



Antisickling Activity and Membrane Stabilizing Effect of Anthocyanins Extracts from *Adansonia digitata* L. Barks on Sickle Blood Cells

P. T. Mpiana^{1*}, F. S. Misakabu², D. S. T. Tshibangu¹, K. N. Ngbolua¹
and D. T. Mwanangombo¹

¹Faculty of Sciences, University of Kinshasa, P.O. Box 190, Kinshasa XI, DR Congo.

²Faculty of Science and Applied Sciences, Official University of Bukavu Bukavu, DR Congo.

Authors' contributions

This work was carried out in collaboration between all authors. Author PTM designed the study and author FSM wrote the first draft of the manuscript. Author KNN performed the statistical analysis, wrote the protocol, author DSTT managed the analyses of the study.

Author DTM managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Background: Parts of baobab tree (*Adansonia digitata*) including especially the barks are commonly used for their medicinal properties.

Aims: The aim of this work is to evaluate the antisickling activity of baobab tree barks, which are used in Congolese traditional medicine to manage Sick Cell Disease.

Study Design: Baobab tree barks was extracted with water by maceration. Phytochemical tests were conducted with standard procedures. Antisickling activity and the minimum concentration of extract required to normalize sickled cells was determined by Emmel test.

Place and Duration of Study: This work was done at department of chemistry, Science Faculty, University of Kinshasa (DR Congo), between November 2012 and February 2013.

Methodology: The barks collected from *Adansonia digitata* were dried and powdered. A chemical screening was performed and extraction of anthocyanins done. Antisickling activity

*Corresponding author: E-mail: ptmpiana@yahoo.fr;

was evaluated by Emmel test, membrane stability by osmotic fragility test and Fe³⁺ evolution by following solution absorbance at 630nm. The rate of sickle cell shape normalization was determined at different plant aqueous extract concentrations in order to determine the minimal concentration of extract required to normalize sickle cells. The shape modification was quantitatively evaluated from the values of parameters such as surface, radius and perimeters of sickle blood cells before or after treatment with plant extract using Motic software.

Results: The aqueous extract of *Adansonia digitata* showed an antisickling activity with a maximal normalization rate of 65.7% and a minimal concentration required to normalize sickled cells of 5.0mg/mL. The cell surface, perimeter and radius were significantly different before and after treatment with plant extract. The chemical screening showed the presence of polyphenols among which anthocyanins. The biological activity of this plant would be due to these pigments. The anthocyanins extract have also shown a stabilization effect on sickle blood red cells membranes and a reduction of methemoglobin to hemoglobin effect

Conclusion: The results obtained show significant antisickling activity of *Adansonia digitata* barks thus justifying the use of this plant by traditional healers in Congolese traditional medicine in the management of Sickle Cell Disease.

Keywords: *Antisickling activity; anthocyanins; adansonia digitata; osmotic fragility test; hemolysis.*

1. INTRODUCTION

Each year approximately 100,000 children in the world are born with sickle cell disease (SCD) which is a genetic disorder. This disease is considered as a public health problem in many countries, but with a major burden in Africa particularly in tropical regions in west and central Africa [1-5]. SCD also known as sickle cell anemia or drepanocytosis, is an inherited illness which is caused by an abnormal hemoglobin. The SCD causal hemoglobin (Sickle hemoglobin or S hemoglobin, Hb S), comes from a mutation at the 6th position of the beta globin chain, which led to the substitution of glutamic acid, a polar amino acid, by valine a non-polar amino acid. This structural modification influences significantly physical and chemical properties of hemoglobin, hemoprotein that are responsible for the transport of oxygen from the lungs to other tissues in the body [1,4-7].

This mutation decreases the affinity of hemoglobin for oxygen. At low oxygen tension, the mutant hemoglobin polymerizes inside the red blood cell into a gel or further into fibers leading to a drastic decrease in the red cell deformability. Polymerization and precipitation of S hemoglobin within the erythrocytes cause the change of the shape of erythrocytes from their normal globular form into one resembling a sickle. Sickling of blood cells is the cause of precocious hemolysis of erythrocytes and various complications of SS subjects [4-7].

Up-to-date, there is no affordable and efficient solution for this disorder. Proposed therapies such as the medullar transplantation remain very expensive for most African population, or struggle with considerable inconveniences such as toxicity of some drugs, the high risk of transmission of other infections, etc. African population and other of developing country in the world have recourse to medicinal plants in order to treat some diseases among which drepanocytosis [2-4,8-16].

In Democratic Republic of the Congo (DRC), our research team has initiated a large ethnopharmacological survey in order to identify plants that are used in the management of sickle cell anemia, and to verify the effectiveness thereof [3,4,13,16-19]. For many plants already investigated, anthocyanins extracts have been found to be highly active, suggesting that anthocyanins are among main active metabolites [20-32].

