



Journal of Medical Sciences

ISSN 1682-4474

science
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P.T. Mpiana
Departement de Chimie,
Faculte des Sciences B.P. 190,
Universite de Kinshasa,
Kinshasa XI, R.D. Congo

Anti Sickle Erythrocytes Haemolysis Properties and Inhibitory Effect of Anthocyanins Extracts of *Trema orientalis* (Ulmaceae) on the Aggregation of Human Deoxyhemoglobin S *in vitro*

¹P.T. Mpiana, ²K.N. Ngbolua, ¹V. Mudogo, ¹D.S.T. Tshibangu,
¹E.K. Atibu, ¹D.D. Tshilanda and ³N.M. Misengabu

Recent findings indicated antisickling activity of anthocyanins from plants used in the management of sickle cell disease in Democratic Republic of Congo. The aim of this study was to evaluate the effect of anthocyanins extracts from *Trema orientalis* on sickle cell. So, Emmel, Itano and hypoxic induced sickle erythrocyte haemolysis bioassays were used to evaluate the influence of these extracts on haemoglobin S aggregation and sickle erythrocyte haemolysis. Anthocyanins extracts were found to possess antisickling activity. Indeed, the treated sickle erythrocyte indicated the re-appearance of the normal and classical biconcave form of red blood cells with a radius value of $3.5 \pm 0.2 \mu\text{m}$ similar to that of normal erythrocytes values. The solubility of Deoxyhemoglobin S and the rate of inhibition of hypoxic induced haemolysis, increased upon treatment with anthocyanins extracts. Anthocyanins would cross the erythrocytes' membrane for either interfering with intracellular polymerization hemoglobin S or to scavenge the free radicals preventing erythrocyte sickling or haemolysis.

Key words: Sickle cell disease, medicinal plants, antisickling activity, anthocyanins extracts, polymerization

¹Departement de Chimie, Faculte des Sciences B.P. 190, Universite de Kinshasa, Kinshasa XI, R.D. Congo

²Departement de Biologie, Faculte des Sciences B.P. 190, Universite de Kinshasa, Kinshasa XI, R.D. Congo

³Institut de Recherche en Sciences de la Santé (IRSS), Kinshasa, R.D. Congo

INTRODUCTION

Red Blood Cells (RBCs) or erythrocytes are made up of two main components, the cytoplasmic proteins and the membrane. Haemoglobin (Hb) constitutes 97.5% (by weight) of the protein system while the other 2.5% proteins provide energy and help regulate water and ionic composition of the cell. The membrane which constitutes a very small percentage of the cell mass, surrounds the intracellular concentrated solution of Hb (Mehanna, 2001). Membrane protein are involved in active ion transport which keeps the intracellular Na^+ and Ca^{++} ions concentration low and that of K^+ and Mg^{++} high. The proteins provide also peculiar flexibility to the RBCs membrane (Elekwa *et al.*, 2003; Osuagwu and Mbeyi, 2007).

In sickle cell anaemia, the shortened RBCs survival is due to increased rigidity of cells and membrane damage caused by intracellular precipitation of HbS. Hb S aggregation induces a panoply of cellular and tissue injuries (substantial loss of membrane flexibility, sickle shaped etc.). The intracellular polymerization of deoxy Hb S occurs as a result of sickle erythrocyte dehydration. Some pathways have been implicated in this dehydration especially the Gardos channel and $\text{K}^+\text{-Cl}^-$ cotransport (Buchanan *et al.*, 2004).

At biochemical level, these pathways are modulating by cellular energy. Indeed, depletion of ATP production causes RBCs abnormalities in both structure and functions. The inactivation of sickle cell membrane-bound (Ca^{++} and Mg^{++}) ATPase leads into the increase of intracellular free Ca^{++} and the loss of K^+ with accompanying movements of Cl^- and water (Mehanna, 2001; Elekwa *et al.*, 2003).

It was postulated that oxidative damage to cells and the decrease of the intracellular free Mg^{++} are believed to be responsible for the activation of $\text{K}^+\text{-Cl}^-$ co transport in sickled erythrocytes (Brugnara, 2000; Girot and Begue, 2003).

