9% served as the vehicle and was used to prepare the doses. Group I served as the control group and received the NaCl 0.9% vehicle only. They were observed for 24 hours for signs of toxicity, mortality and general behaviours. Animals were fed ad libitum with standard feed, and had free access to water. They were also maintained under standard conditions of humidity, temperature, and 12 hours light/dark cycle. The animals were acclimatized for a week before the commencement of the study [27]. A standard protocol was drawn up in accordance with current guidelines for the care for laboratory animals and ethical guidelines

for investigations of experiments in conscious animals [28]. The first day of dosing was taken as day 0 and blood was collected on day 7 and 14 respectively and used for biochemical, haematological and histopathological analysis. During the two-week period, all the animals were observed daily for clinical signs and mortality patterns once before dosing, immediately after dosing and up to two to four hours after dosing.

### 2.2.3 Weekly body weight measurement

The body weight of each rat was expressed using a sensitive balance during the acclimatization period, once before commencement of dosing, once weekly during the period and once on the day of sacrifice [29].

### 2.2.4 Determination of hematological parameters

Blood samples (7 mL) were collected by the orbital technique. Blood sample for hematological determinations was collected from the retro-bulbar plexus of the medial canthus of the eye to puncture the retro-bulbar plexus and thus enable outflow of blood into a sample bottle containing ethylene-diamine-tetra-acetic acid (EDTA). The sample bottle was gently shaken to mix up the blood with EDTA and prevent clothing. The hematological parameters determined were: white blood cells count (WBC), red blood cells number (RBC), hemoglobin levels (Hb), hematocrit volume (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet counts (PLT), red blood cells distribution index (IDR), platelet distribution index (PDI), mean platelet volume (MPV), platecrit (PCT).Hematological analyses were carried out using SYSMEX XS-1000i automate.

### 2.2.5 Determination of biochemical parameters

Blood was collected by orbital technique. The blood sample was kept at room temperature for 30 minutes to clot. Afterwards, the test tube containing the clotted blood sample was centrifuged at 3,000 rpm for 10 minutes using a table centrifuge to enable a complete separation of the serum from the clotted blood. The clear serum supernatant was then carefully aspirated with syringe and needle and stored in a clean sample bottle for the biochemical tests. The values of total serum protein, Serum albumin, Serum alanine aminotransferase (ALT), Serum aspartate aminotransferase (AST), Serum alkaline phosphatase (ALP), Serum urea and Serum creatinine were determined following standard laboratory procedures [24]. All haematological parameters were determined at room temperature.

### 2.2.6 Organ weight

The liver, kidney, intestine and heart of rats in the various groups were excised on the day 14 immediately after blood collection. Following excision, the organs were trimmed of extraneous tissues, placed on a saline soaked gauze pad to retard desiccation and were immediately weighed (paired organs were weighed together). The organ weight ratio was calculated using the following formula:

$$\text{Organ weight ratio} = \frac{\textit{Organ weight (g)}}{\textit{Body weight (g)}} \times 100$$

### 2.2.7 Histopathological examination

For post-mortem, the rats in the group IV (4000 mg/kg) were dissected and careful examination of the liver was carried out. Tissue samples were fixed in 10% formalin and dehydrated overnight using upgraded ethanol series and embedded in paraffin blocks. Ultrathin sections were de-waxed by xylene, hydrated through a degraded ethanol series, and stained with haematoxylin and eosin. A pathologist, blinded to the treatments, performed the histopathologic examination with an optical microscope Nikon Eclipse E600, USA.

## 2.3 Statistical Analysis

Values of treated groups were compared statistically with control by Independent Sample t-test and one-way ANOVA. Inferences were made from findings at 95% confidence level. Data obtained were presented as mean ± standard deviation.

## 3. RESULTS AND DISCUSSION

## 3.1Phytochemical Screening

The chemical screening which was performed on the aqueous and alcoholic extracts of Drepanoalpha® indicated the presence of phenolic compounds, terpenoids, quinones, alkaloids and organic acids.

## 3.2 Antisickling Activity of Lyophilized Aqueous Extract, Anthocyanins and Organic Acids Crude Extracts from Drépanoalpha®

(Figs. 1 and 2a-c) show respectively the microscopic images of sickle cell blood alone in a NaCl 0.9% solution (control, Fig. 1) and the sickle cell blood incubated with the lyophilized aqueous (Fig. 2a), anthocyanins (Fig. 2b) and organic acids (Fig. 2c) crude extracts from Drepanoalpha®.