In the present study, as a continuity of our investigation, we report the *In vitro* antisickling activity of anthocyanin extracts from barks of *Adansonia digitata* L., another plant used by Congolese practitioners for management of sickle cell anemia. *A. digitata* or Baobab is a very long-lived edible tree with multipurpose uses. Every parts of baobab are reported to be used in traditional medicine as immune stimulant, anti-inflammatory, analgesic, antipyretic, febrifuge, astringent etc.; but it's antisickling activity is not enough reported [8,33-35].

2. MATERIALS AND METHODS

2.1 Plant Material

The barks of *Adansonia digitata* L. used in this study were collect from Baobab trees in various sites of the University of Kinshasa and its surroundings during November 2012. Their identification was carried out by botanists of "Institut National d'Etudes et des Recherches Agronomiques (INERA)". Voucher specimen has been deposited at Herbarium of INERA situated to the department of Biology, Faculty of Science, University of Kinshasa in DRC.

2.2 Extraction and Chemical Screening

The dried and powdered plant material (barks 10g) was repeatedly extracted by cold percolation with 95% EtOH and water (100mlx1) for 48hrs. Fractions were filtered and concentrated to dryness under reduced pressure using a rotary evaporator. A chemical screening has been done on this plant using an aqueous or organic fraction following an established protocol [36]. Extraction of anthocyanins was then done by maceration of 100g of dried powdered plant material (bark) with distilled water and diethyl ether following an established protocol as previously reported [22-32,36].

2.3 Biological Material

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 Mixte et d'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 hemoglobin electrophoresis on cellulose acetate gel and then stored at $\pm 4^{\circ}\text{C}$ in a refrigerator. Ethical clearance on the use of Sickle blood cells was strictly observed according to international rules.

2.4 Bioactivity Evaluation

2.4.1 Emmel test

Sickle cell blood was diluted with 150mM phosphate-buffered saline (NaH_2PO_4 30mM, Na_2HPO_4 120mM, NaCl 150mM) and mixed with an equivalent volume of 2% sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$). A drop from the mixture was spotted on a microscope slide in the presence or absence of an anthocyanin extracts and covered with a cover slip. Paraffin was applied to seal the edges of the cover slip completely to exclude air. The red blood cells (RBCs) were analyzed by measuring various parameters including the area, perimeter and radius of each RBC using a computer assisted image analysis system (Motic Images 2000, version 1.3). The data were processed using Microcal Origin 7.1 package software.

2.4.2 Osmotic fragility test

The fragility of RBCs was determined by placing the cells in graded series of hypotonic saline solutions buffered at pH7.4 with 150mM phosphate. Concentrations ranging from 0.2% to 0.9%NaCl were made up in a final volume of 10mL. A 10 μL sample of washed Sickle RBCs was added to 1990 μL of each hypotonic saline solution and immediately mixed by inverting several times. The tubes were allowed to stand for 150min at room temperature. To determine the effect of the anthocyanin extracts, 10 μL of extract (30mg/mL) were added to 1980 μL of each hypotonic saline solution, then 10 μL of RBCs added and the mixture treated as described earlier [17,23,24,37]. The number of RBCs not lysed at each saline concentration was determined using a photonic microscope (OLYMPUS $\times 21$) and a haemocytometer (Neubauer'scell). Hemolysis was calculated using the following equation:

$$\text{Number of RBCs after 150min} \times 100 / \text{number of RBCs inoculated.}$$

The mean corpuscular fragility (determined from the concentration of saline causing 50% hemolysis of the RBCs) was obtained from a plot of lysis (%) *versus* NaCl concentration.

2.4.3 Fe³⁺ profile

The Fe³⁺ profile was determined according to the method of Mpiana et al. [17,23,24]. Briefly this profile was followed using spectroscopic method at 630 nm with time. Whole blood 0.02 mL was diluted in 5mL of distilled water in which 0.02 mL normal saline solution (NaCl 0.09%) or 0.02mL of anthocyanins extracts were added. Solutions were incubated for 60 minutes.