The treatment of SCD is based on pathophysiology: inhibition of both Hb S polymerization and cell dehydration and protection of sickle erythrocyte oxidative induced damage such as hyper haemolysis which is the most clinical manifestation of SCD (Yoshida and Shevkopyas, 2010). According to the first SCD therapy approach, a mathematical modelling has indicated that during aggregation of Hb S process, there is a pronounced delay before the appearance of the Hb S polymer. This delay time is sensitive to total Hb concentration and fractional saturation with ligand. Any changes in these variables which decrease the delay time (for example cell dehydration which lead to the increase of

total Hb concentration or the decrease of Hb solubility), where postulated to be associated with increased clinical severity of SCD. A drug that prolongs the delay time prior to polymerization might be of therapeutic value in SCD therapy because a longer delay time decreases the probability of RBCs sickling and prevents their lyses' (Iyamu *et al.*, 2002).

Based on this knowledge, it appears feasible to search for a drug which will alleviate or prevent all of the symptoms of the SCD and provide perfectly normal lives and life-expectancies for sicklers. This may be achieved with the aid of synthetic product such as hydroxyurea, decitabine and short-chain fatty acids derivatives (Sauntharajah *et al.*, 2003; Cokic *et al.*, 2003), or alternatively, with plant-derived preparations. The use of plants is affordable for people living with SCD in developing countries where medical care is not available for all.

With the aim of finding Congolese plants extracts with antisickling properties, a screening work has been performed in our laboratory. The aqueous and/or ethanolic extracts of some Congolese plants displayed appreciable activity *in vitro*. Phytochemical investigation has enabled the identification of the anthocyanins as responsible for the antisickling activity (Mpiana *et al.*, 2007a-d, 2008, 2009a-e, 2010a-c).

Based on the target of interference, two modes of action of anthocyanins were proposed. Firstly, as Hb S molecules modifiers i.e antigelling agent, anthocyanins enhance the solubility of deoxy Hb S as assessed by a direct non covalent interaction with Hb S molecules thus, increasing their O_2 affinity. Secondly as sickle erythrocytes membrane modifiers, anthocyanins decrease intracellular Hb concentration by inhibiting RBCs dehydration (Mpiana *et al.*, 2007b-d, 2008, 2009a, 2010a).

The main emphasis of the present study was to investigate the inhibitory effect of a tropical medicinal plant *Trema orientalis* on the sickle erythrocytes hypoxic-induced lyses' and deoxy Hb S aggregation *in vitro* using anthocyanins fraction as new lead compound. Moreover, the ability of anthocyanins extracts to prevent sickle erythrocyte haemolysis would be an added advantage in protecting sickle cell blood membrane against oxidative injuries.

MATERIALS AND METHODS

This study was conducted from March 2008 to July 2009 under a bioprospecting program for biodiversity-based antisickling drugs discovery, started

since 2007 (Mpiana *et al.*, 2007a-d, 2008, 2009a-e, 2010a-c) in the Laboratory of Natural products and Medicinal Chemistry, Faculty of Sciences, University of Kinshasa, Democratic Republic of Congo.

Plant material: Leaves of *Trema orientalis* L. were collected from plants growing in Kinshasa, DR Congo and were authenticated by Mr. B.L. Nlandu of the INERA (Institut National d'Etudes et Recherches Agronomiques). Voucher specimen is on deposit at the INERA Herbarium of the Faculty of Science (Université de Kinshasa).

Extraction and chemical screening: The dried and powdered plant material (leaves, 10 g) was repeatedly extracted by cold percolation with 95% Ethanol (EtOH) and water (100 mL×1) for 48 h. Chemical screening was done in aqueous and organic extract according to a well known protocol as previously 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 plant material with distilled water and diethyl ether following an established protocol.

Biological material: The sodium citrate suspension of blood samples used in the evaluation of the antisickling activity of the plant extracts in this study were taken from known sickle cell adolescent patients attending the Centre de Medecine Mixte et d'Anemie 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 at pH 8.5. They were found to be SS blood and were then stored at +4°C in a refrigerator.