As shown in (Fig. 1), the control contains a majority of sickle-shaped erythrocytes. This confirms the blood was taken from sicklers patients. When mixed together with lyophilized aqueous, anthocyanins and organic acids extracts (Fig. 2, a-c), the majority of erythrocytes recovered the normal biconcave shape. The values of normalization rates calculatedwere 79.5% for lyophilized aqueous extract, 80.7% for anthocyanins extract and 88.2% for organic acids extract. This indicates that Drepanoalpha® has antisickling effects, thus justifying the vulgarization of this polyherbal formulation in the management of SCD in Democratic Republic of the Congo. As previously reported, this activity could be due to anthocyanins and/or to phenolic or triterpenoic acids [6,7,13-20,15,23]. The treated SS RBCs demonstrated the same shape as the normalerythrocyte. This indicates that anthocyanins and organic acids are the major antisickling agents of Drepanoalpha®. Indeed, these results confirm those already given by our research team with anthocyanins and organic acids such as betulinic acid, maslinic acid and lunilaric acid from other plants used in traditional medicine for the management of sickle cell anaemia [15,23].

In fact, it is known that anthocyanins have the ability to interact with proteins [3]. Interaction of these pigments with hemoglobin S could compete with the polymerization of this abnormal hemoglobin and prevent the sickling of sickle cells. In addition, anthocyanins (for which intestinal catabolism gives phenolic acids), also known for their antioxidant properties, could

affect the $Fe^{3+}/Fe^{2+}$ higher ratio in sickle cells and the stability of erythrocytes membranes by preventing the oxidation of membranes phospholipids [6,7,28]. As SCD is a chronic disease, using a polyherbal formulation as medicinal foods or nutraceuticals would be a good approach instead of giving pharmaceutical products to sicklers during all their life.