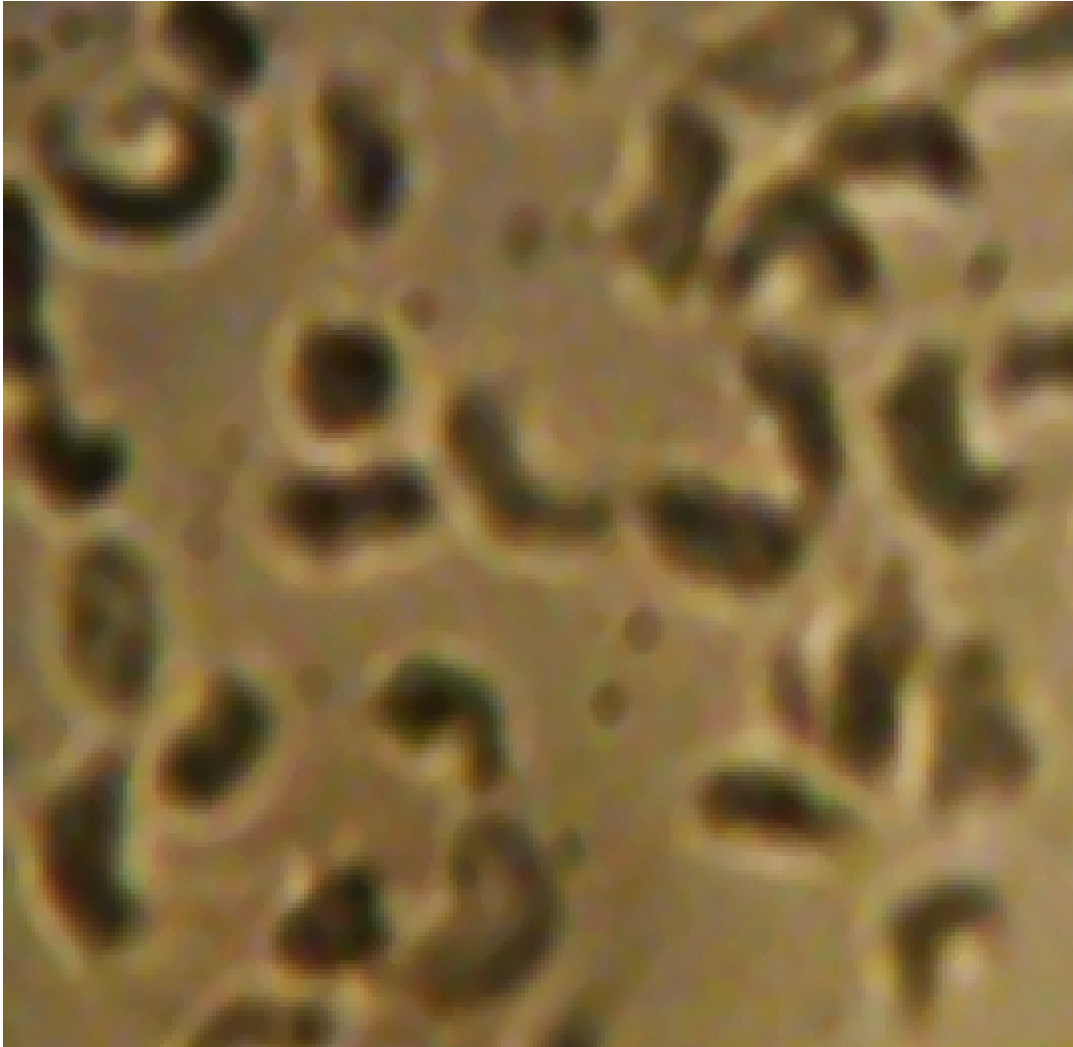
2.5 pH and Optical Density Measurement

The pH values were determined with Metrohm E 604 pH-meter equipped with a glass electrode. This electrode was kept soaked in 3mol. L⁻¹ KCl solution and calibrated with aqueous standard buffer. Absorbance of solutions was measured with HACH DR/4000U UV-Visible spectrophotometer.

3. RESULTS AND DISCUSSION

3.1 Antisickling Activity of the Aqueous Extracts from *Adansonia digitata* Barks

Figs. 1 and 2 show respectively micrographies of sickle RBCs alone in a NaCl 0.9% solution (control) and the sickle RBCs incubated with the aqueous crude extract of *Adansonia digitata* barks.



