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In Vitro Biological Activities of Drepanoalpha® Ethanolic Extract, A *Justicia Secunda* and *Moringa Oleifera*-Based Phytomedicine Proposed for The Symptomatic Treatment of Sickle Cell Disease

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Abstract

Sickle cell disease (SCD) is an autosomal recessive blood disorder characterized by red blood cells that assume an abnormal, rigid sickle shape under low-oxygen conditions. These sickle-shaped erythrocytes tend to lyse, aggregate, and obstruct small blood vessels, leading to major complications. The present study aims to investigate properties that may underlie the activity of Drepanoalpha®, an antisickling herbal formulation developed in the Democratic Republic of Congo (DRC) for the prevention and symptomatic treatment of sickle cell disease crises. The Drepanoalpha® Ethanolic Extract (DEE) is a dry extract (drug-extract ratio, DER, 100/11) prepared from ethanol (96 %, v/v) percolation of a 1:1 mixture of 2 food plants, *Justicia secunda* Vahl and *Moringa oleifera* Lam. Sickling was classically measured by light microscopy on diluted washed erythrocytes obtained from homozygote patients; erythrocytes were treated with 2 % $Na_2S_2O_5$ in the presence of DEE (suspension in 9 ‰ NaCl), 9 ‰ NaCl (negative control) or disodium cromoglycate (DSCG, positive control). For all tested conditions, the sickle hemoglobin polymerization, the Fe^{2+}/Fe^{3+} ratio, and the median corpuscular fragility were measured by spectrophotometry. The DEE reversed sickling by 89.1 %, comparable to DSCG (87.7 %; 60.3 µg/mL), inhibiting sickle cell hemoglobin polymerization of 77.8 % and 74.4 %, respectively. The Fe^{2+}/Fe^{3+} ratio was improved by 18.0 % for DEE and 15.9 % for DSCG. The median corpuscular fragility values were 0.602, 0.714, and 0.732 for NaCl 9 ‰, DSCG, and DEE, respectively. The measured in vitro parameters validate an effective antisickling effect of DEE and confirm the value of this improved traditional herbal formulation for the management of SCD.

Keywords: antisickling activity; drepanoalpha®; erythrocytes; Fe^{2+}/Fe^{3+} ratio; hemoglobin polymerization; sickle cell disease

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INTRODUCTION

Sickle cell disease (SCD), or sickle cell anemia or drepanocytosis, is a genetic disease that affects hemoglobin and leads to the synthesis of hemoglobin S (HbS) instead of normal hemoglobin (HbA). In their oxygenated forms, the solubility of HbS decreases 50 times, resulting in its precipitation and intracellular polymerization, which modifies the structure of red blood cells that take a "sickle shape," which tend to lyse but also aggregate and obstruct small blood vessels, leading to major complications.[1,2] The disease is not only most prevalent in black people from Africa but is also prevalent around the Mediterranean and in India.[3] It is estimated that more than 300,000 babies are born worldwide each year with severe forms of this hemoglobinopathy.[1,4,5]

Management of the disease is difficult in developing countries, particularly in the Democratic Republic of Congo (DRC), where, with some 25 % AS genotypes, nearly 2 % of the population is affected with SS genotypes.[6] Indeed, poverty conjugates to the absence of a welfare system, with huge difficulties in meeting medical costs.[5,7] As for many diseases, the relative costs of treatments and their associated adverse effects make the recourse to herbal medicines attractive or even essential, especially for rural populations.[4,8] Effectively, an estimated 80 % of the Sub-Saharan population uses traditional medicine for health care; some plants have already proven their effectiveness, and bioactive molecules have been identified.[1,4,9,10] The growing interest and use of phytomedicines to treat sickle cell disease are also probably linked to the assumption that medicinal plants are *"natural"* and *"safe"*. [4,11–13]

In DRC, a vast bio-prospecting program has identified a hundred antisickling plants; based on an *in vitro* antisickling assay, the most active plants were developed in Drepanoalpha®, an improved traditional herbal formulation.[1,5,14,15] The Drepanoalpha® powder is a mixture 1:1 (w/w) of 2 edible plant leaves powder, *Justicia secunda* Vahl (Acanthaceae) and *Moringa oleifera* Lam.

Justicia secunda, a native tropical herbaceous plant originating from South America, is nowadays grown in tropical or subtropical African countries. In the past, this plant was considered ornamental until locals discovered the medicinal properties of its leaves, notably for the treatment of anemia, hypertension[16,17] and sickle cell disease.[14,18] The phytochemical study of leaves from various *J. secunda* cultivars revealed the presence of polyphenols, including tannins, leucoanthocyanins, anthocyanins and, mainly, flavonoids.[16] The *Moringa* genus comprises 14 species, among which *M. oleifera* is sometimes designated as the *"tree of life"* or *"miracle vegetable"*. This tropical tree, native to the sub-Himalayan mountains, is widely distributed in tropical and sub-tropical areas, both dry and humid.[19–21] The leaves used for animal and human feeding, given their alleged richness in proteins, vitamins, b-carotene, and amino acids, are now considered a functional food.[14,19,21–25] A wide variety of medicinal uses have been attributed to *M. oleifera's* various organs for anti-inflammatory, anti-infectious, cardiovascular, gastrointestinal and hematological properties, including the management of sickle cell disease.[14,19,21,24] Phytochemical studies reported the highest level of phenolic compounds, mainly flavonoids, in *M. oleifera* leaves.[26]

The originality of this study resides in the extraction that allowed to prepare of an

improved phytomedicine, easier to manage compared to traditional decoctions, and the combination of a series of anti-sickling tests (on cells and hemoglobin) to evaluate, *in vitro,* the effectiveness of this extract.