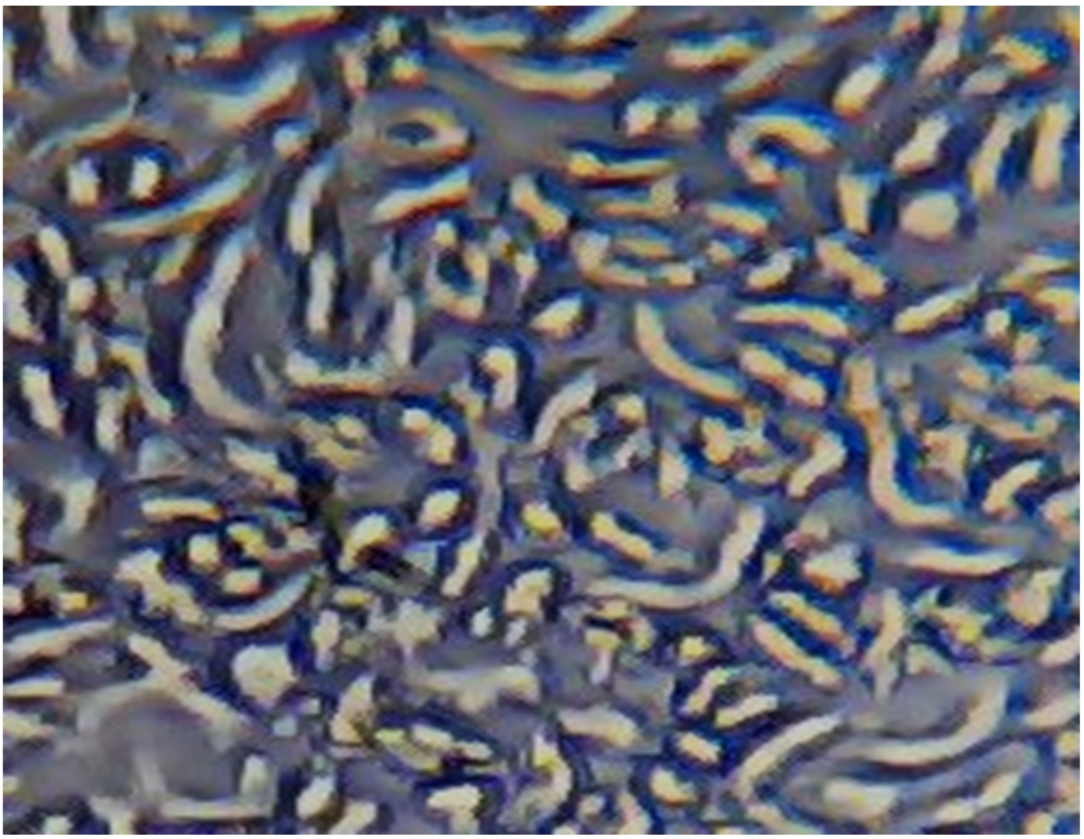

**Fig. 1. Phenotype of sickle cells of untreated SS blood (control) (x500) [NaCl 0.9%; $Na_2S_2O_5$ 2%]**

## 3.3 Free Radical Scavenging Activity

The antioxidant activity of Drepanoalpha® and its bioactive fractions is given in (Table 1).

**Table 1. The antioxidant activity of lyophilized aqueous, anthocyanins and organic extracts from Drepanoalpha®**

| Fraction | $ED_{50}$ (µg/mL) | Antioxidant activity (mL/µg ) |
|---|---|---|
| L-Ascorbic Acid (Positive control) | 0.562±0.212 | 1.779 |
| Drépanoalpha® (lyophilized) | 0.604±0.028 | 1.655 |
| Anthocyanins extract | 1.590±0.211 | 0.628 |
| Organic acids extract | 1.949±0.212 | 0.513 |

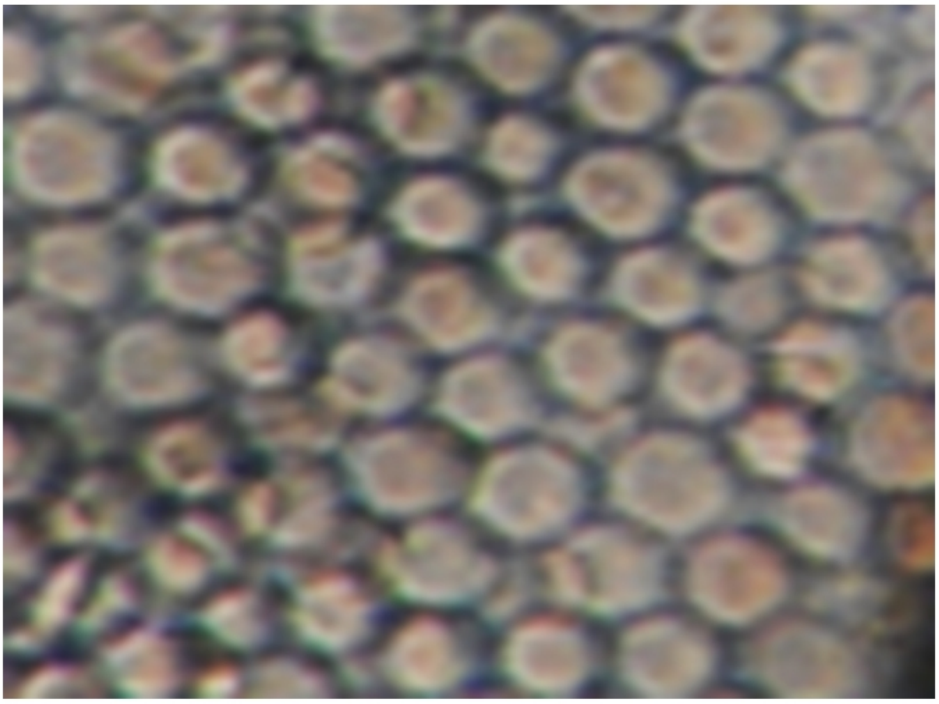

**Fig. 2a. Phenotype of sickle cells treated with 50 µg/ml of lyophilized aqueous extract of Drepanoalpha® (X500), [NaCl 0.9%; $Na_2S_2O_5$ 2%]**