**Fig. 1. Morphology of drepanocytes of untreated sickle RBCs (control) (x500)
[NaCl 0.9%; Na₂S₂O₄ 2%,]**

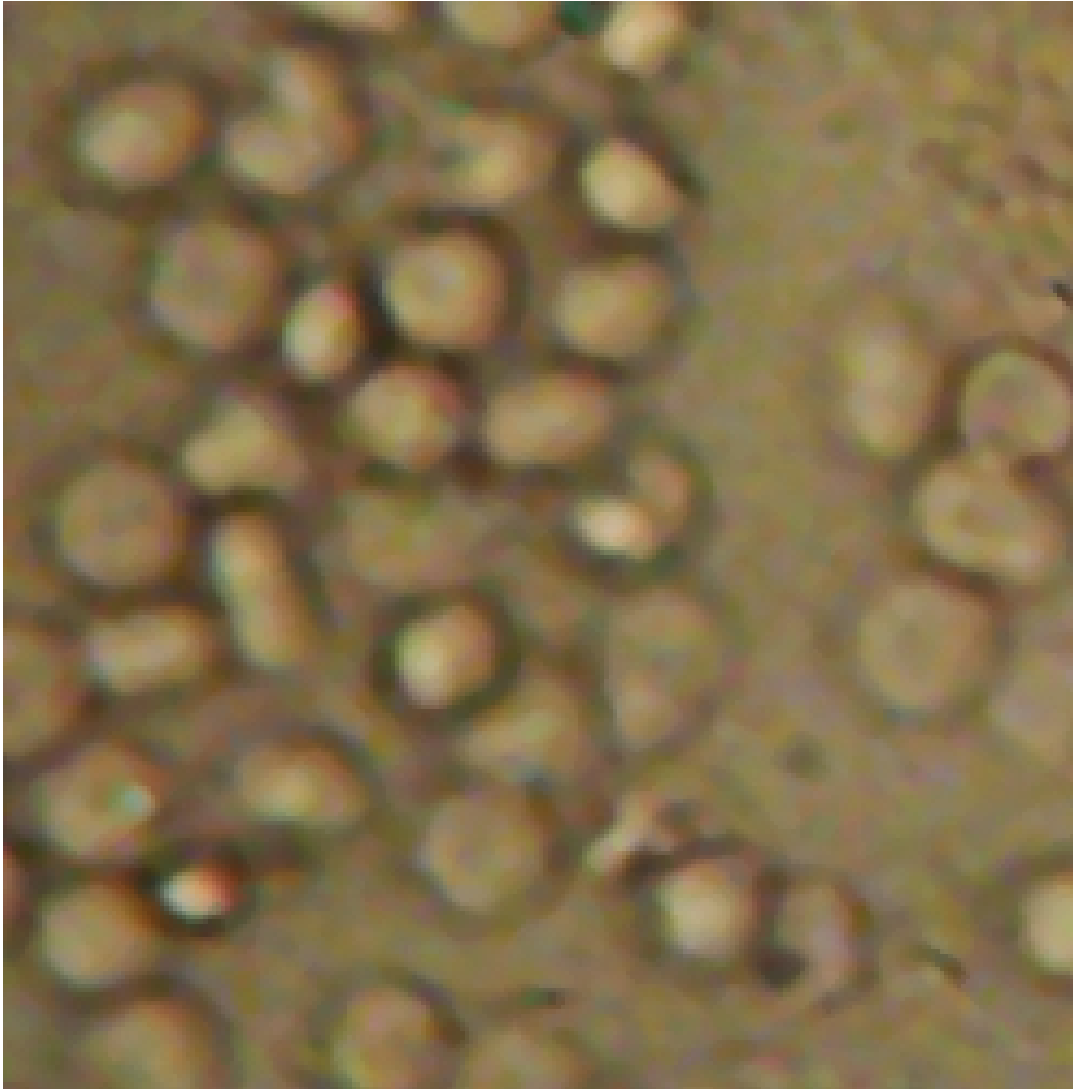


Fig. 2. Morphology of drepanocytes treated with 5mg/mL of aqueous extract of *Adansonia digitata* barks (X500), [NaCl 0.9% ; Na₂S₂O₄ 2%,]

Fig. 1 shows clearly that the control contains the majority of sickle-shaped erythrocytes, confirming the sickle cell nature of the blood. Mixed together with total aqueous plant extract from *A. digitata* barks Fig. 2, the majority of erythrocytes are reversed into normal-shape. This fact indicates that the crude aqueous extract of this plant has an antisickling activity on drepanocytes. This normalization of sickle erythrocytes in hypoxic condition is experimental evidence justifying the use of this plant in Congolese traditional medicine in the management of sickle cell disease. These results are in perfect agreement with those of previous studies [3,4,16-32]. Indeed, aqueous and alcoholic extracts of more than 70 plants used by Congolese traditional healers for the management of this chronic disease have shown an interesting *In vitro* antisickling activity.

Shape normalization can be quantitatively evaluated from the values of parameters such as the area, the radius and the perimeters of treated and untreated sickle blood cells. The Table 1 gives the average values of different measures on untreated sickle blood cells (control) and treated with plant extracts.

Table 1. Mean values of perimeters, surfaces and radius of untreated and treated sickle blood cells

	Cells perimeter (μm)	Cells surface (μm^2)	Cells radius (μm)
Untreated RBCs (control)	25.1 \pm 1.3	33.2 \pm 2.3	-
RBCs treated with <i>A. digitata</i>	15.6 \pm 0.9	29.3 \pm 1.6	3.1 \pm 0.4

It can be seen from this table that the perimeter and area of untreated RBCs are higher than those of the treated one with *A. digitata* barks extracts. This difference can be justified by the stretched out shape (sickle) taken by the sickle cell in hypoxic conditions ($P_{\text{O}_2} < 45$ mm Hg). In the untreated RBCs, the used software could not give the radius because of the sickle shape of drepanocytes. But, in the presence of plant extracts, the normal biconcave shape conducts to the reappearance of radius values. These results are in agreement with our previous findings on other Congolese bioactive plants and confirm the antisickling activity of *A. digitata* [3,4,16-32].