The present study was carried out on a polar extract of the 2 plants mixture to investigate properties that may underlie the use of Drepanoalpha® in the prevention and symptomatic treatment of sickle cell disease crises.

METHOD

Chemicals and reagents

All reagents were of analytical grade. 2-aminoethyl diphenylborinate (97 %) and quercetin hydrate (≥ 95 %) were purchased from Sigma-Aldrich (Merck); polyethyleneglycol 400 (PEG 400) (for laboratory use), methanol (99 %), absolute ethanol (≥ 99.8 %), methyl ethyl ketone (GPR Reactapur), formic acid (98 %) and ethyl acetate (ACS reagent) were obtained from VWR Chemicals. A 10 g/L solution of diphenyl boric acid 2-amino ethyl ester (NP reagent) and a 50 g/L solution of PEG 400 were prepared in methanol to detect polyphenols.

Herbal material

The Drepanoalpha® powder, a 1:1 (w/w) mixture of the leaves from 2 food plants, *Justicia secunda* Vahl (Herbarium MNHN-P-P00719831) and *Moringa oleifera* Lam. (Herbarium MNHN-P-P05401821) has been provided by Research for Sustainable Development (RESUD, Kinshasa, DRC), approved by producer and distributor of the phytomedicine.

Extraction

The dry extract (drug-extract ratio, DER, 100/11) was obtained by percolating 100 g of the herbal material mixture with 1000 mL ethanol 96 % for 48 h and drying under vacuum at 40°C. The resulting Drepanoalpha® ethanolic extract (DEE) was stored at ± 4°C for a maximum of 3 months. The leaves of *J. secunda* and *M. oleifera* were individually extracted similarly.

High-performance thin-layer chromatography (HPTLC)

HPTLC was performed according to the procedure of the European Pharmacopeia 10,[27] using Automatic TLC Sampler (ATS 4), Automatic Developing Chamber 2 (ADC 2), Derivatizer and TLC Visualizer 2 (Camag, Muttenz, Switzerland). The systems were driven by the software visionCATS version 2.5. The HPTLC was performed on silica gel 60 F_{254} HPTLC plates (Merck, Germany); 2 µL of quercetin (1 mg/mL MetOH), 10 µL of DEE (30 mg/mL MetOH) and 5 µL of *J. secunda* and *M. oleifera* (30 mg/mL MetOH) extracts were applied in 6-mm wide bands, the plates were activated on $MgCl_2$ (~ 33 % RH) and the tank saturated for 20 min; the solvent system was ethyl acetate - methylethylketone - formic acid -water (60:30:5:5, V/V/V/V) and the plate was developed over a path of 60 mm (Fig.1). The plate was heated at 100 °C for 3 min, sprayed with 2 mL of the NP reagent (Derivatizer with green nozzle, level 3) and PEG (Derivatizer with blue nozzle, level 2), and photographed immediately after derivatization under UV_{365nm}, using the Visualizer system.

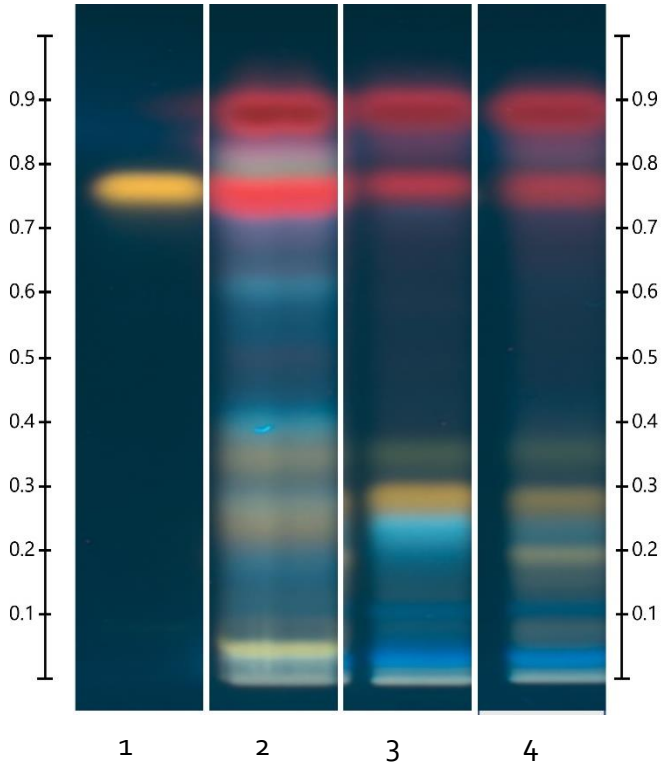

Figure 1. HPTLC profile of the ethanolic extracts of Drepanoalpha® and its constituent plants.
Stationary phase: HPTLC plate (Silica gel 60 F254)
Mobile phase: ethyl acetate - methylethylketone - formic acid - water (60:30:5:5, V/V/V/V);
Detection: NP-PEG-400; 1% solution of 2-aminoethyl diphenylborinate in methanol, followed by 5% polyethylene glycol in ethanol; examination under UV_{365nm}.
Legend: Tracks: 1-Quercetin, 2-*Justicia secunda*, 3-*Moringa oleifera*, 4- Drepanoalpha®.