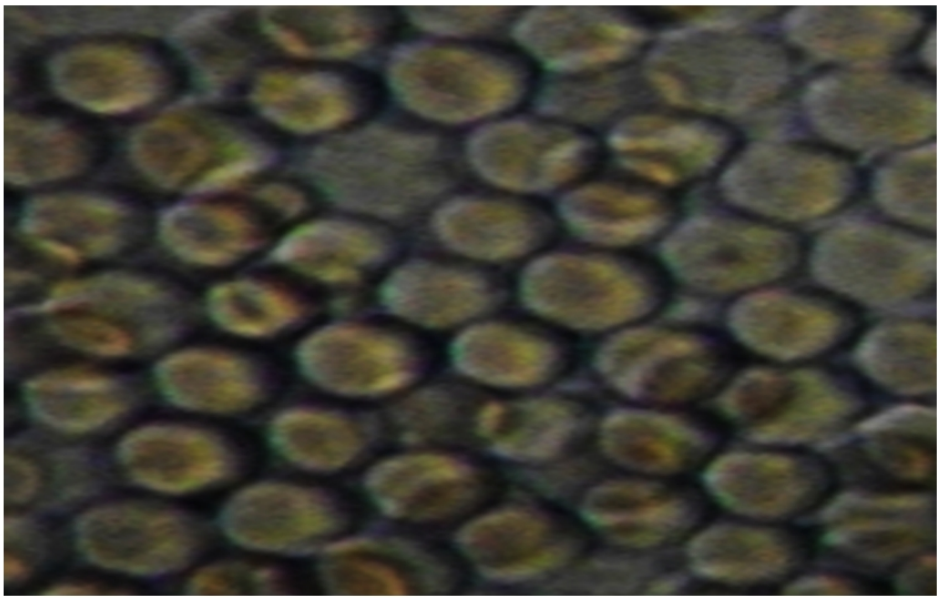

**Fig. 2b. Phenotype of sickle cells treated with anthocyanins extracts (10 µg/mL) from Drepanoalpha® (X500), [NaCl 0.9%; $Na_2S_2O_5$ 2%]**

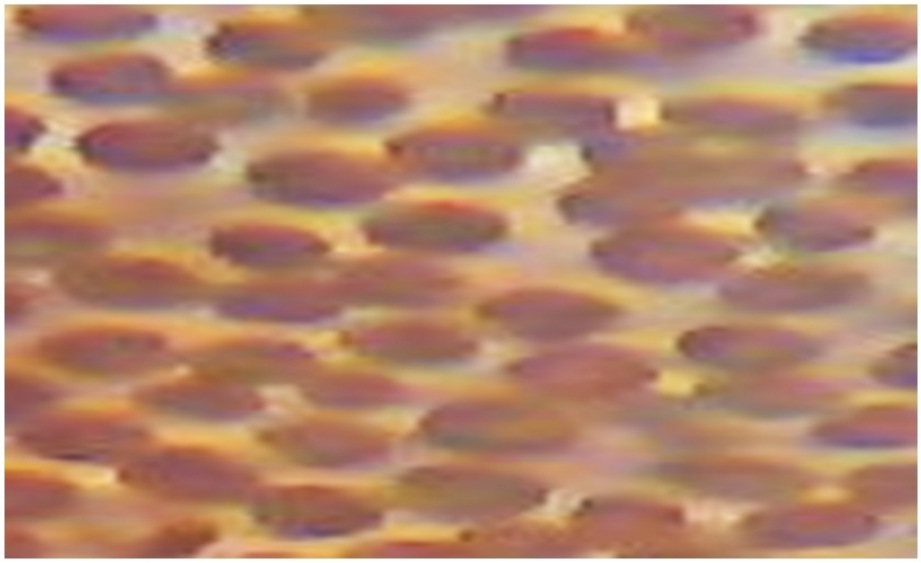

**Fig. 2c. Phenotype of sickle cells treated with organic acid extracts (10 µg/mL) from Drepanoalpha® (X500), [NaCl 0.9%; $Na_2S_2O_5$ 2%]**

As shown in (Table 1), lyophilized aqueous extract of Drepanoalpha® possess the lowest $ED_{50}$ value, compared to the positive control (high antioxidant activity). This high antioxidant activity of Drepanoalpha® could be correlated to the presence of anthocyanins and organic acids. Therefore, the antisickling activity of Drepanoalpha® could be due to the presence of these secondary metabolites known for their antioxidant properties as displayed in the (Table 1).

