



## **Mineral Content and Antisickling Activity of *Annona senegalensis*, *Alchornea cordifolia* and *Vigna unguiculata* Used in the Management of Sickle Cell Disease in the Kwilu Province (Congo, DR)**

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### **Authors' contributions**

This work was carried out in collaboration among all authors. Authors JMK, KNN and PTM designed the study and wrote the protocol. Authors CLI, EML and DDT wrote the first draft of the manuscript. Authors DSTT, KMT, BMM and BS managed the literature searches. All authors read and approved the final manuscript.

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### **ABSTRACT**

**Aims:** To determine the mineral composition of some plants (*Annona senegalensis* Pers., *Alchornea cordifolia* (Schumach. & Thonn.) Müll. Arg. and *Vigna unguiculata* (L.) Walp.) used in the management of sickle cell disease by traditional practitioners in Kwilu province and to evaluate their antisickling activity *in vitro*.

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**Study Design:** Plant collection in the Kwilu province, sample preparation, antisickling tests and fluorescence spectrometric analysis.

**Place and Duration of Study:** This work was performed at the Faculty of Science, University of Kinshasa, Congo DR, from October 2016 to January 2018.

**Methodology:** These three plants were harvested in the province of Kwilu in Democratic Republic of the Congo. The mineral composition analysis was carried out using the fluorescence spectrometric method while the *in vitro* antisickling activity was evaluate using Emmel and hemolysis tests.

**Results:** Twenty three mineral elements were identified in each of these three plants: Potassium (K), Phosphorus (P), Calcium (Ca), Sodium (Na), Magnesium (Mg), Sulphur (S), Chlorine (Cl) and trace elements as: Aluminum (Al), Silicon (Si), Vanadium (V), Chromium (Cr), Manganese (Mn), Iron (Fe), Nickel (Ni), Copper (Cu), Zinc (Zn), Selenium (Se), Brome (Br), Molybdenum (Mo), Tin (Sn), Iodine (I), Barium (Ba) and Lead (Pb). *Annona senegalensis* Pers., *Alchornea cordifolia* (Schumach. & Thonn.) Müll.Arg. and *Vigna unguiculata* (L.) Walp. aqueous extracts showed the capacity to prevent the sickling and the hemolysis of red blood cells.

**Conclusion:** The obtained results confirm the antisickling activity thus justifying the use of these plants in Traditional Medicine for the management of sickle cell disease. The presence of some mineral elements like Fe, Zn, Mg and Se are useful for sickle cell disease patients.

**Keywords:** *Annona senegalensis*; *Alchornea cordifolia*; *Vigna unguiculata*; sickle cell disease; mineral elements; fluorescence; antisickling.

## 1. INTRODUCTION

Medicinal plants are an important source of health care worldwide [1]. Traditional Medicines are widely used due to population growth and inaccessibility to modern medicines [2]. In Africa, this demand is not only the result of the inaccessibility of modern medicines and the high cost of conventional medicines but also due to the fact that Traditional Medicine is very often considered a more appropriate treatment method [3]. Indeed, nearly 80% of populations depend on traditional medicine for their primary health care, according to the World Health Organization [4].

In the Democratic Republic of Congo (DRC), urban and rural populations are increasingly turning to the use of medicinal plants to solve their health problems [3]. Sickle cell disease is an inherited disease that affects nearly 5% of the world's population [1]. It is due to a mutation in the hemoglobin beta globin gene and is characterized by severe anemia, vaso-occlusive attacks and high susceptibility to both viral and bacterial infections [5]. This disease is characterized by a loss of mineral elements that are important for the body's functioning [6].

Minerals are natural chemical elements that the body uses to activate certain biochemical

reactions. They are part of functionally important inorganic compounds such as iron (Fe) in hemoglobin and Cytochrome or zinc (Zn) in insulin [7].

Recently our research team showed that some medicinal plants used in Congolese Traditional Medicine have *in vitro* antisickling activity [8], [9], [10]. Anthocyanins and organic acids and their derivatives were found to be most active phytochemicals [11], [12]. But few works are done on the composition of mineral elements of these plants in order to evaluate their possible effect on sickle cell disease.