Antisickling and antihaemolytic assays

- **EMMEL test:** Sickle cell blood was diluted with 150 mM phosphate buffered saline (NaH₂PO₄ 30 mM, Na₂HPO₄ 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 anthocyanins extracts and covered with a cover slip. Paraffin was applied to seal the edges of the cover completely to exclude air (Hypoxia). The RBCs were analysed 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 6.1 package software

- **Haemoglobin S solubility test:** RBCs were washed twice in physiological saline solution (NaCl 0.9%) by centrifugation at 3000 rpm for 10 min, re-suspended in hypotonic medium. After that, the hemolysate of RBCs was centrifuged and an equivalent volume of 2% metabisulfite was added to supernatant. It was then incubated at ambient temperature for 45 min

At fixed time points aliquots (50 µL) of the 2% sodium metabisulfite pre-treated hemolysate were diluted with 500 µL of phosphate buffer (pH 7.5) containing (NH₄)₂SO₄ 30%, Saponine 1% and K₂HPO₄ 1.2%. 50 µL of anthocyanins crude extracts were added to the test sample mixed and incubated for 10 min. The equivalent volume of Phosphate Buffered Saline (PBS) was added to the control sample instead of the drug. At predetermined time intervals aliquots of test or control samples were removed and centrifuged at 3500 rpm at ambient temperature for 5 min. The absorbance of the supernatant was measured at 700 nm. The solubility of the deoxygenated sickle cell hemoglobin was expressed as the decrease of the optical density at 700 nm (Mpiana *et al.*, 2010b).

The rate of polymerization inhibition (% PI) versus time was calculated using the following formula:

$$\%PI = \frac{\text{Absorbance of untreated HbS} - \text{Absorbance of treated HbS}}{\text{Absorbance of untreated HbS}} \times 100$$

The zero time corresponds to 30 min after pre-incubation of HbS with anthocyanins extract (treated Hb S) or NaCl 0.9% (untreated HbS) in hypoxic conditions.

Before zero time (period of pre-incubation), the HbS which is soluble in aqueous medium is converted into deoxy-Hb form for which solubility is much reduced after chemical treatment by the sodium metabisulfite 2% (hypoxia) thus initiating a beginning of polymerization. At time zero, the absorbance of untreated HbS increases (due to the formation of polymer or tactoid which absorbs at 700 nm) whereas that of treated Hb decreases at the same wave length (inhibition of polymerization).

- **Hypoxic induced haemolysis assay:** RBCs were washed twice in physiological saline (NaCl 0.9%, 1:5 v/v) by centrifugation at 3000 rpm for 10 min, re-suspended in phosphate buffer (150 mM, pH 7.4) containing 2% sodium metabisulfite and incubated in the absence (control) or presence of anthocyanins extracts ($50 \mu\text{g mL}^{-1}$ of NaCl 0.9%) at 37°C for 60 min. At fixed time points, aliquots of the blood samples were removed and centrifuged at 3000 rpm at ambient temperature for 5 min. The absorbance of the supernatant was measured at 540 nm and was expressed as the degree of haemolysis (Iyamu *et al.*, 2002)

The rate of hemolysis inhibition (% HI) versus time was calculated from the absorbance of sickle erythrocyte (SS RBCs) suspension by the relation:

$$\%HI = \frac{\text{Absorbance of untreated SS RBCs}}{\text{Absorbance of untreated SS RBCs suspension}} \times 100$$

The zero time corresponds to 30 min after pre-incubation of SS RBCs with anthocyanins extract (treated SS RBCs) or NaCl 0.9% (untreated SS RBCs) in hypoxic conditions. During the pre-incubation time, the SS RBC used its initial enzymatic stock to prevent the oxidative stress. From time zero one attends with a progressive hemolysis of sickle erythrocytes. The treatment of the SS RBCs by the anthocyanin extracts reduce this hemolysis (decrease of absorbance at the wave length of 540 nm).

pH and optical absorption measurements: The pH values were determined with Metrohm E 604 pH-meter equipped with a glass electrode. This electrode was kept soaked in 3 mol L^{-1} KCl solution and calibrated with aqueous standard buffers. A Perkin Elmer Lambda 2 UV-Visible spectrophotometer was used.

The *in vitro* bioassays were performed in triplicate and the number of observed erythrocytes was determined using Neubauer's cells counter as previously reported (Mpiana *et al.*, 2010b). All results presented are Mean \pm SD.