Blood samples

Blood samples were left-overs of specimens sampled for the regular monitoring of known sickle cell disease homozygote patients attending the *"Centre de Médecine Mixte et d'Anémie SS"* (Kalamu district, Kinshasa, DRC) and the *"Hôpital Civil Marie Curie"* (Charleroi, Belgium). None of these patients had experienced a recent transfusion with Hb AA blood. All antisickling experiments were carried out with blood freshly collected on citrate and stored at ± 4 °C for a maximum of 72 h. Red cell pellets, obtained by centrifugation (1500 g, 10 min) of 0.5 mL of SS blood, were washed thrice with NaCl 9 ‰, in a 1:10 (v/v) ratio, centrifuged (1500g, 10 min) and resuspended in 4 mL NaCl 9 ‰.

The study was conducted after receiving the approval of the ethical and scientific committee of the School of Public Health, Faculty of Medicine, University of Kinshasa, Kinshasa-DRC (Approval No.: ESP/CE/237/2019) and of the I.S.P.P.C. OM008 ethical committee of "C.H.U. Charleroi", Charleroi, Belgium (Approval No P19/55-23/10 CHRAU: UMONS CCB: B325201941714).

***In vitro* antisickling activities**

Induction of sickling and inhibition of falciformation

Samples of 950 µL of blood, obtained from homozygote patients, diluted 1:10 in 9 ‰ NaCl, were added with 10 µL of Drepanoalpha® Ethanolic Extract (DEE) (suspension in 9 ‰ NaCl), 9 ‰ NaCl

(negative control) or disodium cromoglycate (DSCG, positive control) and homogenized. Upon adding 50 µL of 2 % $Na_2S_2O_5$ and homogenizing, 5 µL of samples were placed on a microscope slide, covered and smeared with clear varnish to isolate from oxygen and induce hypoxia and sickling. The sickled/normal erythrocyte ratios were measured in light microscopy at times 0 and 60 min on images of 5 different microscopic fields acquired with a digital camera (Olympus U-CMAD3):

$$\mathrm{F}\ (\%) = \frac{\mathrm{SRB}}{\mathrm{TRB}} x100$$

Where F: Sickling Cell Rate; SRB: Sickled Red Blood Cell Count and TRB: Total Red Blood Cell Count.

The proportion of sickle cell inhibition SI was calculated as follows (28):

$$SI = \frac{F_0 - F_n}{F_0}\ x\ 100$$

Where SI is the percentage of sickling inhibition, F_0 is the % of sickling of the mixture [SS blood + $Na_2S_2O_5$] (negative control) and F_n is the % of sickling of the mixture [SS blood + tested extract or compound + $Na_2S_2O_5$].

For a test to be considered valid, the ratio $TRB_{60\ min}/TRB_{0\ min}$ should be over 80 $\pm$ 5 % to control that only limited hemolysis has been induced by the tested extract or compound.

The capacity of extracts to prevent, reverse or protect against falciformation

To understand whether the tested extract or compound prevents or reverses falciformation, 3 procedures were assessed: a) A mixture of 950 µL diluted blood and 50 µL $Na_2S_2O_5$ was incubated for 60 min in a closed vial, then added with 10 µL of DEE, NaCl 9 ‰ or DSCG and homogenized; b) A mixture of 950 µL diluted blood and 10 µL DEE, NaCl 9 ‰ or DSCG was incubated for 60 min in a closed vial, then added with 50 µL $Na_2S_2O_5$ and homogenized; c) A mixture of 950 µL diluted blood, 10 µL of DEE, NaCl 9 ‰ or DSCG and 50 µL $Na_2S_2O_5$ was incubated for 60 min in a closed vial.

For the different procedures, the proportions of sickle cell inhibition were assessed as previously described.

Determination of the Minimum Reversibility Concentrations (MRC)

For the MRC determination, the proportions of sickle cell inhibition (= reversibility rate) were measured as described here above by varying the concentrations of DEE and DSCG (50 to 250 µg/mL). For each concentration, the proportion of sickle cell inhibition (SI) was calculated as above to determine the rate of reversibility R as:

$$R\ = \frac{SI_0 - SI_n}{SI_0}\ x\ 100$$

Where R is the reversibility rate (%) and SI_0 and SI_n are the proportions of sickling inhibition for the control (NaCl 9 ‰) and the tested concentration, respectively.

Dose-effect curves were obtained by fitting data to the equation $y = \frac{A_1 - A_2}{1 + (x/x_0)^p} + A_2$ using the Origin 8.5 software (OriginLab, Northampton, MA, United States).

Inhibition of sickle hemoglobin (HbS) polymerization

According to the original method of (29), the HbSS polymerization was assessed at 700 nm from the turbidity of a polymerizing mixture. 200 µL of a red cell pellet were hemolyzed by adding 400 µL of distilled water, incubated for 30 min in the presence or absence of the drug (400 µL) and primed for polymerization by deoxygenating with 3000 µL of a 2 %

sodium metabisulphite solution. The optical densities were measured after centrifuging at 3500 rpm for 5 min. Their difference yields the measure of turbidity. The rate of polymerization inhibition was estimated by the tangent of the graph *"absorbance versus time"*. The relative polymerization and relative inhibition were determined concerning the control (24) as

$$R_p = \frac{OD_t - OD_i}{t}$$

Where R_p = rate of polymerization, OD_t = Optical Density at time t, OD_i= initial Optical Density, t=time

Determination of erythrocyte membrane stability

The osmotic fragility of erythrocytes allows the measurement of eventual membrane-stabilizing effects by a 60 min incubation in osmotic stress conditions. 10 µL of a red cell pellet were diluted in 1990 µL of a series of buffered hypotonic saline solutions at different concentrations (0.2 - 0.8 % NaCl), added with 10 µL of DEE, DSCG, or NaCl 9 ‰ and homogenized. The effect of the different extracts on hemolysis was observed in light microscopy with a digital camera (Olympus U-CMAD3). Total cells were counted from 5 different fields across each slide at 0 and 60 min. For each NaCl concentration and extract, the percentage of hemolysis was calculated as follows:

$$\%\, Hemolysis = \frac{N_0 - N_{60}}{N_0}\, x\, 100$$

where N_0 and N_{60} are the numbers of red blood cells at 0 and 60 min, respectively. The median corpuscular fragility (MCF), the NaCl concentration that causes 50 % erythrocyte hemolysis, was estimated from the linear regression *"% hemolysis versus NaCl concentration"* using the ORIGIN 8.5 software.