The objective of this work is to confirm the *in vitro* antisickling activity of *Annona senegalensis* Pers., *Alchornea cordifolia* (Schumach. & Thonn.) Müll.Arg. and *Vigna unguiculata* (L.) Walp, three medicinal plants used in the management of sickle cell disease in Kwilu province, DRC and to evaluate their mineral elements composition mainly that related to sickle cell disease. Toxicological studies have shown that *Annona senegalensis* is relatively safe, but prolonged ingestion could induce oxidative stress and impair ATP synthesis through the modulation of the activity of mitochondrial succinate dehydrogenase [13] while *Alchornea cordifolia* was found to be no toxic [14]. *Vigna unguiculata* is widely consumed in DRC as well as across Africa [15].

## 2. MATERIALS AND METHODS

### 2.1 Biological Samples

The leaves of *Annona senegalensis* and *Alchornea cordifolia* and the seeds of *Vigna unguiculata* used in this study were collected in May and July 2016 in the Masi-Manimba territory, Kwilu province, DRC whose geographical coordinates are: 5° 02' 01" South, 18° 50' 01" East.

Blood samples used for antisickling evaluation were collected from known sickle cell disease patients treated in "Centre de Médecine Mixte et d'Anémie SS" in Kinshasa, DRC. None of the patients had been transfused 4 months before the assay, with Hb AA blood. All antisickling experiments were carried out with freshly collected blood. Only one type of human blood sample was used at a time for all plant extracts. In order to confirm their sickle cell nature, the above-mentioned blood samples were first characterized by hemoglobin electrophoresis on cellulose acetate gel, as previously reported [8], [15]. They were found to be Sickle cell blood and were then stored at  $\pm 4$  °C in a refrigerator. Stratified sampling method was used as follow: five blood samples was firstly submitted to Emmel test for a primary checking and sample presenting good sickling rate ( $\geq 90\%$ ) was selected for bioassay after further confirmation by electrophoresis.

### 2.2 Mineral Composition Determination

The plant material samples were prepared (washed, dried and ground) at the basic sciences laboratory of the University of Kikwit. The detection and quantification of the mineral composition was done by the fluorescence spectrometric method (XEPOS 3) at the University of Kinshasa. Five grams of the powder of each plant sample was compressed into pellets through the hydraulic press for each plant and the resulting pellets were introduced into the fluorescence spectrophotometer for reading.

### 2.3 Antisickling Assays

The evaluation of antisickling activities of plant extract samples was done using two *in vitro* antisickling tests: Emmel and hemolysis tests respectively.

#### 2.3.1 Emmel test

Emmel test was performed as previously described [16]. Briefly, sickle cell blood was diluted with 150 mM phosphate buffered saline ( $\text{NaH}_2\text{PO}_4$  30 mM,  $\text{Na}_2\text{HPO}_4$  120 mM, NaCl 150 mM) and mixed with an equivalent volume of 2% sodium metabisulfite. A drop from the mixture was spotted on a microscope slide in the presence or absence of plant extracts and covered with a cover slip. Paraffin was applied to seal the edges of the cover completely to exclude air (Hypoxia condition). The red blood cells images were treated with a computer assisted image analysis system (Motic Images 2000, version 1.3).

#### 2.3.2 Hemolysis test

Washed sickle cell erythrocytes are contacted with 2% sodium metabisulfite solution in the presence or absence of the extract. At determined time intervals, an aliquot part is sampled, diluted with 0.9% NaCl solution and centrifuged (15 sec, 4000 rpm). The optical density of the supernatant is read at 540 nm using an UV-vis spectrometer PERKIN ELMER Lambda 7. An increase in optical density indicates an increase in hemoglobin content outside the cells and therefore red cells hemolysis [8].

### 2.4 Statistical Analysis

All assays were carried out in triplicate and the values were obtained by calculating the average of three experiments and data are presented as Mean  $\pm$  SD (Standard Deviation). Data were subjected to analysis of variance (ANOVA) at probability threshold level of 0.05, using Origin 8.5 Pro software package. For anti-hemolytic assay, the two sample T test was used to evaluate the difference between the treated and untreated sickle red blood cells population.

## 3. RESULTS AND DISCUSSION

### 3.1 Antisickling Effect

The Fig. 1 shows a micrograph of the sickle cell blood alone and the Fig. 2 give for illustration a micrograph of sickle cell blood in the presence of aqueous extract of *Annona senegalensis* leaves.