RESULTS

- **Phytochemical screening:** The Phytochemical screening of leaves of *Trema orientalis* L. revealed the presence of alkaloids and polyphenols such as flavonoids, tannins, leuco-anthocyanins, quinones and anthocyanins
- **Antisickling activity**
Effects of plant and anthocyanins extracts on sickle erythrocytes morphology: Figures 1-3 show the

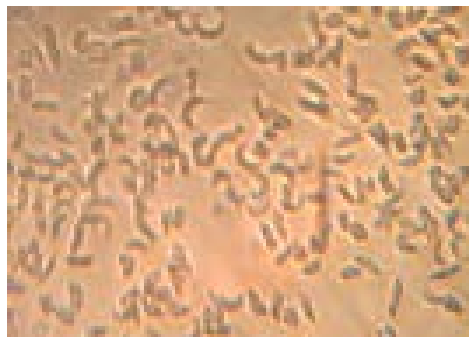


Fig. 1: Morphology of drepanocytes of untreated SS blood (control) (X500), [NaCl 0.9% ; $\text{Na}_2\text{S}_2\text{O}_4$ 2%]

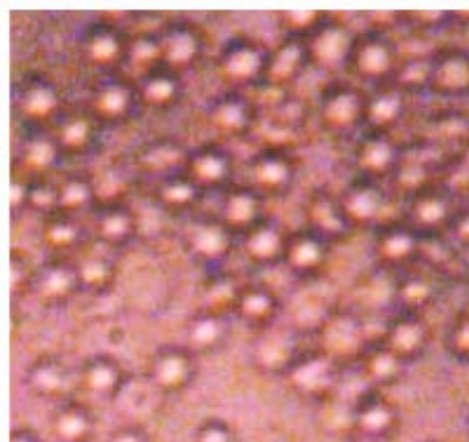


Fig. 2: Morphology of drepanocytes treated with $50 \mu\text{g mL}^{-1}$ of ethanolic extract of *Trema orientalis*, [NaCl 0.9% ; $\text{Na}_2\text{S}_2\text{O}_4$ 2%, X500]

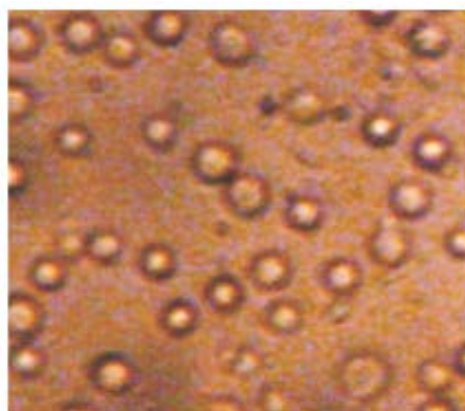


Fig. 3: Morphology of drepanocytes treated with anthocyanins extract ($10 \mu\text{g mL}^{-1}$) of *Trema orientalis*, [NaCl 0.9% ; $\text{Na}_2\text{S}_2\text{O}_4$ 2%, X500]

Table 1: Average values of radius, perimeter and surface of erythrocytes before and after treatment with ethanolic extract and anthocyanins extract of *V. unguiculata*

Measured parameters	Untreated SS RBCs	SS RBCs (+ACE)
Radius (μm)	-	3.5 \pm 0.2
Perimeter (μm)	33.0 \pm 1.3	19.8 \pm 1.4
Area (μm^2)	20.6 \pm 1.2	31.9 \pm 1.6

ACE: Anthocyanins extract

morphology of untreated Sickle erythrocytes (control) and that of Sickle erythrocytes treated with plant and anthocyanins extracts of *Trema orientalis*. These optical micrographics are characteristic and are usually observed during the *in vitro* treatment of drepanocytes by antisickling plant extracts (Fig. 2, 3) or in the absence of antisickling plants (Fig. 1).

Calculated average value of radius, perimeter and surface of drepanocytes before and after treatment with anthocyanins extracts of *Trema orientalis* are given in Table 1. This indicates that untreated SS red blood cells are not circular, so, it's not possible to calculate the radius due to the sickled shape of SS red blood cells in hypoxic conditions. By the other way, in the presence of plant extracts; drepanocyte shapes become normal. This phenomenon is confirmed by the appearance of radius with same values with that of normal erythrocytes and the modification of both perimeter and area of treated cells. These results confirm that tested plants have antisickling effect ($p < 0.05$).