Increasing evidence accumulated over the last decade indicates that reactive oxygen species (ROS) play a key role in the pathophysiology of various ischemic diseases including SCD. The oxidative stress in SCD is likely the result of intravascular sickling and transient vaso-occlusive event leading to the decrease of nitric oxide (NO) probably due to consumption of NO by free oxygen radicals, and/or by cell-free plasma heme as a result of hemolysis [6,7,30]. The results outlined in this paper, indicate the antisickling and scavenging effects of Drepanoalpha®, as attractive potential candidate for SCD therapy for improving the quality life of sicklers. As reducing agent, Drepanoalpha® could prevent in vivo oxidative reactions, often by scavenging ROS before they can damage cells.

## 3.4 Acute Toxicity Study

Table 2 shows the result of the acute toxicity and the changes in bodyweight of rats of the effect of lyophilized aqueous extract of Drepanoalpha®

There was no obvious sign of toxicity and mortality up to the dose of 4000 mg/kg. However at higher doses 2 000 mg/kg and 4 000mg/kg, the animals showed reduced physical activities and increased respiratory rate which returned to normalcy after one hour of extract administration. Overall, 14 days after a single dose exposure to Drépanoalpha® on day 0, an increase in growth in all the rat groups was observed.

Table 3 gives the organ weight and relative organ weight of rats.

**Table 2. Acute toxicity and weight change caused by lyophilized aqueous extract of Drepanoalpha®**

| Dose (mg/kg) | Death (n=3) | Behavioural change | Day 0 (n=3) | Day 7 (n=3) | Day 14 (n=2) | Weight mean gain (%) |
|---|---|---|---|---|---|---|
| Control | 0 | nothing | 111.92±27.23 | 130.71±25.44 | 130.74±19.28 | 14.39 |
| 500 | 0 | nothing | 139.66±1.59 | 158.91±5.53 | 160.56±0.86 | 14.96 |
| 1000 | 0 | nothing | 148.65±1.71 | 171.22±22.41 | 162.46±7.98 | 9.29 |
| 2000 | 0 | hypoactivity | 159.19±7.07 | 168.32±1.68 | 168.23±2.72 | 5.37 |
| 4000 | 0 | hypoactivity | 188.71±12.67 | 199.64±13.52 | 201.29±17.34 | 6.67 |

**Table 3. Organ weight (g) and relative organ weight in parenthesis (%) on day 14**

| Dose mg/kg | Liver | Kidney | Intestine | Heart |
|---|---|---|---|---|
| Control | 4.97±0.32 | 0.89±0.02 | 11.61±2.10 | 0.61±0.01 |
| | (3.71±0.15) | (0.68±0.12) | (8.86±0.29) | (0.47±0.07) |
| 500 | 6.13±0.45 | 0.89±0.02 | 11.70±2.81 | 0.60±0.06 |
| | (3.81±0.26) | (0.55±0.01) | (7.29±1.79) | (0.37±0.03) |
| 1000 | 6.30±0.20 | 0.99±0.02 | 10.11±0.53 | 0.63±0.07 |
| | (3.87±0.04) | (0.60±0.01) | (6.22±0.60) | (0.39±0.06) |
| 2000 | 5.46±0.08 | 1.11±0.12 | 10.47±2.02 | 0.61±0.04 |
| | (3.24±0.10) | (0.66±0.08) | (6.32±0.94) | (0.36±0.03) |
| 4000 | 7.64±0.10 | 1.36±0.30 | 13.47±0.04 | 0.71±0.04 |
| | (3.69±0.21) | (0.67±0.09) | (6.71±0.55) | (0.35±0.01) |

As it can be seen on the (Table 3), there was no significant ($p>0.05$) difference in liver relative weight of untreated rats (control) and rats treated with Drepanoalpha® on day 14 post-treatment. No significant ($p>0.05$) difference was also observed in kidney, intestine and heart relative weight for the control group and groups treated with Drepanoalpha®.

This result suggested that Drepanoalpha® did not affect the weight of rats. However, at the dose 2000 mg/kg of body weight, we observed a temporary decrease in rat weight gain (0.33%) between the days D7 and D14.This weak transitory decrease of rat growth can be explained by the altered physiological processes in this group for transient period due to administered dose.

Changes in the body weight can be used as an indicator of adverse effects of phytochemicals [31]. In the present study, we observed the increase in weight at the onset of the 14 day observation suggesting that Drepanoalpha® does not exert any deteriorative effect on the weight and growth of the animals. The increase in weight of the animals suggests that they increasingly accumulated calories from the normal rat diet. Although the rats used in this study were fed with normal diet, the lyophilized aqueous extract of Drepanoalpha® might have allowed a good absorption and utilization of the nutrients. Drepanoalpha® may have stimulated appetite and increased feed utilization resulting in increased weight gain.