The Fig. 1 shows that the majority of erythrocytes in sickle cell blood have sickled and elongated forms. It means and confirms that the collected blood is from sickle cell disease patient. Fig. 2

indicates that all blood cells, even in hypoxic condition, return to the normal circular form.

This modification of the morphology of sickle cells indicates an antisickling activity of the aqueous extracts of *Annona senegalensis* leaves used as an illustration. Aqueous extracts from *Alchornea cordifolia* and *Vigna unguiculata* showed similar images.

This behavior has already been observed for ethanolic extract of the same plants and for other medicinal plants used in the management of sickle cell disease in DRC [8], [10], [17]. It was shown that this effect was due to anthocyanins and phenolic or terpenic acids. These results therefore confirm those obtained in our previous studies [8], [9], [18].

It was postulated that these bioactive compounds could interact with hemoglobin S and then compete with the polymerization of this abnormal hemoglobin that conducted to the sickling of drepanocytes [8], [12].

### 3.2. Antihemolytic Effect

Sickle cell disease is also characterized by a precocious hemolysis of red blood cells. A benefit effect of a plant on sickle cell disease can also be evaluated by its capacity to prevent hemolysis [8]. Fig. 3 gives the evolution of the optical density at 540 nm with time in isotonic solution (NaCl 0.9%).

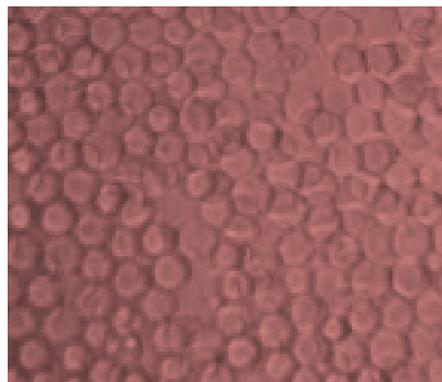
Fig. 3 shows that the optical density of sickle cell blood alone increases over time at 540 nm. In fact, it is the heme of hemoglobin that constitutes the chromophore group that absorbs at this wavelength [19]. The fact that the optical density increases over time indicates that the hemoglobin concentration is increasing in solution, meaning that it leaves the inside of the erythrocyte envelope that has hemolyzed. But in the presence of plant extracts, it can be noticed that the optical density does not increase with time but rather decreases. At the 0.05 level, the difference of the treated and untreated sickle red blood cells population means was significantly different with the test difference.

This behavior can only be explained by the action of these extracts on sickle cells by preventing their hemolysis. This was observed with aqueous extracts of all the three plants under study. The same effect has already been

observed for extracts from *Justicia secunda* leaves [10]. Indeed, sickle cell disease is known as hemolytic anemia because of the early hemolysis of red blood cells in sickle cell blood, which, by evacuating the hemoglobin in an unfavorable environment, destroys it early and leads to a decrease in the level of this vital blood protein, conducting to anemia.



**Fig. 1. Optical microphotography of untreated SS blood erythrocytes (control)[NaCl 0.9%, Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> 2%, Magnification 500 X]**



**Fig. 2. Optical microphotography of SS blood erythrocytes treated with aqueous extracts of *Annona senegalensis* leaves (50 µg/mL) [NaCl 0.9%, Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> 2%, Magnification: 500 X]**

Any substance that prevents or reduces this hemolysis would be beneficial for sickle cell patients [8], [10]. This, combined with the normalization of sickle cells, would explain the use of these plants in the management of sickle cell disease in Kwilu province.

### 3.3 Mineral Content

#### 3.3.1 Macroelements

Table 1 gives the macroelements content of the three plants under investigation.