3.2 Determination of Minimal Concentration of Normalization

Fig. 3 shows the evolution of the normalization rate of sickle cells shape with the concentration of *A. digitata* bark aqueous extracts.

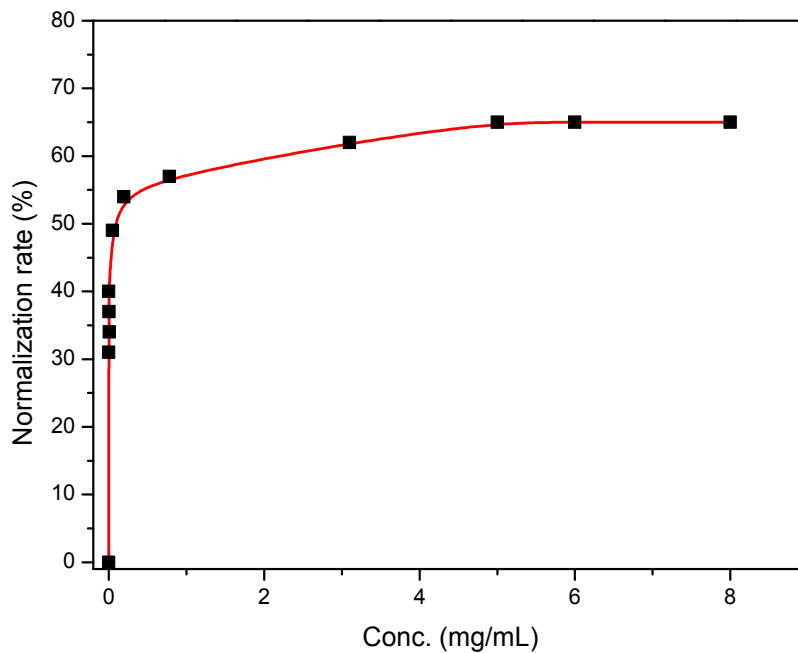


Fig. 3. Evolution of normalization rate of sickle cell shape with the concentration *A. digitata* bark aqueous extracts

This curve shows that the normalization rate or the percentage of sickle cell which regain the normal shape in hypoxic condition increases with the concentration in plants extracts to achieve the maximum threshold above which the normalization rate remains constant regardless of the increase in concentration. The Minimum Concentration of Normalization (MCN) value was 5.00 mg mL^{-1} corresponding to a maximal normalization rate (NRmax) of 65.74% with an concentration of extract for which 50% of the sickled erythrocytes are reversed (ED_{50}) equal to 0.54 mg mL^{-1} .

So, the antisickling activity of *Adansonia digitata* aqueous extract is dose dependent. This result shows that extract of *A. digitata* strongly inhibit the sickling of drepanocytes induced by 2% sodium metabisulfite and is significant if compared with the results obtained by other plants. Indeed, Iyamu et al. [38] have obtained 50% of reversion of erythrocytes sickling with 5 mg mL^{-1} of NIPRISAN[®], natural occurring potent antisickling based plants, *Thomandersia hensii* for instance has shown a MCN of 12.5 mg mL^{-1} and *Centella asiatica* a MCN of 25 mg mL^{-1} [3]. Nevertheless, it can be noted that the MCN of *A. digitata* barks is lower than that obtained for some other plants like *Bombax pentadrum* (0.34 mg mL^{-1}), *Ricinodendron heudelotii* (0.39 mg mL^{-1}), *Ipomoea batatas* leaves (0.54 mg mL^{-1}), *Trema orientalis* (2.1 mg mL^{-1}) and the NRmax obtained is less than that of *Ricinodendron heudelotii* (76%), *Thomandersia hensii* (75%) *Centella asiatica* (71%) and *Bombax pentadrum* (78%) [3,20,21,24].

3.3 Phytochemical Composition of *Adansonia digitata*

The chemical screening performed on the aqueous and ethanolic extracts of *A. digitata* bark revealed an abundant presence of polyphenols among which anthocyanins and tannins. The presence of anthocyanins in this plant is an interesting indication, since previous works of our research team indicated that these secondary metabolites are responsible of the antisickling activity of others Congolese plants [20-32]. Therefore, anthocyanins from *A. digitata* barks were extracted and their antisickling activity tested.

3.4 Antisickling Activity of Anthocyanins Extracts from *A. digitata* Barks

Fig. 4 gives microphagy of sickle cell blood in the presence of anthocyanins extracted from *A. digitata* barks.

This Fig. 4 shows that in presence of anthocyanins extracted from *A. digitata* barks, the majority of sickle-shaped erythrocytes in sickle RBCs Fig. 1 reversed their shapes to the normal biconcave form. This indicates that the anthocyanins extract of *A. digitata* have the capacity to reverse sickle-shaped erythrocytes into their normal biconcave form. These results confirm those already obtained by our research team with anthocyanins extracts from other plants used in Congolese traditional medicine against sickle cell anaemia [20-32].