Organ weight was reported as an important index of physiological and pathological status in animals. The relative organ weight is fundamental to establish whether the organ was exposed to the injury or not. It is known that, the heart, liver, kidney, spleen, intestine and lungs are the primary organs affected by biochemical reaction caused by toxicant. The liver, being a key organ in the metabolism and detoxification of xenobiotics, is vulnerable to damage induced by toxicant [32]. The present study revealed that oral administration of Drepanoalpha® (500-4000 mg/kg body weight) to rats is safe.

### 3.5 Effect of Lyophilized Aqueous Extract of Drepanoalpha® on Biochemical Markers and Hematological Parameters

(Table 4 and Table 5) show the effect of lyophilized aqueous extract of Drepanoalpha® on hematological parameters (WBC, MCV, PLT, IDR, PDI, MPV, PCT) and on other biochemical markers such as AST, ALT, ALP, creatinine, urea, total proteins, albumin measured in the blood collected on day14 post-treatment.

As shown in (Table 4), no significant difference ($p>0.05$) was observed between the untreated rats and the rats treated with the various concentration of Drepanoalpha® (500, 1000, 2000 and 4000 mg/kg) for MCV, MCH, MCHC, PDI, and MPV. For other hematological parameters, we observed that Drepanoalpha® increased significantly the values of RBC, Hb, PCT, PLT, HCT and WBC. The values significantly reduced comprised of IDR-SD and P-RGC.

Meanwhile, the effect of Drepanoalpha® on liver and kidney function parameters is not significant for all parameters but ALT and ALP (Table 5).

**Table 4. Effect of lyophilized aqueous extract of Drepanoalpha® on hematological markers measured in post-mortem blood samples**

| Blood parameters | Group control | Dose of Drepanoalpha® aqueous lyophilized extract (mg/kg) | | | | Sign. |
|---|---|---|---|---|---|---|
| | | 500 | 1000 | 2000 | 4000 | |
| WBC (x $10^3$ /µL) | 5.44±0.62 | 4.51±0.88 | 4.87±3.31 | 6.67±3.70 | 6.85±4.53 | ↑S |
| RBC (x $10^6$ /µL) | 4.79±2.27 | 7.86±0.07 | 8.29±0.13 | 8.53±0.50 | 8.39±0.24 | ↑S |
| Hb (g/dL) | 9.10±3.67 | 14.00±0.14 | 14.2±0.42 | 15.50±1.13 | 14.65±0.91 | ↑S |
| HCT (%) | 27.35±2.23 | 43.70±0.98 | 45.00±0.56 | 47.35±2.47 | 45.75±1.90 | ↑S |
| MCV (fL) | 57.55±1.76 | 55.60±1.83 | 54.90±2.40 | 55.50±0.42 | 54.50±0.70 | NS |
| MCH (pg) | 19.35±1.48 | 17.80±0.10 | 17.30±0.09 | 18.15±0.21 | 17.45±0.63 | NS |
| MCHC (g/dL) | 33.65±1.62 | 32.05±1.06 | 31.55±1.34 | 32.70±0.70 | 32.02±0.70 | NS |
| PLT (x $10^3$ /µL) | 366.00±145.70 | 649.00±110.30 | 799.00±223.40 | 806.50±12.00 | 979.50±61.50 | ↑S |
| IDR-SD (fL) | 30.35±1.48 | 28.90±0.42 | 28.25±1.06 | 26.55±0.35 | 28.55±0.21 | ↓S |
| IDR-CV (%) | 15.70±1.13 | 17.90±0.42 | 17.35±1.76 | 17.70±1.27 | 18.40±0.03 | ↑S |
| PDI (fL) | 9.85±0.91 | 9.70±0.14 | 8.90±0.98 | 8.60±0.28 | 8.65±0.21 | NS |
| MPV (fL) | 8.80±0.56 | 8.80±0.28 | 8.20±0.70 | 7.85±0.07 | 8.20±0.14 | NS |
| P-RGC (%) | 17.55±4.17 | 18.20±2.96 | 12.85±4.31 | 10.15±1.06 | 12.90±1.13 | ↓S |
| PCT (%) | 0.32±0.14 | 0.56±0.07 | 0.66±0.24 | 0.63±0.01 | 0.79±0.03 | ↑S |