Figure 4 shows the dose dependent antisickling activity of ethanolic extract of *Trema orientalis*.

Figure 4 indicates that the rate of normalization of the drepanocytes in hypoxic conditions increases with the plant extracts dose until reaching the maximum threshold of which this rate of normalization remains constant despite the increase in the dose of drug (normalization dose-dependent). The weakest dose of extracts for which the rate of normalization is maximum is called Minimal Concentration of Normalization (MCN).

Inhibitory effect of anthocyanins extracts on the aggregation of deoxy-HbS: The effect of anthocyanins on the aggregation of deoxy-HbS can be determined in following the evolution of Deoxy-HbS solubility in the absence and in the presence of anthocyanins extracts. This can be done by monitoring the optical density of polymerized HbS at 700 nm at different times. Figure 5 gives the *in vitro* effect of anthocyanins extracts of *Trema orientalis* leaves on the aggregation of deoxy-Hb S. Each point represents a mean of three independent determinations (Mean \pm SEM). Figure 5 shows the evolution of absorbance of tactoids polymer which is formed in hemoglobin aqueous solution. indicates that, in the untreated hemoglobin aqueous solution (control)

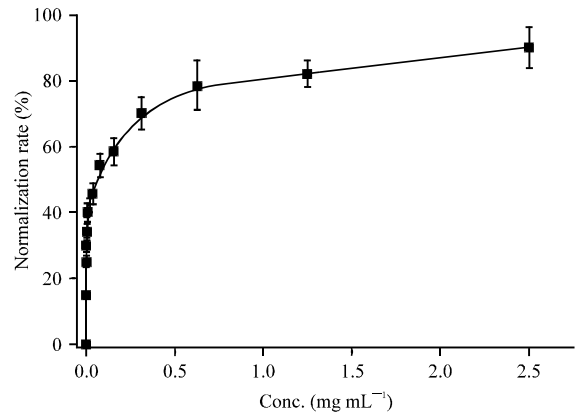


Fig. 4: Concentration-dependent antisickling effect of ethanolic extract of *Trema orientalis*

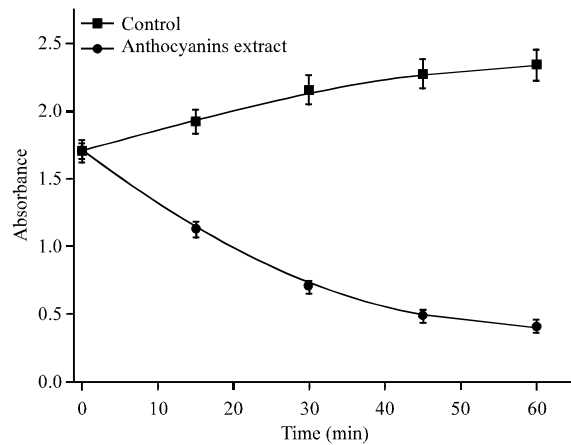


Fig. 5: *In vitro* effect of anthocyanins extracts ($50 \mu\text{g mL}^{-1}$) of *Trema orientalis* leaves on the aggregation of deoxy-Hb S (Phosphate buffered saline 150 mmol L^{-1} ; pH 7.4; 2% Sodium metabisulfite, wave length: 700 nm)

the absorbance at wave length of 700 nm increase with the time as a result of the loss of hemoglobin solubility in hypoxic conditions. However, after addition of the anthocyanins extract, one notes a reduction in the absorbance at 700 nm in the course of time. These results indicate that anthocyanins inhibit the polymerization of hemoglobin S into tactoids (anti gelling effect). The antisickling effect of anthocyanins would be achieved through a no covalent interaction with HbS molecules. The oxygen carrying capacity of sickle erythrocyte would increase improving tissue respiration.