The Anthocyanins, because of their properties to adsorb themselves on proteins would block the polymerization of the desoxyhemoglobin S in tactoids; this could reduce the sickling process and thus, induce the return to the normal biconcave form of the erythrocytes as the Emmel test reveals it.

Another probable mode of anthocyanins' action is their possible effects on the erythrocyte membrane hydration. This can be evaluated using osmotic fragility of blood cells.

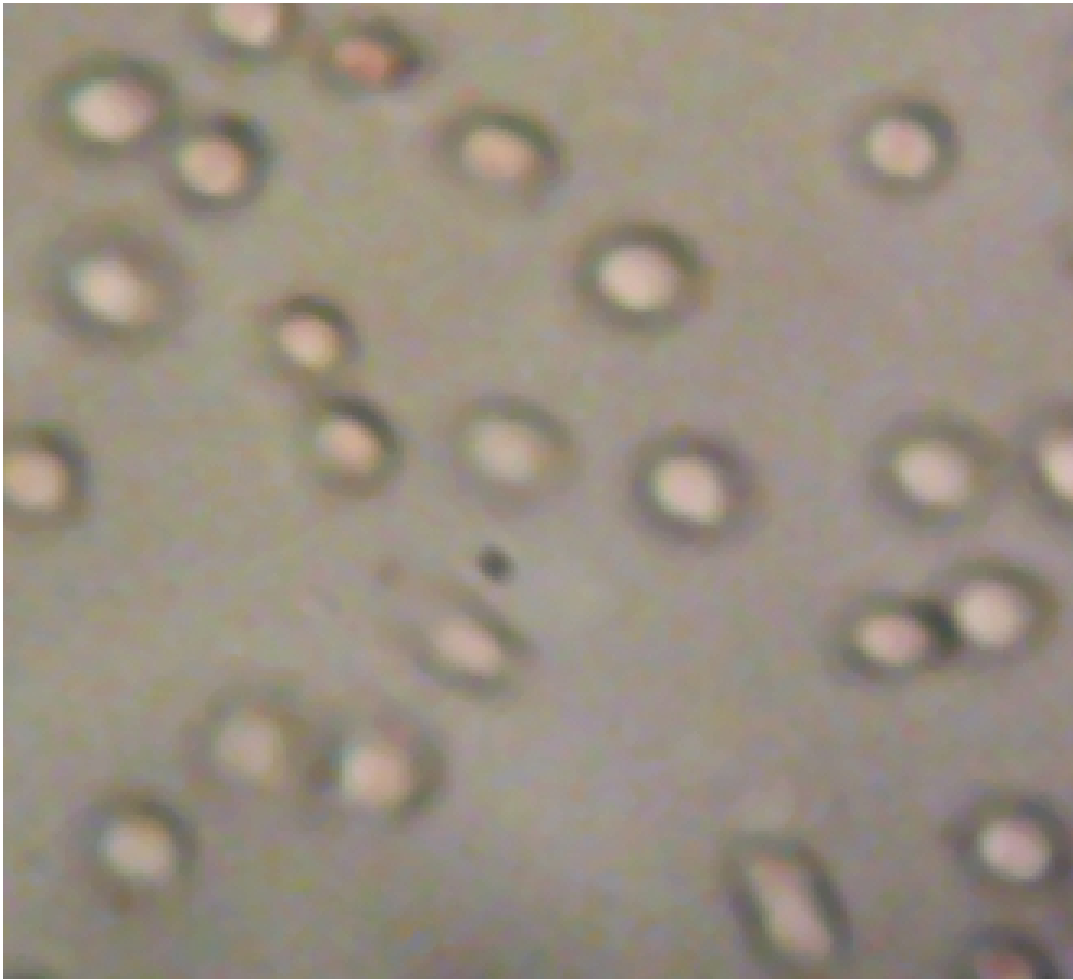


Fig. 4. Morphology of sickle erythrocytes treated with anthocyanins (10µg/mL) from *A. digitata* bark

3.5 Effect of Anthocyanins Extracts on Membrane Stability of RBCs

The effect of anthocyanins on the membrane stability of RBCs can be evaluated by comparing the hemolysis rates of untreated and treated sickle RBCs with anthocyanins using the osmotic fragility test. Fig. 5 gives comparative hemolysis rates of untreated and treated sickle RBCs with the NaCl concentration.