Determination of methemoglobin concentration

The red cell pellet was hemolysed with distilled water in a 1: 2 ratio (v/v) and centrifuged (1500 g, 10 min). The hemolysate was incubated at room temperature in the presence or absence of the DEE/DSCG. The evolution of absorbances was measured at 540 and 630 nm for hemoglobin (Fe^{2+}) and methemoglobin (Fe^{3+}), respectively.

The proportion of methemoglobin was calculated at each time as follows:

$$Fe^{3+} = \frac{(A_{630})^2}{(A_{540})^2 + (A_{630})^2} x100$$

Fe^{3+}, A_{540} and A_{630} are the proportion of methemoglobin and the absorbances at 540 and 630 nm, respectively.

To appreciate the kinetics of the reaction in the presence or absence of extract, Fe^{3+}-time curves were obtained by fitting data to the equation $y = \frac{A_1 - A_2}{1+(x/x_0)^p} + A_2$ using the Origin 8.5 software.

Statistical analysis

All the experiments were conducted in triplicate; the data were expressed as mean ± standard deviation (S.D) and analyzed using Origin 8.5 software with a Chi-square test. The level of significance was classically set at 0.05.

Results and Discussion

Sickling induction

Table 1: Sickling induction assay (60 min contact with sodium metabisulphite in air-tight conditions; measurement over 5 microscopic fields)

Patient	Date of assay	Sample code	Total red blood cells	Sickled red blood cells	% Sickling	Test considered as
1	31/10/19	SS_70	168	139	80.4	Positive
2	31/10/19	HETERO_40	136	4	4.4	Negative
3	31/10/19	HETERO_58	185	6	3.2	Negative
4	05/11/19	SS_91	189	21	11.1	Negative
5	05/11/19	SS_54	240	240	100.0	Positive
6	08/11/19	SS_40	152	14	9.2	Negative
7	12/11/19	SS_59	144	6	4.2	Negative
8	12/11/19	SS_759	138	14	10.1	Negative
9	12/11/19	SS_36	93	78	83.9	Positive
10	12/11/19	SS_68	127	15	11.8	Negative
11	14/11/19	SS_07	100	13	13.0	Negative
12	18/11/19	SS_396	126	117	92.9	Positive
13	18/11/19	SS_597	195	162	83.1	Positive
14	12/12/19	SS_237	234	207	88.4	Positive

Fourteen samples were received from Hôpital Civil Marie Curie and tested for their ability to falciform in deoxygenation conditions (Table 1). Figure 2 shows phenotypic micrographs of representative samples. As expected, the samples from heterozygote patients yielded a very low proportion of sickled cells in our experimental conditions. However, 6 samples from homozygote patients were also weakly falciform, indicating a possible treatment by hydroxyurea (induction of non-sickling fetal hemoglobin) and antioxidants (scavenging $Na_2S_2O_5$). These heterozygotes and non-falciform samples (<50 % sickling) were not considered for the following experiments.

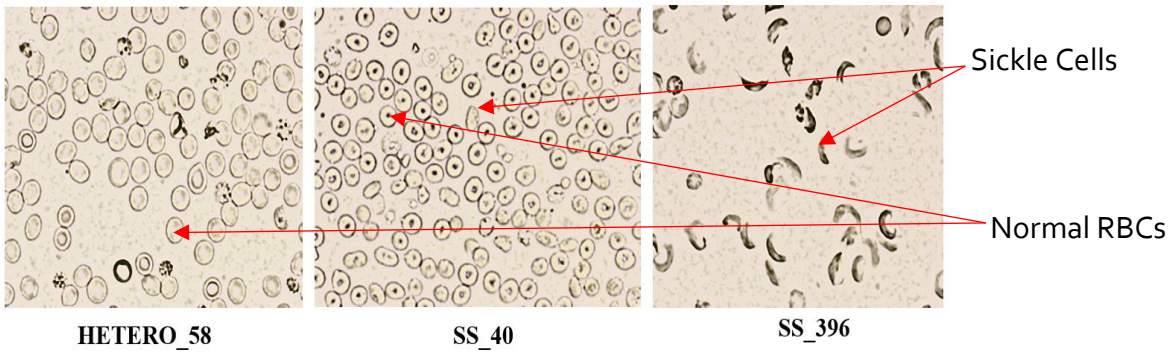

Figure 2. Sickling induction assay. Phenotypic micrographs of representative samples (60 min contact with sodium metabisulphite in air-tight conditions; 500 X)

Legends: HETERO: heterozygous blood; SS: SS homozygous blood; 40, 58, 396 are the identification numbers of the samples attributed by the laboratory of the supplying hospitals.

Reversibility assay

Table 2 details the sickling reversal of SS patient erythrocytes untreated (control) and treated with DSCG and *J. secunda*, *M. oleifera*, and DEE under hypoxic conditions. Figure 3 shows representative phenotypic micrographs of untreated and treated erythrocytes.