Effect of anthocyanins extracts on the haemolysis of sickle erythrocytes: Effect of anthocyanins on hypoxic induced membrane damage of RBCs can be evaluated by

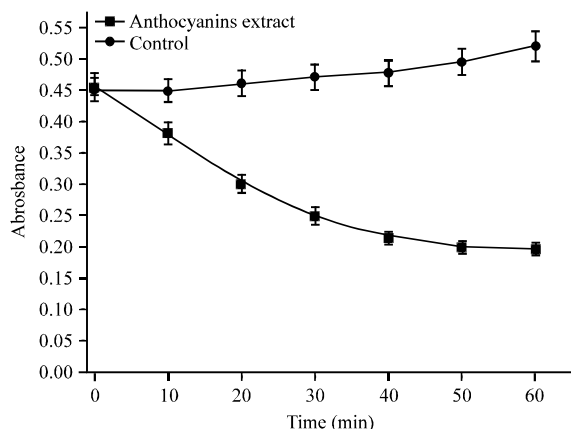


Fig. 6: *In vitro* effect of anthocyanins extracts ($50 \mu\text{g mL}^{-1}$) from *Trema orientalis* leaves on the haemolysis of Sickle erythrocytes (NaCl 0, 9%; 2% Sodium metabisulfite, wave length: 540 nm)

comparing % of haemolysis of untreated and treated SS RBCs in isotonic medium (NaCl 0.9%) by monitoring the optical density of released Hb S at 540 nm at different times.

Figure 6 shows the rate of inhibition of haemolysis of the RBCs compared to time and indicates that although it is low in the initial time, this one increases and reach a rate of 20% after 60 min of incubation. Each point represents a mean of three independent determinations (Mean \pm SEM). Figure 6 indicates that the rate of hemoglobin (expressed in terms of absorbance at the wave length of 540 nm increases with the time in the suspension of untreated SS red blood cells (control). The rate of hemoglobin can be increase only in the context of haemolysis of RBCs. However, after addition of anthocyanins extracts we observe a decrease of the absorbance at the wave length of 540 nm at which hemoglobin absorbs. These results show that anthocyanins extract possess anti-hemolytic effect. Indeed, if it is admitted that the anthocyanins extracts are not actives, it should thus expect that the rate of inhibition of haemolysis of the sickle erythrocytes falls to zero in the course of time owing to the fact that these cells are unable to renew their enzymatic stock which would enable them to fight against oxidation injuries.

DISCUSSION

As it can be noticed from micrographies on Fig. 1-3, the control (Fig. 1) contains sickle-shaped erythrocytes, confirming the SS nature of the blood as predicted by haemoglobin electrophoresis test. Mixed together with plant or anthocyanins extracts (Fig. 2, 3), the sickle

erythrocytes are reversed normal-shape. These morphological SS blood cells were observed in anoxic conditions and are similar to those currently observed or previously reported (Mpiana *et al.*, 2007a-d, 2008, 2009a-d, 2010a).

As it can be seen, a normalization of sickle erythrocytes treated with plant or anthocyanins extracts of *Trema orientalis* indicates the inhibitory effect of the extract on the sickling of RBCs. The same results are also reported for anthocyanins from some others Congolese plants used by traditional healers for the management of SCD.

Statistical treatments according to Tukey's multiple range tests (Krishna *et al.*, 2010) enabled the determination of a significant difference between the average values of both the perimeter and the surface of the untreated and treated erythrocytes (Table 1, $p < 0.05$ and $p < 0.01$). Thus, confirming the antisickling effect of anthocyanins extracts. Indeed, in anoxic condition, the RBCs were observed to change from the sickled shape to normal biconcave cells. These values were in agreement with previously reported values by Mpiana *et al.* (2007a-d, 2008, 2009a-d, 2010c). The treated sickle erythrocyte demonstrated a remarkable similarity to normal blood values.

The computer software used in this study did not give the average radius for drepanocytes (Table 1), as sickled cells of untreated SS blood were not circular. The average radius appeared after treatment of SS blood cells with Ethanolic (EtoH) and anthocyanins extracts, indicating the re-appearance of the normal and classical biconcave form of RBCs.

The normalization of sickled cells with the ethanolic extract concentration increased with the extract concentration and reached a maximum and constant value at 2.5 mg mL^{-1} (Minimal Concentration of Normalization, MCN). This corresponds to a normalization rate of 91% with an ED_{50} (concentration of extract for which 50% of the sickled erythrocytes are reversed) equal to 4, 8 $\mu\text{g mL}^{-1}$. So, the antisickling activity of *Trema orientalis* ethanolic extract is dose dependent.