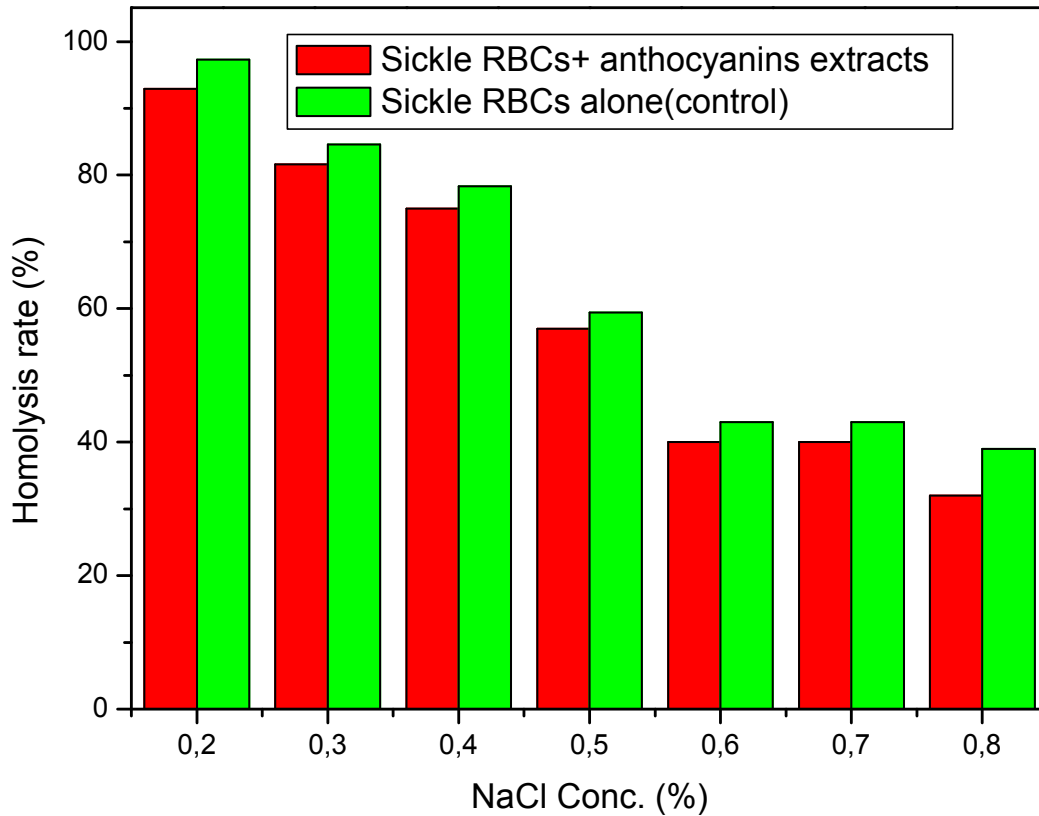


Fig. 5. Hemolysis rates of untreated and treated sickle RBCs with the NaCl concentration evolution

As it can be seen from this figure, the hemolysis rate of sickle RBCs decreases with increasing of NaCl concentrations. This curve can permit to determine values of the mean corpuscular fragility (or the NaCl concentration for which 50% of erythrocytes are hemolysed) for untreated and treated sickle RBCs are respectively 0.647 and 0.606. This indicates that the anthocyanins extract improved the ability of drepanocytes to take up water without lysis occurring. This stabilization effect could be explained by the fact that anthocyanins rendered the sickle RBCs capable of withstanding higher concentrations of NaCl by increasing the volume of the RBCs, reverting the sickling to produce biconcave cells, and thereby, maintaining membrane integrity.

The sickling modifies the membrane flexibility, which would make it more fragile and would increase the precocious risk of hemolysis. But it is as possible as the anthocyanins, according to their antioxidant or free radical scavenger effect, prevent hemoglobin from oxidizing in methemoglobin and inhibit the generation of free radicals. It is thus probable that the anthocyanin extracts exert this protective effect according to their reducing properties preventing that the lipids membrane, hemoglobin and the enzymatic equipment are destroyed or inactivated by oxidation [39-43].

It is so, interesting to see the effect of anthocyanin extract from *A. digitata* on methemoglobin levels in sickle RBCs.

3.6 Effect of Anthocyanin Extracts on Methemoglobin Levels in Sickle RBCs

Fig. 6 shows the evolution of methemoglobin (Fe^{3+}) profile for untreated and treated sickle RBCs.