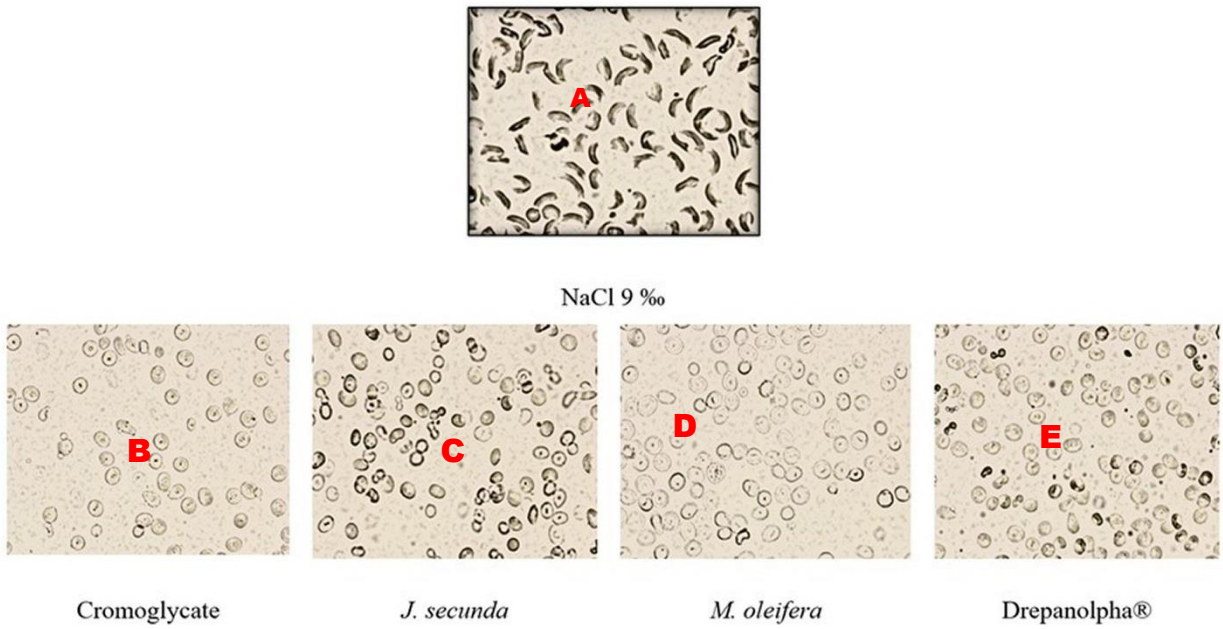

Figure 3. Morphology of drepanocytes of SS blood (Sample SS_54), A: untreated (0.9% NaCl), and upon treatment with sodium Cromoglycate (B: 250 µg/mL); ethanolic extracts of J. *secunda* (C), M. *oleifera* (D) and Drepanolpha® (E) (125 µg/mL); 60 min contact with sodium metabisulphite in air-tight conditions; 500 X)

Table 2. Anti-sickling effects on SS erythrocytes (60 min contact with sodium metabisulphite in air-tight conditions; measurement over 5 microscopic fields)

Blood sample	Negative control			Cromoglycate			Ethanolic extracts								
							Justicia secunda			*Moringa oleifera*			Drepanoalpha®		
	TRB[a]	SRB[b]	SI[c]	TRB	SRB	SI	TRB	SRB	SI	TRB	SRB	SI	TRB	SRB	SI
SS_70	168	139	82.7	159	31	19.5	168	10	6.0	162	11	6.8	156	30	19.2
SS_54	240	240	100.0	186	28	15.1	204	18	8.8	225	20	8.9	219	24	11.0
SS_36	93	78	83.9	93	9	9.7	95	7	7.4	95	7	7.4	93	9	9.7
SS_396	126	117	92.9	177	11	6.2	177	8	4.5	120	8	6.7	102	8	7.8
SS_597	195	162	83.1	174	18	10.3	189	11	5.8	189	18	9.5	183	15	8.2
SS_237	234	207	88.5	183	15	8.2	231	15	6.5	234	20	8.6	210	18	8.6
Mean			88.5			11.5			6.5			8.0			10.8
(SD)			*(6.9)*			*(4.9)*			*(1.5)*			*(1.2)*			*(4.3)*
P vs. negative control[e]			---			<0.001			<0.001			<0.001			<0.001

Description

[a] TRB: Total red blood cell count

[b] SRD: Sickled red blood cells count

[c] SI: percentage of sickling induction

[e] Anova one-way with posthoc t-tests (Tukey); there were no statistical differences between the treatments cromoglycate - *Justicia secunda* - *Moringa oleifera* - Drepanoalpha®

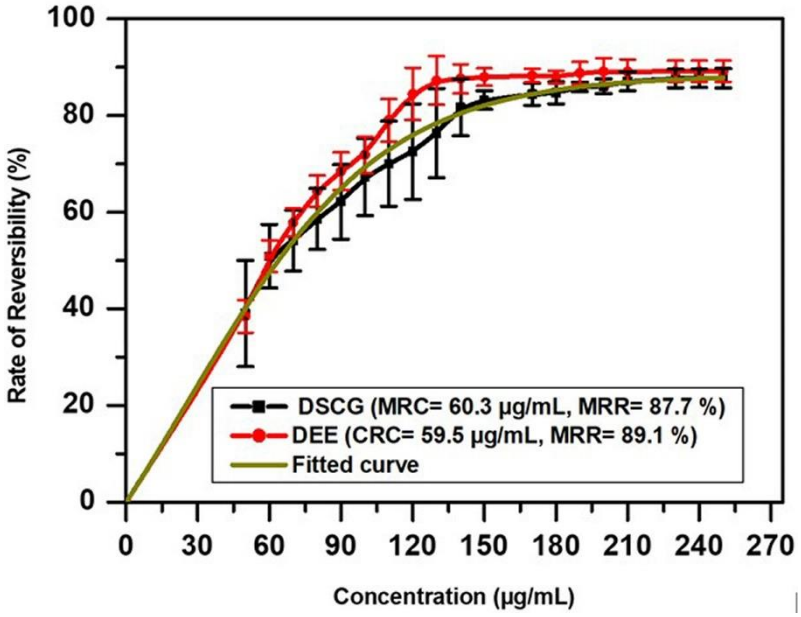

Figure 4. Reversibility rate of sickled red blood cells according to the concentration of DEE and DSCG. (60 min contact with sodium metabisulphite in air-tight conditions). (Data from 3 biological tests in triplicate, Bars represent the mean ± SD)