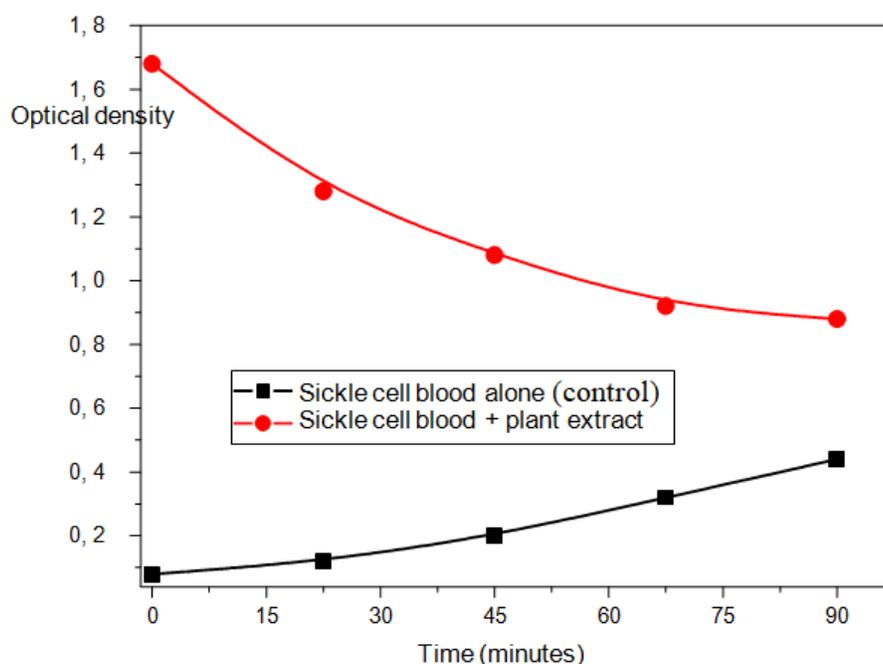


Fig. 3. Evolution of the optical density (540 nm) with time of Sickle cells treated with 50 µg/mL of *Annona senegalensis* aqueous extract in isotonic solution (NaCl 0.9%)

Table 1. Macroelements content of the three plants

Element	Concentration (mg/kg)		
	<i>V. unguiculata</i>	<i>A. senegalensis</i>	<i>A. cordifolia</i>
K	7291.51±311.04	7695.07±385.06	9262.06±482.14
Na	1300.86±59.08	1815.29±90.68	ND
Mg	1937.52±82.23	1693.12±88.46	1852.08±93.74
P	1333.57± 67.12	1566.57±79.69	1258.37±61.72
Ca	15865.50±763.45	13747.27±635.23	9441.10± 473.18
S	2095.30±107.86	1659.00±87.83	2124.68±113.34
Cl	128.98± 6.86	118.03±6.03	533.02±26.62

Legend: ND: none detected

The results obtained in the Table 1 show that these plants contain seven macroelement (K, Na, Mg, P, Ca, S, Cl), the Ca content is higher than that of the other elements identified in the three plants when Cl has the lowest content in the plants. It can be noticed that Na was not detected in *Alchornea cordifolia* while the mineral content varies from plant to plant. Magnesium and Calcium are most abundant in *Vigna unguiculata*; Na and P in *Anona senegalensis* when K, Cl and S are most abundant in *Alchornea cordifolia*.

The values obtained are of the same order of magnitude but generally slightly lower than those found for three other plants from the same region, namely *Hura crepitans*, *Alternanthera*

*bettzickiana* and *Dissotis brazzae* [20]. The values of Calcium and Potassium concentrations obtained in this work are also at the same order of magnitude as that of *Ocimum basilicum* [21]. But the K and Ca content of *Alchornea cordifolia* in this work is higher than that obtained by Ebenyi et al. [22] when K, Ca, P, Mg and Cl content of *Vigna unguiculata* vary from an author to another [23]. The difference in content obtained from these plants could be due to the methods used and ecological factors [20].

Calcium that is the most abundant mineral element in all the three plants under investigation constitutes a large proportion of the bone, human blood and extracellular fluid. It is necessary for

the normal functioning of cardiac muscles, blood coagulation and the regulation of cell permeability. Calcium is also involved in sickle cell disease related to the hydration of sickle cells. Indeed, the membrane of sickle cells is generally more rigid than that of normal erythrocytes, this result in a decrease in the efflux of  $Ca^{2+}$  ion out of the cell. The intracellular accumulation of calcium ions leads to cellular dehydration and consequently an intracellular increase in the concentration of HbS. This increase in the concentration of HbS inside the red blood cells accelerates the polymerization of the HbS which precipitates on the membrane and thus reinforces its rigidity. It is also known that the  $Ca^{2+}$  ion is involved, like potassium, in the functioning of the Gardos channel [24]. Calcium from these plants could therefore disrupt the transmembrane transport of  $Ca^{2+}$  ions to the membrane of sickle cells and improve cell hydration, which would be beneficial for sickle cell patients and would enhance the antiskinking activity of these plants.