This result shows that extracts of *Trema orientalis* strongly inhibit the sickling of drepanocytes induced by 2% sodium metabisulfite and is very significant if compared with the results obtained by other authors. Indeed, Iyamu *et al.* (2002) have obtained 50% of reversion of erythrocytes sickling with 5 mg mL^{-1} of NIPRISAN[®], naturally occurring, potent antisickling based plants. It can be also noted that the ED_{50} obtained in this study is lower than those obtained by Joppa *et al.* (2008) for *Morinda lucida* BENTH

(2.2 mg mL⁻¹) and *Newbouldia leavis* P.BEAUW. (8.9 mg mL⁻¹) attesting thus that *Trema orientalis* is more effective than these two plants.

The pathophysiology of SCD is attributed to both sickle haemoglobin and/or erythrocyte membrane behaviour. In this regard, more than two mechanisms of action would be suggested to explain the antisickling activity of anthocyanins. Because of their property to bind to proteins, the anthocyanins would be inhibiting the polymerization of deoxyHbS into tactoids inside the RBCs. Experimentally; it can be studied by the Itano solubility test using a UV-Visible spectrophotometer (Mpiana *et al.*, 2010b). Apart from the inhibition of haemoglobin S polymerization, endothelial injury and the erythrocyte membrane, free radicals production have been defined as new target in SCD therapy (Buchanan *et al.*, 2004). Because of the reduced glucose metabolism and the low activity of both the glutathione reductase system and methemoglobin reductase which are involved in the protection of hemoglobin and membrane from oxidative breakdown, methemoglobin, a biomarker of oxidative stress and Radical Oxygen Species (ROS) are build up spontaneously in sickle erythrocyte (Osuagwu and Mbeyi, 2007; Nagababu and Rifkind, 2000). These ROS would act as biological nucleophile in the de-esterification of membrane lipids leading into the hyperhemolysis of sickle erythrocyte (Yoshida and Shevkopyas, 2010).

Such a significant activity of anthocyanins extracts can be explained by the fact that they would cross the erythrocytes' membrane for either interfering with intracellular polymerization while being adsorbed on hemoglobin S or to scavenge the free radicals. It is thus probable that the anthocyanins 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 (Mpiana *et al.*, 2009c).

The ability of anthocyanins extracts in this study to reduce the sickle erythrocytes haemolysis can be deduced as antioxidative free radical properties. Therefore, from the above results, anthocyanins might be potential antisickling drug candidate for sickle cell patients, because they do not alter the structure stability or the biological properties of hemoglobin.

Plants have shown to be beneficial in the prevention of various ailments (Bangou *et al.*, 2011; Hemalatha *et al.*, 2011; Rahman *et al.*, 2011; Koneru *et al.*, 2011; Jain *et al.*, 2011). In the Democratic Republic of Congo (DRC), both urban and rural populations depend on medicinal plants for their health care needs because the costs of conventional drugs are unaffordable (Ngbolua *et al.*,

2011). So, biodiversity and the associated indigenous knowledge systems are an indispensable basis for developing solutions to DRC's development challenges. In order to manage sickle cell disease chemotherapeutically, plant based therapeutic agents such as anthocyanins can constitute a valuable alternative for the developing world because many antisickling agents did not show promising success for clinical use (Mpiana *et al.*, 2010b).

CONCLUSION

Results in this study suggest that anthocyanins extract may play a role in both inhibiting polymerization of S haemoglobin and haemolysis by scavenging free radicals produced spontaneously within the sickle erythrocytes. This provides additional possible putative mechanism for earlier reports on the antisickling properties of anthocyanins of some Congolese plants and their use in the management of sickle cell disease by Congolese traditional healers. It should be indicated that anthocyanins plant derived have not yet been reported to exhibit antisickling and antihemolytic effects before these findings.

ACKNOWLEDGMENTS

The authors are grateful to the International Foundation for Science (IFS, Stockholm, Sweden) and the Organization for the Prohibition of Chemical Weapons (OPCW) for the IFS Research Grant F/4921-1 attributed to Dr NGBOLUA KN for a study on antisickling plants.

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