Description
DEE: Drepanoalpha® ethanolic extract
DSCG: disodium cromoglycate
MRC: minimum reversibility concentration
MRR: maximum reversibility rate

Figure 4 presents the reversibility rate of sickled red blood cells according to DEE and cromoglycate concentration. The rate of reversibility of sickle red blood cells in hypoxic conditions increased with the concentration of DEE or DSCG until reaching a maximum threshold (MMR, maximum reversibility rate), above which the reversibility remained constant, regardless of the increase in concentration. The minimum reversibility concentration (MRC) was defined as the extract concentration for which 50 % of the sickled cell population was normalized. MMR and MRC were evaluated by non-linear regression using ORIGIN 8.5 software

The capacity of extracts to prevent, reverse, and protect against falciformation

To verify whether DEE prevents or reverses falciformation, the SS_54 sample was treated in 3 different protocols (DEE treatment followed by deoxygenation, i.e., prevention; deoxygenation followed by DEE treatment, i.e., reversal; concomitant DEE treatment and deoxygenation, i.e., protection) compared with cromoglycate. Figure 5 shows the phenotypic micrographs of treated samples.

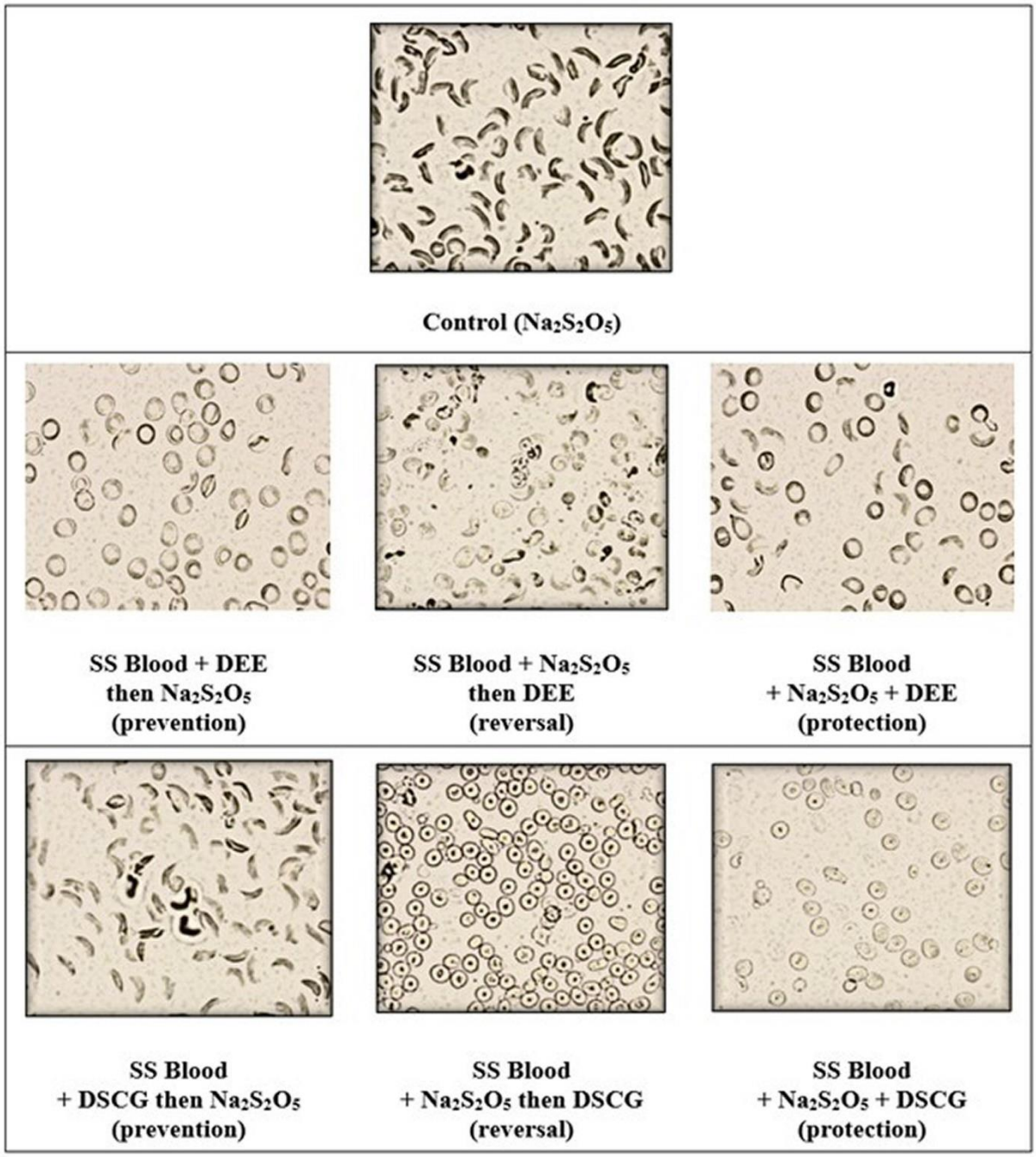

Figure 5. Morphology of drepanocytes of SS blood (Sample SS_54) upon different schemes of treatment with Drepanolpha® ethanolic extract (DEE) or cromoglycate (DSCG)
(125 µg/mL; 60 min contact with sodium metabisulphite in air-tight conditions; 500 X)

These experiments indicated that the DEE had the ability to prevent, reverse and protect against erythrocyte sickling. Although DSCG also reversed and protected against sickle cell formation, the preventive DSCG treatment appeared inefficient in averting sickling.