In humans, Mg is required in the plasma and extracellular fluid, where it helps maintain osmotic equilibrium. In addition, it may play an important role as co-factor of enzymes, reduces the number of abnormal erythrocytes in sickle cell disease and improves the hydration of red blood cells. In general, sickle cell patients have a magnesium deficiency [6], [25]. This deficit is involved in red blood cell dehydration. Mg supplementation reduces the number of abnormal erythrocytes and improves the hydration of red blood cells in sickle cell patients [24], [25]. The high content of these plants in this macro-element could explain their use in the management of sickle cell disease in Congolese traditional medicine.

Potassium and sodium are electrolytes needed for the body to function normally and help maintain fluid and blood volume in the body. According to Martinez-ballesta et al. [26], the sodium content in food plants generally varies between 0.04 to 277 mg/100g or 0.4 to 2770 ppm which is consistent with the content of most of our plants.

In sickle red blood cell, an abnormal activation of potassium chloride (KCl) co-transport is reported as the cause of cell Potassium loss and dehydration while deoxygenation of sickle red cell increases Sodium and Calcium intracellular level [27,28]. The polymerization of HbS stretches the cell membrane by interfering with the activity of Calcium<sup>+</sup>-activated Magnesium<sup>+</sup>-

dependent ATPase (Calcium<sup>+</sup> pump) responsible for maintaining membrane integrity. Declining  $Ca^{2+}$  pump efficiency may lead to premature ageing and formation of rigid sickled red blood cells [29]. Medicinal plant species containing such minerals are of great importance as supplier minerals for the patients. Calcium can restore 25-hydroxy-vitamin D level in treated patients [30]. Indeed, Calcium plays an important role in strong bone and teeth formation, the regulation of muscle contractions, and the transmission of nerve impulses in the human body; thus, its presence in human diets is a necessity. Calcium also plays a crucial role in nerve impulse transmission and in the mechanism of neuromuscular system [31]. It plays an important role in blood clotting, muscles contraction, and neurological function and also helps in enzymatic metabolic processes [32]. Potassium plays a very crucial role in the body by helping maintain body fluid and osmotic balance, as well as helping in the regulation of nerve signals and muscle contractions [33]. As previously reported [34], Magnesium is a major constituent of bones and teeth alongside with Calcium and Phosphorus; also, it is necessary for tissue respiration, the release of parathyroid hormone and for its action in the backbone, intestine, and kidney. Sodium plays a vital role in the conduction of nerve impulses alongside potassium [35].

The trace element levels of the three plants studied are summarized in Table 2.

Mn is the most abundant element in all three plants except in *A cordifolia* where silicon is more abundant. Mo was not detected in any of these three plants while Pb, which is a toxic element, was detected at concentrations below 1.1 ppm. The values of the concentrations found are of the same order of magnitude as those of our previous work for other plants in the same region [20].

But the concentrations of these trace elements in the same plants vary slightly from one author to another [20,21]. This is probably due to ecological factors and the techniques used [20].

Iron is the second most abundant trace element in all three plants with a slightly higher concentration in *A cordifolia*. This element is essential for the survival of living beings because it is thanks to the iron in hemoglobin that oxygen is carried. Sungu et al. [36] proved that during their crisis, sickle cell patients lose mineral elements including iron. A plant that has a good

Table 2. Trace elements content of the three plants

Element	Concentration (mg/kg)		
	<i>V. unguiculata</i>	<i>A. Senegalensis</i>	<i>A. Cordifolia</i>
Al	35.71±1.56	27.89±1.45	48.13±2.37
Si	57.40±2.92	153.85±7.83	696.48±35.03
V	1.14±0.05	< 1.00	1.25±0.06
Cr	1.54±0.07	1.33±0.65	2.33±0.12
Mn	475.36±22.06	428.46±20.92	197.92±9.76
Fe	128.19±6.45	123.68±6.22	164.15±8.19
Ni	4.38±0.28	4.27±0.22	3.59±0.17
Cu	9.41±0.47	16.60±0.81	11.06±0.54
Zn	15.29±0.77	28.55±1.43	20.35±1.12
Se	1.02±0.06	< 0.80	ND
Br	54.00±2.74	7.27±0.35	9.07±0.43
Mo	ND	ND	ND
Sn	1.08±0.05	0.99±0.05	0.75±0.03
I	2.66±0.13	2.41±0.11	2.00±0.09
Ba	4.46±0.25	7.24±0.34	4.07±0.18
Pb	< 1.00	< 1.10	< 1.02

Legend: ND: none detected

iron content is beneficial for sickle cell patients because it increases hemoglobin levels. The high iron content in these plants used by traditional healers in Kwilu proves its role in increasing hemoglobin levels in sickle cell patients. The studies carried out by Itoua et al. [37] on *Phytolacca dodecandra* or wild spinach gave results similar to those obtained in this research for the content of iron.

