The anti-sickling mechanism of *Amphimas pterocarpoides* (Cesalpiniaceae) against sickle cell Anemia

**ABSTRACT**

Sickle cell anaemia (SCA) is a genetic pathology which forms haemoglobin S (Hb S). Hb S under hypoxia condition polymerizes, leading to the free radicals production and the destruction of red blood cells’ (RBCs) membrane. Considering the insufficiency and some adverse effects of existing treatments, the use of medicinal plant extracts as alternative therapies could be efficient and helpful. The objective of this study was to investigate *in vitro* the anti-sickling mechanism of *Amphimas pterocarpoides*. *A. pterocarpoides* was collected in the West region of Cameroon and then submitted to a hydroethanolic extraction at pH 3. The sickling of red blood cells (RBCs) was induced using sodium metabisulphite (2%) followed by treatment with extracts at different concentrations. Cell sickling reversal, the deoxyhaemoglobin solubility test and the osmotic fragility test were conducted to explore the effect of *A. pterocarpoides* extracts on the sickle cells (SS cells) polymerization and the membrane stability. *A. pterocarpoides* possess the sickling reversal activity by reducing the sickling up to 37.03% and allow the Hb S to become soluble by increasing the solubility of the deoxy-haemoglobin S. This plant also allows the rehydration of SS cells by reinforcing their capacity to resist against osmotic fragility (from 100 to 18.42%). *A. pterocarpoides* has previously showed its capacity to fight against induced hemolytic anaemia, reverse the polymerization, solubilize the deoxy-haemoglobin S and maintains the integrity of SS cells. Then, this extract could be a potential agent against SCA.

**Key words:** Sickle cell anaemia, deoxygenated Hb S, polymerization, cell fragility, *A. pterocarpoides*.

**INTRODUCTION**

Sickle cell anaemia (SCA) is a genetic pathology which creates abnormal haemoglobin called haemoglobin S (Hb S). It mostly affects people of African ancestry, but also occurs in other ethnic groups, including people who are of Mediterranean and Middle Eastern descent (Frömml et al., 2014). In Cameroon, the carrier rate ranged from 20-25% and the prevalence is between 2-3% (Drepavie, 2013).

Due to the substitution of a polar amino acid (glutamic acid) to a hydrophobic aminoacid (valine), the presence of HbS creates (Iyamu *et al.*, 2003) hypoxic conditions which lead to the sickling, forming rigid and sickled cells. This, in turn, causes the deformation of the normal disc biconcave RBC and the haemolysis (Iyamu *et al.*, 2003). The rigidity and distortion Sickle cells reduce the resistance to haemolysis. A rigid cell cannot expand, meaning that it is not flexible and therefore cannot easily move along the narrow human blood vessels. When the osmotic fragility decreases, the resistance increases and *vice versa*. Then, antisickling agents having the capacity to reduce the osmotic fragility is an advantage since it increases the RBCs’ resistance to lysis. In other words, a rigid and distorted red blood cell with low elasticity can be fragile and may break.
with little stress (Allison, 1957). Sickled erythrocytes, also called pro-oxidant machine, is one of the important pro-oxidant sources in SCD. Then the unstable antioxidative HbS cause increased ROS generation (Banerjee and Kuyper, 2004). The increased and unremitting pro-oxidant generation in SCD result in excessive antioxidant consumption and thus antioxidant deficiency (Banerjee and Kuyper, 2004).