Inhibition of sickle hemoglobin (HbS) polymerization

The polymerization rates of HbS and its inhibition are presented in Table 3

Table 3. Polymerization rates of HbS and its inhibition by the Drepanoalpha® extract (DEE) and cromoglycate (DSCG) (n = 3 technical replicates)

Sample	Rate of polymerization	Relative % Inhibition vs. negative control
Negative control	0.65 ± 0.01	----
DSCG	0.17 ± 0.0	74.4 ± 0.0
DEE	0.14 ± 0.02	77.8 ± 0.0

Table 3 indicates that the polymerization of sickle hemoglobin (HbS) is partly inhibited in the presence of either DSCG or DEE in a similar proportion. This property is well known to contribute to antisickling activities.

Stabilization of erythrocyte membranes

Figure 6 indicates an effective hypoosmolarity-induced lysis of sickle erythrocytes when decreasing the NaCl concentration. Although this effect was less marked in the presence of Drepanoalpha® extract or cromoglycate, the protection afforded was not statistically significant.

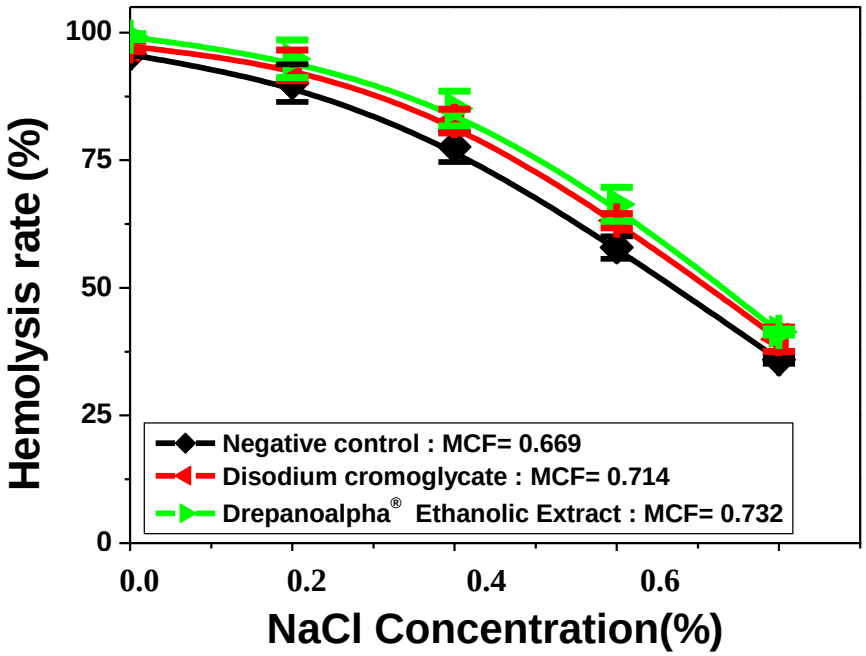

Figure 6. Lysis susceptibility of sickle erythrocytes, according to osmolarity, in the presence of Drepanoalpha® ethanolic extract or cromoglycate (125 µg/mL; 60 min incubation at room temperature)

Description

MCF: median corpuscular fragility

Modulation of methemoglobin formation

Figure 7 and Table 4 show the evolution of methemoglobin as a function of time; DEE and DSCG significantly reduced the formation of methemoglobin in HbSS blood, preventing the oxidation of Fe^{2+} into Fe^{3+}.

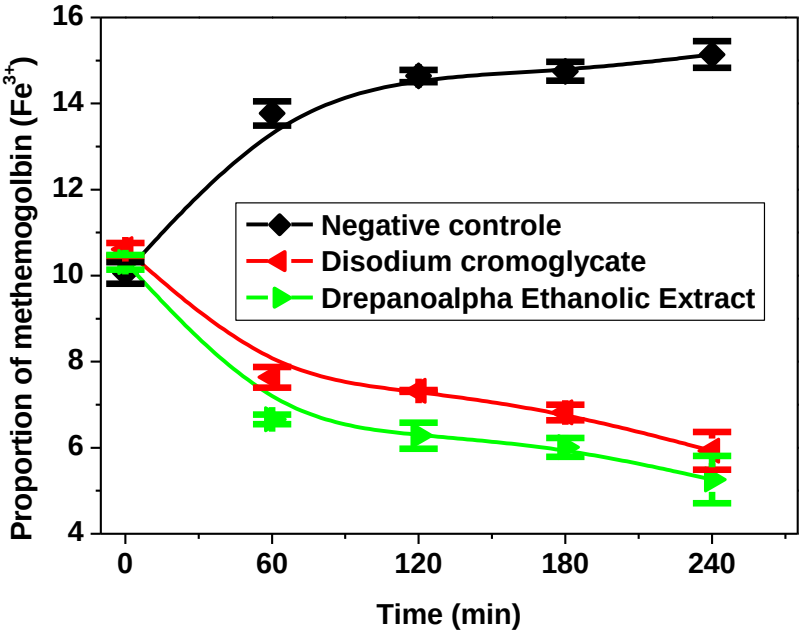

Figure 7. Evolution of methemoglobin proportion versus time. Bars represent the mean ± SD for N=3 technical replicates.