*Mean values ±SD (n=3); ↑S significant increase of values; ↓S significant decrease of values (p<0.05)*

**Table 5. Levels of biochemical markers of liver and kidney profile in rats administered lyophilized aqueous extract on day 14 post-treatment**

| Biomarkers | Group control | Dose of Drepanoalpha® aqueous lyophilized extract (mg/kg) | | | | Sign. |
|---|---|---|---|---|---|---|
| | | 500 | 1000 | 2000 | 4000 | |
| ALT (UI/L) | 21.41±2.82 | 24.50±1.64 | 16.15±1.20 | 13.95±1.20 | 25.20±1.54 | NS |
| AST (UI/L) | 35.00±1.40 | 30.70±8.62 | 26.90±1.00 | 24.90±4.80 | 16.62±3.31 | ↓S |
| ALP (UI/L) | 85.00±6.87 | 60.55±3.26 | 60.00±2.62 | 38.00±4.24 | 48.50±3.77 | ↓S |
| Creatinine (mg/dL) | 0.56±0.08 | 0.62±0.04 | 0.68±0.05 | 0.65±0.03 | 0.55±0.02 | NS |
| Urea (mg/dL) | 32.25±5.16 | 28.80±0.84 | 33.05±3.84 | 26.10±0.98 | 30.75±4.45 | NS |
| Total protein (g/dL) | 6.15±0.63 | 6.70±0.42 | 6.70±0.46 | 6.45±0.07 | 6.65±0.07 | NS |
| Albumin (mg/dL) | 3.91± 0.12 | 3.85±0.27 | 4.14±0.28 | 3.85±0.77 | 4.25±0.07 | NS |
| Globulin (mg/dL) | 2.25±0.63 | 2.85±0.35 | 2.45±0.21 | 2.61±0.84 | 2.40±0.14 | NS |
| Albumin/Globulin | 1.74±0.19 | 1.35±0.14 | 1.64±0.25 | 1.62±0.82 | 1.77±0.12 | NS |

*Mean values ±SD (n=3); ↑S significant increase of values; ↓S significant decrease of values (p<0.05)*

As it can be seen on Table 5 above, on day 14 post-treatment, there was no statistically significant ($p>0.05$) difference in AST, ALT, ALP, creatinine, urea, total proteins, albumin and globulin in all the groups compared to the control. The ratio of albumin to globulin was elevated in all the rat groups but not significantly ($p>0.05$) different in treated groups compared to the control. It is known that, when liver cell plasma membrane is damaged, a variety of enzymes normally located in the cytosol (ALT, AST) or membrane bound enzymes such as ALP are released into the blood stream. Their measurement provides information on liver function (ALT and AST) and extrahepatic bile obstruction and intrahepatic cholestasis [24]. The present study revealed that, Drepanoalpha® is no toxic toward liver and kidney and the biosynthetic capability of liver and excretory capability of kidney are maintained.

## 3.6 Haematological Parameters

Table 4 shows the effect of lyophilized aqueous extract of Drepanoalpha® on haematological parameters in rats.

On day D14 post-treatment, no significative difference was not observed between the untreated rats (group I, control) and the rats treated with the various concentration of Drepanoalpha® (500, 1000, 2000 and 4000 mg/kg), concerning hematological parameters such as WBC, MCV, PLT, IDR, PDI, MPV, PCT ($p>0,05$).The fact that Drepanoalpha® does not alter the values of these parameters confirms that this poly-herbal formula is not toxic towards immune cells and blood clotting factors. We also observed that Drepanoalpha® increases the values of PCT PLT, RBC, Hb and HCT. This result shows that Drepanoalpha® could help in erythropoiesis and justify it use in the management of SCD.

## 3.7 Results of Histopathological Assessment of Liver

Fig. 3 shows the phenotype of liver section from group I (control, Fig. 3a) and group V (treated with 4000 mg/kg, Fig. 3b) rats.