Recent therapeutic strategies against SCA have focused on the inhibition of HbS polymerization. In fact, interactions among the amino acids in the hypoxic states explain the phenomenon of polymerization. Haemoglobin exists in normal RBCs as a tetrameric protein with two alpha- and two beta-chains. The interactions among the tetramers of haemoglobin molecule can lead to polymerization. In occurrence, in homozygous SS patients, both beta-chains contain valine (Val) at the 6th position. Val6 interacts hydrophobically with Phe85 and Leu88 of the other haemoglobin molecules (beta-2 of the first Hb molecule interacts with beta-1 of the second Hb molecule). This interaction constitutes the basis for polymerization. The same beta-2 chain of the first Hb molecule also contains glutamic acid at position 121, which interacts with Gly16 of the beta-1 chain of a third Hb molecule. Meanwhile, between the first and the third Hb molecules, His20 of the first Hb molecule of alpha-2 chain interacts with Glu6 of beta-1 of the third Hb molecule. Another interaction is that beta-2 Val6 of the first Hb is interacting with Phe85 and Leu88 of the second Hb molecule. In addition, the Asp73 of Beta-2 of the first Hb interacts with Thr4 of beta-2 of the fourth Hb molecule. There is also an interaction between Glu121 of the first Hb molecule on beta-1 chain and proline of alpha-2 and His116 of beta-2 chain of the fifth Hb molecule. This interaction model is supported by earlier reports on the nature of polymerization of sickle cells (Ngolet et al., 2016).

These complex molecular interactions among the haemoglobin tetramers and with neighboring haemoglobin molecules result in the polymerization of the HbS cells as well as the deformation or sickled shape (Ferronne et al., 2002). The subsequent consequence of polymerization is the decrease of the cell membrane fluidity which affects the red blood cell permeability, the cell dehydration and then the cell membrane fragility and haemolysis. The role of Ca\textsuperscript{2+} in preserving the low permeability of the red cell to K\textsuperscript{+} and Na\textsuperscript{+} thereby maintaining the normal low rate of cation leakage from the cell, had been reported (Sarkadi et al., 1979). In addition, elevated intracellular Ca\textsuperscript{2+} had been suggested to destroy the normal cell shape and plasticity, leading to increasing K\textsuperscript{+} leakage hence inhibiting Na\textsuperscript{+}, K\textsuperscript{+}-pump (Bewaji et al., 1985). Indeed, it had been suggested that sickling of red cells could be reversed if the excess Ca\textsuperscript{2+} in the red cells is pumped out (Bewaji et al., 1985). There are reports that pharmacological agents that are capable of altering the permeability of cell membranes could be useful in the management of sickling, a major manifestation in sickle cell disease (Bewaji et al., 1985). It is clear that substance that can interfere with the complex interactions among the haemoglobin tetramers could be a new strategy of therapy. A series of treatment have been taken into consideration. The use of hydroxyurea (an antioxidant substance) which protects the body against oxidative stress by increasing the amount of reduced glutathione, stimulates the induction of haemoglobin F (Hb F) and substitutes the Hb S effects (Flaurentino et al., 2011). Previous research on Hydroxyurea in 1984 showed the induction of Hb S in two sickle cell adult patients while a subsequent report showed the efficacy and tolerability of the drug in the patients. Many agencies have authorized the use of Hydroxyurea against SCA. In 1998 and in 2007, the US Food and Drug Administration and the European Medicines Agency have respectively approved its use in order to alleviate the frequent painful crises observed in sickle cell patients. Furthermore, the Agency for Healthcare Research and Quality as well as the National Institutes of Health Consensus Development Conference in 2008 have also allowed the use of hydroxyurea for SCA (Halsey and Roberts, 2003). Hydroxyurea also reverse the sickling state of the cells by acting on the membrane in regulating the Ca\textsuperscript{2+} ion flow between the inner and the outer red cell membrane. Several reports are available online on the in vitro effects of hydroxyurea on the erythrocyte membrane deformability (Athanasiiou et al., 2006). Piracetam drug reduces the incidence of sickling crises by reversing the sickling process of erythrocytes (Al Hajeri et al., 2011). However, the use of hydroxyurea in a long period can be toxic and produces some side effects such as leg ulcer, mouth ulcer and squamous cell carcinoma (Flaurentino et al., 2011). Stem cell transplant are another opportunity to manage SCA complications but the cost is very high.