Table 4. Modulation of methemoglobin formation in the presence of DEE and DSCG (mean ± SD; n = 3 technical replicates)

Sample	%Hemoglobin (Fe^{2+})	%Methemoglobin (Fe^{3+})	Fe^{2+}/Fe^{3+}	% increase
Negative Control	84.9±0.3	15.1 ± 0.3	5.6 ± 1.0	-----
DSCG	94.1±0.4	5.9 ± 0.4	15.9 ± 1.0	64.64±1.3
DEE	94.7±0.6	5.3 ± 0.6	18.0 ± 1.0	68.85±1.0

RESULTS AND DISCUSSION

The bioactivity was assessed based on the phenotypic sickling of SS red blood cells (RBCs) in the presence of an oxygen-scavenging agent. The individual plant extracts and DEE displayed remarkable sickling inhibitory effects, reverting sickle erythrocytes to a typically normal morphology in the same proportions as cromoglycate, a well-known positive control (18,24,30). It confirms that the DEE extract conserves the previously shown antisickling effects of *Moringa oleifera* extracts[24] and Drepanoalpha® herbal powder.[1] The sickling of red blood cells is a process that results from the polymerization of Hb S under conditions of hypoxia and cellular dehydration by loss of ions (K^+, Cl^-, Mg^{++}) and water (31,32). The antisickling effect indicated that DEE could rehydrate deoxygenated sicked red cells, thus preventing the increase of intracellular Hb S concentration. Anthocyanins, part of DEE secondary metabolites[1], have been shown to be

responsible for most plants' biological activities against sickle cell disease in traditional Congolese medicine by weakening hydrophobic interactions at the intermolecular contact sites of different deoxyhemoglobin S molecules.[1,15,33–37] The antisickling effect of DEE is higher compared to some antioxidants and micronutrients often combined in the management of sickle cell disease, i.e., magnesium (0.1 mM;48.4 % reversal of *in vitro* falciformation), zinc (0.1 mM; 89.7 %), vitamins A (100 IU; 30.9 %), C (1 mg/mL; 38.1%) and E (1 mg/mL; 30.9 %) (38). The action of DEE on the inhibition of polymerization could be a synergistic effect of its (phyto)chemical constituents such as anthocyanins, flavonoids and micronutrients, including the Mg^{++}, Zn^{++}, and Vit A .[1] However, the copper identified among the micronutrients of this phytomedicine (1) is a negative agent for sickle cell disease whose level should be controlled in the final product.

It is well established that dense and dehydrated red blood cells can contain HbS polymers under conditions of moderate hypoxia, and even in arterial blood, due to the particularly high intracellular concentration of HbS. These dense and dehydrated red blood cells thus play a central role in sickle cell disease's acute and chronic manifestations based on reduced blood flow and vaso-occlusions in small vessels (31). The erythrocyte membrane stability test performed on sickled red cells in hypotonic NaCl condition indicates a slight but insignificant increase of sickle cell resistance as measured by the MCF. It would be interesting to repeat the test with the preincubation of erythrocytes before hypotonic stressing to evaluate an eventual membrane protective effect.

Given the high red cell oxidative status (39,40), hemoglobin can oxidize to methemoglobin and thus lose the property of combining with oxygen (41–44). In normal blood, only a very small amount of methemoglobin exists. An effective system based on nicotinamide adenine dinucleotide phosphate (NADPH), methemoglobin reductase and cytochrome B_5 to reduce the heme Fe^{3+} to Fe^{2+}, the metabolic shunt pathway of pentose phosphates in erythrocyte is necessary for the synthesis of NADPH that protects hemoglobin and membrane lipids from oxidation.[45] However, this reduction system is less efficient in cases of glucose-6-phosphate dehydrogenase (G-6-PD) deficiency, an erythrocytic enzymopathy often associated with sickle cell disease. A treatment inducing a decrease in methemoglobin level would indicate an effective antioxidant effect on sickle red blood cells,[46] likely to protect from sickling and senescence. As shown here, both DSCG and DEE effectively improve the Fe^{2+}/Fe^{3+} ratio, a mechanism likely to increase the oxygen affinity of drepanocytes and so to reverse sickle improving the Fe^{2+}/Fe^{3+} ratio erythrocytes to their original biconcave structure. Our results indicated that, *in vitro,* DEE effectively prevented both erythrocyte sickling and hemoglobin oxidation. Here again, this activity could be the result of the DEE content in polyphenols, including flavonoids, and trace elements such as Mg^{++}, Zn^{++} and Vit A (1,38,47).

This study will likely impact transfusion treatments as our previous clinical studies on the phytomedicine decoction have shown an increase in Hb level and protection against early hemolysis, which would prevent anemia and avoid transfusion (48,49).

CONCLUSION

This research depicted *in vitro* anti-sickling activity of Drepanoalpha® ethanolic extract. The results revealed that DEE preventing sickling and reversing sickled HbSS red blood cells had a membrane stabilizing effect on sickled red blood cells, possessed abilities to inhibit sickle cell hemoglobin polymerization, and improved the oxidant status of erythrocytes by increasing the Fe^{2+}/Fe^{3+} ratio in a sickled red blood cell. It highlighted that this traditional improved herbal formulation had medicinal benefits, confirming its use in managing sickle cell disease (SCD). Future studies are suggested to identify and isolate the active principle of this phytomedicine by bio-guided fractionation, which could enhance the standardization of this anti-sickling recipe.

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