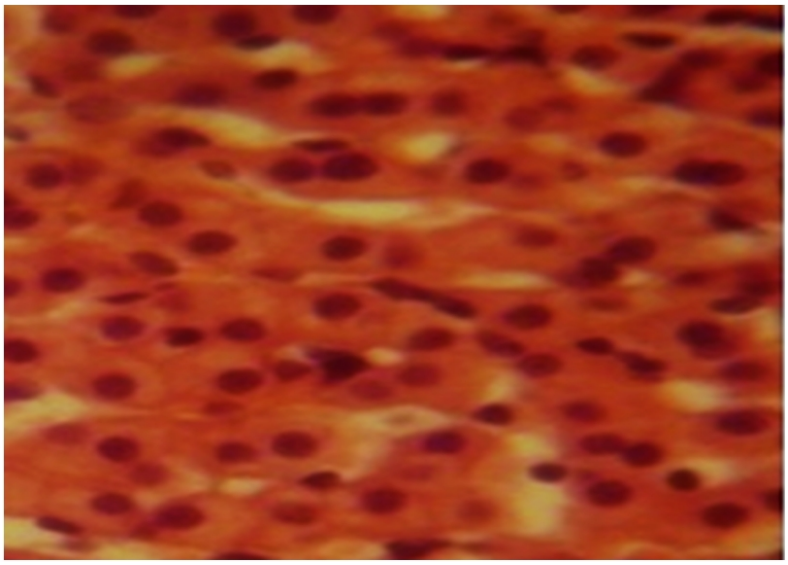

**Fig. 3a. Histological architecture of the liver of the control animals (X500)**

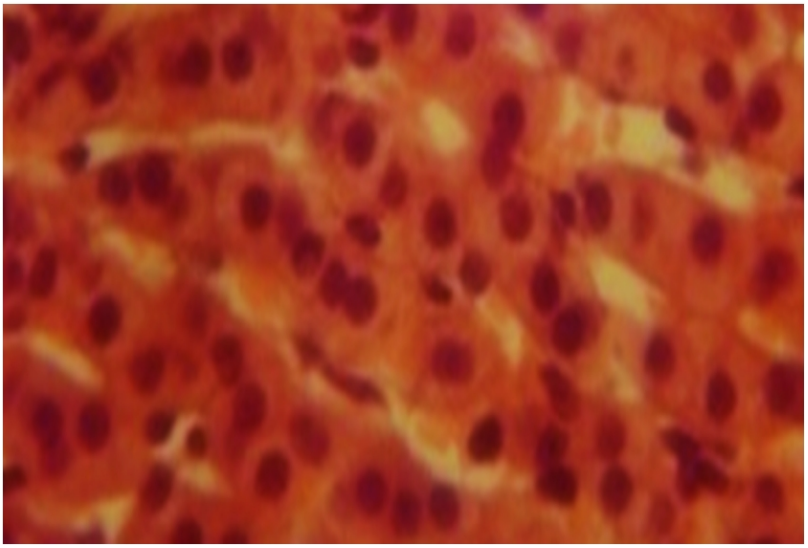

**Fig. 3b. Histological architecture of the liver tissue of rat treated with lyophilized aqueous extract of Drepanoalpha® (per os, 4000 mg/kg) (X500)**

As it can be seen, Fig. 3b shows that the animals that received the high dose of lyophilized aqueous extract of Drepanoalpha® orally displayed a normal histo-architecture of the liver cells (sinusoidals cells with well-preserved cytoplasm and prominent nucleus) as well as the control (Fig. 3a). The normal architecture observed in the histopathological examination of the liver is an indication that this poly-herbal formula did not have any adverse effect.

## 4. CONCLUSION

This study performed the *in vitro* antioxidant and antisickling activity and acute toxicity study of Drepanoalpha®. This polyherbal showed promising antisickling and radical scavenging effects *in vitro*. The acute toxicity study of lyophilized aqueous extract of Drepanoalpha® did not show any mortality up to the dose of 4000 mg/kg during the observational period of 48 hours. These results showed that in single dose, there was no adverse effect of Drepanoalpha®, indicating that the medium lethal dose ($LD_{50}$) is higher than 4000 mg/kg. The mean values of biochemical markers (ALT, AST, Urea, Creatinine, total protein) and haematological parameters (WBC, RBC, Hb, HCT, MCV, PLT, IDR, PDI, MPV, PCT) and the normal architecture of the liver of treated rats revealed that Drepanoalpha® is potentially safe.

## CONSENT

Not applicable.

## ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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