Herbal medicine has its own role against SCA and is encouraged by WHO. Phytomedicine investigation is up to date associated with the scientific-based known therapeutic properties for the management of SCA. Some common plant constituents found to fight against SCA are terpenes and polyphenols: Phytochemical examination of the extract of Scoparia dulcis (Vanhaelen-Fastre et al., 1999), epicatechin from Theobroma cacao (Giovanni et al., 2016). The presence of polyphenolic compounds in some plants characterizes their capacity as antioxidants compounds. Alternative therapy such as the use of antioxidant is known to be the efficient one in 21 century (Takasu et al., 2002) against SCA. In fact, oxidative damage to cells is believed to be responsible for activation of KCl-cotransport in sickled erythrocytes (Brugnara, 2000). The sickle cell erythrocytes
are fragile and dehydrated and it is important that minerals and antioxidants are constantly supplied to maintain hydration and membrane integrity. Therefore, many tropical plants have been investigated for their antioxidative properties in relation with antisickling properties (Nanfack et al., 2013; Biapa et al., 2018). Moreover, preliminary studies revealed (Takasu et al., 2002) the effect of aged garlic extract on SCD patients with a reduction in aggregates that adhere to red blood cells and damage it without significant changes to RBC, haemoglobin, and reticulocyte count. Furthermore, Epigallocatechin gallate, an antioxidant found in green tea, has been shown to reduce sickling of RBC by 30%. Antioxidant compounds also act on several enzymes such as ribonucleotide reductase and guanylyl cyclase by respectively inhibiting the reaction that leads to the production of deoxyribonucleotides (in fact it is well known that the deoxyribonucleotides production requires a free radical called tyrosyl group; this compound captures these free radicals thereby preventing the production of deoxyribonucleotides) and by increasing the nitric oxide levels through the activation of the second enzyme (the activation thereby increase in the cyclic GMP). Hydroxyurea as antioxidant activates gammaglobulin synthesis, which is required for the production of fetal haemoglobin by removing the rapidly dividing cells that preferentially produce sickle haemoglobin (Cokic et al., 2008).

Many plant extracts have shown some anti-sickling mechanism against SCA. The extract of *Pterocarpus santalinoides* and *Aloe vera* was reported to increase the gelling time of sickle cell blood and inhibits sickling in vitro (Ugbor, 2006). The reversal of sickling by root extracts of *Fagara zanthoxyloides* has also been reported (Sofowora and Issac-Sodeye, 1971). *Cajanus cajan* leaf and seed, *Zanthoxylum zanthoxyloides* leaf, and *Carica papaya* inhibit the induction of haemolysis of human erythrocytes and reverse the cell sickling (Ismaila et al., 2017). Moreover, *Garcinia kola*, known as “bitter kola”, has an effect on membrane stabilization than phenylalanine (Orhue et al., 2005). Furthermore, the ried fish (Tilapia) and dried prawns (Astacus red) extracts were established to have the ability to inhibit polymerization of sickle cell haemoglobin (HbS), improve the $\text{Fe}^{2+}/\text{Fe}^{3+}$ status and lower the activity of lactate dehydrogenase (LDH) in the blood plasma. LDH is a sensitive indicator of haemolysis and its level in sickle cell blood determines the severity of crises (Nwaoguikwe and Uwakwe, 2005).

*Amphimas pterocarpoides* are trees found in the forests of Cameroon used in traditional Cameroonian medicine to prevent anaemia and allow the reconstitution of blood. Previous research that showed *A. pterocarpoides* to contain compounds such as flavonoids, tannins, glycosides, phenols groups which are responsible for its antioxidiant capacity observed through the complete inhibition of DPPH with an intermediate kinetic (Biapa et al., 2007; Biapa et al., 2011; Saah et al., 2013) identified some flavonoids (bis-isoflavone derivative, named amphiisoflavone (1), 8-methoxysysoformononetin (2), 6-methoxyisoformononetin (3) and isoformononetin). Furthermore, the bark extract of this plant has revealed the anti-anaemia activity by increasing the Hb, RBC and HCT concentrations in rats (Biapa et al., 2011). The objective of this study was to investigate *in vitro* the anti-sickling mechanism of *A. pterocarpoides*.

**MATERIALS AND METHODS**

**Plant materials and collection**

*A. pterocarpoides* barks were collected in the peripherical zones in Yaoundé (Center region of Cameroon) between October and December 2015. They were subsequently identified under reference number 52563 / HNC at the National Herbarium of Yaoundé-Cameroon.