Even if the deficiency of iron is considered as the main factor in the pathogenesis of anemia, only 40-60% of anemia cases are responsive to treatment with Fe containing medications and a large proportion of anemia does not respond to iron supplementation [38]. Several studies have shown that three other trace elements are strongly involved in hemoglobin deficiency or anemia. These include Zinc, Copper and Selenium [39].

Zinc has a concentration slightly higher than that of copper in all the plants under study. Zinc is an essential element in human, animal and plant nutrition. It plays a major role in growth and development, in testicular maturation, in neurological functions and in immunocompetence. It is involved in the activity of many enzymes. Zinc supplementation has been shown to reduce the incidence of infections and pain crisis of vaso-occlusive origin for sickle cell patients, the generation of tumor necrosis factor alpha and the decrease in the level of markers of oxidative stress [39]. Zinc is the key component of GATA-1, also called as erythroid

transcription factor. It is required in different stages of erythropoiesis.

Copper is an essential trace element that plays a vital role in various metabolisms. It is known to be involved in particular in the quality of cartilage, bone mineralization, the synthesis and regulation of neurotransmitter peptides, immunity and iron metabolism. Copper also has an important role in the oxidative metabolism of glucose and is therefore essential for the functioning of the myocardium. Hypocupremia is known to be responsible for dysregulation of human pluripotent stem cell proliferation and an impediment in the cellular differentiation in the bone marrow. The deficiency in this trace important element can cause anemia [38].

The highest selenium content in all three plants is found in *V. unguiculata* with 1.02 mg/kg. It should be noted that the selenium content depends on the soil. The selenium content of plants growing in normal soil is usually less than 3 mg/kg. Selenium is an important component of glutathione peroxidase, and its concentration in erythrocytes shows its protective effect on erythrocytes against oxidative damage. This suggests that increased oxidative stress could be another contributing factor for the development of anemia due to selenium deficiency. In fact, it should be noticed that sickle cell anemia is characterized by high oxidative stress which not only converts hemoglobin (with Fe<sup>2+</sup>) into methemoglobin (with Fe<sup>3+</sup>) thus reducing the capacity of this protein to bind to oxygen, but this

stress also leads to peroxidation of membrane lipids leading to precocious hemolysis of sickle cells [10].

The contents of other trace elements vary slightly from one plant to another. Although their importance in human, these trace element are not known to be directly related to sickle cell anemia.

#### 4. CONCLUSION

The objective of this study was to determine the mineral composition and antisickling activity of extracts of the three medicinal plants used in traditional Congolese medicine in the province of Kwilu in the management of sickle cell disease. Obtained results showed the effect of these plants in erythrocytes shape and in their hemolysis *in vitro* confirming their antisickling activity and justifying their traditional use. Concentration of 23 mineral elements was determined. Calcium was the most abundant macroelement in the three plants and could be involved with Mg in the antisickling effect of these plants due to their effect on the hydration of red blood cell. Concerning the trace elements, apart from Fe that has crucial role in hemoglobin level, other element such Zn, Mg, Cu and se could also have an important impact on the beneficial effect of these plants on the life of the sickle cell patients.

Other plants used in the management of sickle disease are under investigation and bio-guided fractionations of these three plants are in progress for the identification and characterization bioactive molecules.

#### CONSENT AND ETHICAL APPROVAL

An informed consent was obtained from all the patients above the age of 18 years or from the parents/tutors for patients under the age of 18 years selected in the study. Ethical clearance on the use of sickle blood cells was strictly observed according to international rules and the study was approved by the Ethical Committee of the Department of Biology (Faculty of Science, University of Kinshasa) under the registration number N/Ref. 006-2020/CD-CDB.

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#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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