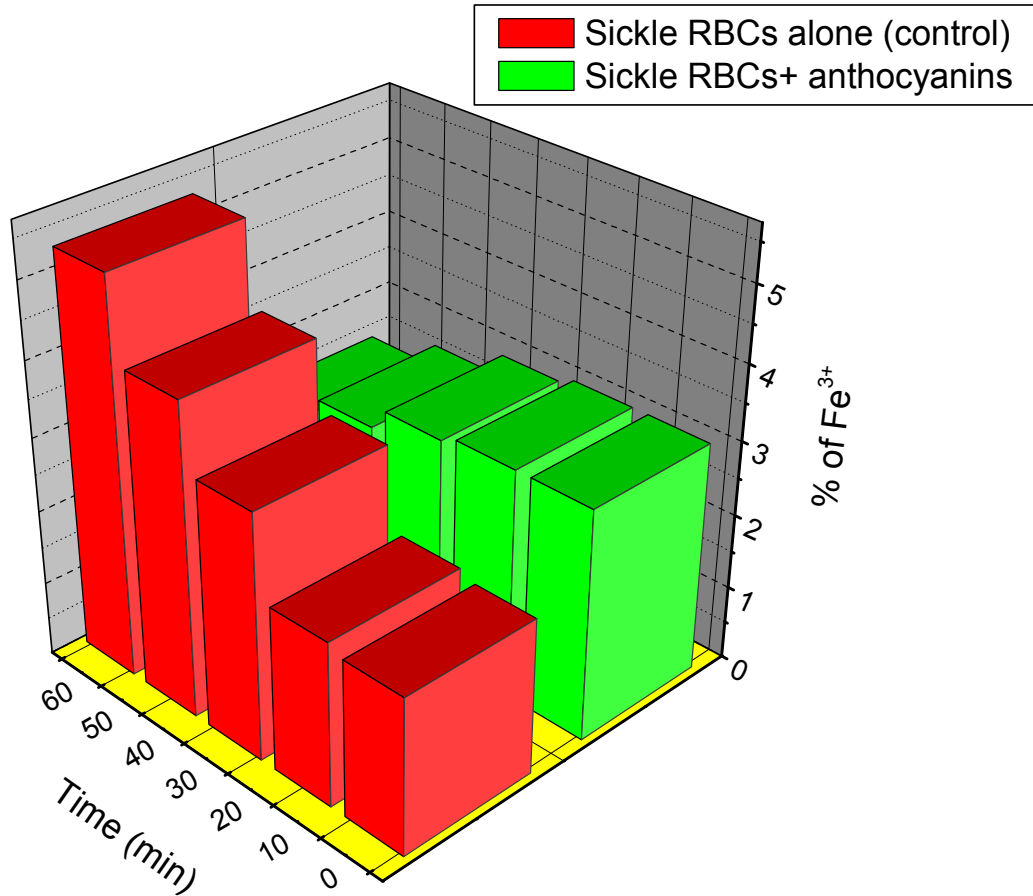


Fig. 6. Evolution of Fe^{3+} rate with time for untreated and treated sickle RBCs

This figure shows that the rate of Fe^{3+} increases with time in sickle blood cells (control); in contrast when treated with anthocyanins extracted from *A. digitata*, the Fe^{3+} decreases with time. These results show the reduction of methemoglobin content in solution after treatment with anthocyanins extracts, thus reflects the antioxidant effect of anthocyanins. In normal blood, only a very small amount of methemoglobin is present since the erythrocyte contains a system of reducing Fe^{3+} of the heme in Fe^{2+} . This system includes the Nicotinamide Adenine Dinucleotide Phosphate (NADPH), methemoglobin reductase and cytochrome B₅. NADPH enables the synthesis of reduced glutathione (GSH) to reduce the cytotoxic action of hydrogen peroxide. The metabolic shunt pathway of pentose phosphate in erythrocytes is necessary for the synthesis of NADPH (reducing power) that protects hemoglobin and membrane lipids against oxidation. This, by preventing the formation of hydrogen peroxide considered as one of the more important generating sources of oxygen free radicals that are

harmful to cellular function [42,43]. Therefore, the concentration of methemoglobin can be exploited as bio indicator of oxidative stress in sickle erythrocytes. The ability of anthocyanins in this study, to reduce methemoglobin to hemoglobin *in vitro* indicates their antioxidant activity. They could protect erythrocytes against premature aging and apoptosis induced by free oxygen radicals in sickle cell patients [39,40]. The anthocyanins reducing of methemoglobin may indirectly increase the affinity of hemoglobin for oxygen. This would correct a consequence of sickle cell anemia, decreased affinity of hemoglobin S for oxygen. Indeed, it is known that the hemoglobin containing Fe^{2+} can easily bind the oxygen, while its oxidized form or methemoglobin (Fe^{3+}) can't bind the oxygen.

4. CONCLUSION

Adansonia digitata L. or Baobab is a multi-purpose tree species. Several parts (fruits, leaves, seeds, barks) of this edible plant have shown interesting nutritional and therapeutic potentials. This work show that *Adansonia digitata* have also antisickling activity justifying the use of this plant in Congolese traditional medicine for the management of SCD. The anthocyanins extracted have shown a good effect in the stabilization of sickle cell membranes and on $\text{Fe}^{3+}/\text{Fe}^{2+}$ ratio. This plant could be used as a nutraceutical in the treatment of sickle cell disease. Indeed, sickle cell anaemia is a chronic illness; the best approach in the management of such hemoglobinopathy would be the use of edible medicinal plants to alleviate or suppress some symptoms of the disease instead of giving medications to patients throughout their live.

CONSENT

All authors declare that written informed consent was obtained from the patient before taking blood samples.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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