**Preparation of plant extracts of *T. cacao***

The modified method of Benhammou et al. (2008) was used for the extraction. Briefly, 195 g of the powdered of *A. pterocarpoides* were macerated in the mixture of ethanol-water (70v/30v) for 48 h at pH 3 by the addition of a few drops of acetic acid. The mixture was stirred several times a day to maximize extraction. After 48 h, the mixture was filtered using Whatman paper N° 4 and 1, and then the filtrate dried in an oven for 24 h at 45°C. The same procedure was repeated in order to maximize the extraction.

**Blood samples collection**

Blood samples have been collected after obtaining an ethical clearance issued by the Regional Committee for Ethics Research for Human Health Center (number N° 0282-CRERSHC / 2016) as well as the authorization of the Central hospital of Yaoundé. Six confirmed sickle cell blood samples were collected (three women and three men between the ages of 16 and 40 who had been attending routine consultations in the Hematology Department of Central Hospital, Yaoundé) and stored at 4°C.

**Test of sickling reversal**

The method of Nanfack et al. (2013) slightly modified was used. As modifications, different concentrations of the extract were used instead of one, cells were read using *malassez* haemocytotometer (*malassez* cell) instead of slides. Incubations were done at 37 °C for 1 h instead of 25°C for 24 h.
Blood samples were washed twice into 5 volumes of 0.9% physiological solution (1:5 in ratio) by centrifugation at 3000 rpm for 5 min. Into a clean eppendorf tube, to 100 μL of washed SS blood, were added 100 μL of sodium metabisulfite 2% and incubated for 1 h at 37°C. Thereafter, 100 μL of each extract at different concentrations (500, 1000, and 2000 μg/mL in 0.9% NaCl solution) was added and incubated for 1 h at 37°C. The 10 μL of this mixture were then transferred using a dropping pipette on malassez cell and observed under light microscope (40X magnification). The percentage of sickle cells has been obtained. A negative and positive controls group was performed in the same way but the extract was replaced by either 100 μL of 0.9% NaCl solution or phenylalanine (standard). The method described by Nanfack et al. (2013) slightly modified was used. As modifications, different concentrations of the extract were used instead of one, cells were read using Malassez haemocytometer (Malassez cell) instead of slides. Incubations were done at 37°C for 4 h instead of “at room temperature” for 24 h.

The osmotic fragility of the erythrocytes is based on the measurement of the stabilizing effect of their membrane after 4 h of incubation at 37°C with the extract. Cell lysis is determined by observing the turbidity shift using spectrophotometry method at 540 nm. Blood sample was washed as mentioned above. Briefly a serial concentration of extracts (500, 1000, 2000 μg/mL) were diluted in NaCl (0.9%). In 800 μL of different NaCl concentrations (0-0.85%), were added respectively 200 μL of extracts and 10 μL of blood. After 4 h of incubation, the supernatant absorbance from the mixture was read at 540 nm against the blank made up of 0.85% NaCl. Hemolysis was expressed as follows:

\[
\text{% Hemolysis} = \frac{\text{DO}_{\text{test}}}{\text{DO}_{\text{control}}} \times 100
\]

### Evaluation of the haemoglobin S solubility

The method described by Itano (1953) was used with slight modification (Incubation was done at different fixed time points). The washed RBC (as described above) was resuspended in hypotonic medium and the haemolysate of RBC was centrifuged at 3000 rpm during 10 min. The supernatant was equally mixed with 2% of metabisulfite freshly prepared. The mixture was then incubated for 45 min at room temperature. An aliquot of 50 μL was subsequently diluted with 500 μL of phosphate buffer at pH 7.5 containing (NH₄)₂SO₄ 30%, saponin 1% and K₂HPO₄ 1.2%. Afterward, 50 μL of the extract were added, mixed and then incubated (0, 30 and 60 min). With regards to the control, an equivalent volume of the phosphate buffer was used. After a fixed time, aliquots of control and test were removed and centrifuged at 3500 rpm for 5 min at room temperature. The absorbance of the supernatant was then read at 540 nm. The solubility of Hb S was expressed as the increase of the optical density.

### RESULTS

#### Properties of *A. pterocarpoides* on the sickling reversal experiment

The effect of *A. pterocarpoides* on the Reversal of Sickling is shown in Table 1. The control group showed that when putting together SS blood cells with 2% metabisulfite only, after 1 h, the sickled cells increased up to 80.11%. The use of *A. pterocarpoides* considerably decreased the process significantly (P<0.05) with concentration-dependent (between 49.98 ± 0.52 and 43.08 ± 0.67). The Reversal of Sickling in term of percent sickling reduction varied from 30 to 43.59%. It is clear

<table>
<thead>
<tr>
<th>Antisickling agents</th>
<th>Percent sickled RBCs (%)</th>
<th>Percent sickling reduction (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>80.11 ± 0.24 a</td>
<td>0</td>
</tr>
<tr>
<td><em>A. pterocarpoides</em></td>
<td>C1</td>
<td>49.98 ± 0.52 b</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>48.03 ± 0.26 b</td>
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<tr>
<td></td>
<td>C3</td>
<td>43.08 ± 0.67 c</td>
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<tr>
<td></td>
<td>Phe</td>
<td>36.52 ± 9.3 c</td>
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Kruskal-wallis followed by Dunnet. Values with different letter represents significant difference at 0.05. Phe: phenylalanine (standard). *A. pterocarpoides*: *A. pterocarpoides*.
that the standard (Phe) reversed best the cell sickling with Percent sickling reduction of 43.59%.

**Effect of A. pterocarpoides on the red blood cell membrane fragility**

The influence of the plant extract on the red blood cell fragility is given as the percentage of cell haemolysis as a function of the salt concentration and the concentrations of various extracts (Figure 1). Figure 1 shows that the curve of *A. pterocarpoides* as well as that of the standard (Phe), was shifted to the left of the control. The haemolysis percentage decreases significantly (P < 0.05) with both the increase of extract concentrations (500, 1000 and 1500 μg/mL) and the salt concentrations. Based on 100% initial total haemolysis, *A. pterocarpoides* reduced significantly (P < 0.05) the cell fragility up to 18.42% as compared with that of Phe which is 12%. When fixing the concentration of the extract, *A. pterocarpoides* influenced significantly the decreasing of haemolysis percentage with salt concentration dependent. The standard showed the best decrease in cell haemolysis but with no significant difference as compared with *A. pterocarpoides*.

**Properties of A. pterocarpoides on the haemoglobin S solubility**

Table 2 shows the effect of *A. pterocarpoides* on the haemoglobin S solubility. In general, this table indicated that the use of *A. pterocarpoides* significantly (P < 0.05) increased as a function of time, the extract concentrations and the absorbance or solubility of Hb S as compare with the control group (SS blood + 2% metabisulfite). This materialized by the absorbance values found which were between 0.260 ± 0.05 to 4.991 ± 0.07. This observation was furthermore found when fixing either the time or the extract concentrations.

**DISCUSSION**

To fight against cell sickling, the biochemical main approach remains to inhibit the Hb S polymerization tendency or to reverse it. In fact, it is well known that the quaternary structure of haemoglobin exists in two states: the tense or T state with a deoxygenated conformation and the R state called relaxed state with an oxygenated conformation. Sickling occurs only in the deoxygenated
conformation due to the sickling tendency of the Hb S (Sunday et al., 2012). Hb S induces many cellular and tissue injury particularly substantial loss of membrane flexibility and sickle shape. The intracellular polymerization of deoxyHb S occurs as a result of sickle erythrocyte dehydration. Two major events explain the physiopathology of SCA: the sickle haemoglobin and the RBC membrane integrity. Subsequently, two mechanisms could explain the role of *A. pterocarpoides* against SCA.

In the present study, deoxyhaemoglobin S has been initiated using 2% metabisulphite which induced the SS cell polymerization. Plant extract has been added at a fixed point in order to study the reversal of sickling. Results showed that *A. pterocarpoides* inhibit polyrimezation by reversing it. In fact, during polymerization, many interactions among the amino acids in the hypoxic states explain the phenomenon of polymerization. Mainly, in homozygous SS patients, Val6 interacts hydrophobically with Phe85 and Leu88 of the other haemoglobin molecules (beta-2 of the first Hb molecule interacts with beta-1 of the second Hb molecule) which form complex fibers (Ngolet et al., 2016). The reversal of sickling means fibers "melt" as oxygen is taken up by the Hb S and the normal discoid shape returns (Stuart et al., 2004). It is then clear that *A. pterocarpoides* reverse the sickling using a mechanism of melting complex fibers. By reversing the polymerization process, *A. pterocarpoides* allows the initial rigid and deoxygenated Hb S to become more soluble as we observed during the test of Hb S solubility. This result is in line previous study on *Justicia seconda* which demonstrated the implication of this plant in the inhibition of polymerization with the same mechanism (Mpiana et al., 2010). Furthermore, according to Ismaila et al. (2017) and Nanfack et al. (2013), *C. cajan*, *Z. zanthoxiloides*, *Carica papaya* and *Z. heidzii* reverse the sickling after several hours of incubation.

This present study also showed the capacity of *A. pterocarpoides* to help red cells resist against fragility or haemolysis. There exist two main well explained physiopathological manifestations of SCA: vaso-occlusive crisis and haemolysis. The later (haemolysis) come after the following situation: deoxygenation, polymerization and cell dehydration of red blood cells (Virgilio et al., 2005). In fact, dehydration of human RBCs in vivo may result from the activation of one or more of the three transporters expressed in their plasma membranes: the Ca2+-sensitive, small-conductance, K+-selective channel (Gardos channel), (Gardos, 1958, Hoffman et al., 2003), a K+-Cl cotransporter (KCC), regulated by internal pH and cell volume (Lauf and Theg, 1980), functionally active in reticulocytes and much less so in mature AA and SS RBCs; and the Na+ pump (Sarkadi et al., 1976). The three transporters differ considerably in their dehydrating modalities, potencies, and distributions among RBCs. It has been shown in SS patients (Tosteson et al., 1952) that upon deoxygenation of fresh, heparinized whole blood, SS RBCs had a much larger gain of Na+ and loss of K+ than RBCs from normal controls. On reoxygenation, SS cells gained K+ and lost Na+, indicating that the effects of deoxygenation were reversible. Polymerization induces the rigidity and distortion of red cells which fragilize the cells and subsequently reduce the resistance to haemolysis. Eaton et al. (1973) independently reported the high ca++ in SS cells and suggested that the Gardos channel might be the main dehydration pathway.

According to Ismaila et al. (2017), the presence of some plants extracts (*C. cajan*, *Z. zanthoxiloides* and *C. papaya*) reduced the SS cells haemolysis by increasing the resistance of SS cells and decreasing its osmotic fragility. Then, antisickling agents having the capacity to reduce the osmotic fragility is an advantage since it increases the RBCs’ resistance to lysis. The presence of increased free radicals mainly produced by Hb S (Banerjee and Kuyper, 2004; Hebbel et al., 1983) are well known to be also responsible for the cell fragility, dehydration and then haemolysis (Brugnara, 2000). This allegation could explain the role of agents with high capacity of antioxidants potential in the management of SCA. Previous studies showed that *A. pterocarpoides* contains antioxidants compounds (Biapa et al., 2007; Saah et al., 2013). Furthermore, previous studies have also shown the implication of antioxidant content of *Theobroma cacao* against the fragility of SS cells (Biapa et al., 2018; Yembeau et al., 2018).

### Conclusion

At the end of this study, it is clear that *A. pterocarpoides* reversed the polymerization, solubilized the deoxy-
haemoglobin S and maintained the integrity of SS cells. In the future, other studies should be carried out such as the Bio-guide fractionation in order to determine the responsible substance against SCA as well as other anti sickling mechanism of *A. pterocarpoides*. These will give the studied plant its real implication against SCA.

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