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Anti-sickling activity of *Daniellia oliveri* (Rolfe) Hutch. & Dalziel. bark aqueous extracts in the management of sickle cell disease in Benin.

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ABSTRACT

*Objective***:** *Daniellia oliveri* (African copaiba balsam tree) is a plant used in Benin in the treatment of sickle cell disease. It is a traditional plant, which not all virtues have been scientifically proved. This work was carried out to evaluate the action of the bark of *Daniellia oliveri* in the treatment of sickle cell crises.

Methods and Results: The aqueous extract of the bark of *Daniellia oliveri* was preincubated at different concentrations with cells of the SS phenotype before or after the Emmel Test. Methaemoglobin was assayed after incubation of the extract with haemoglobin. *In vivo*, the action of the extract on haematopoiesis was evaluated in Wistar rats. At a dose of 40 mg / ml of blood, the extract significantly inhibited and reversed the formation of sickle cells ($P < 0.05$). It lowered the production of methaemoglobin at a dose of 10 mg / ml. Haemoglobin level, MCV, and platelet count did not significantly change in treated rats.

Conclusion and Application of results: The aqueous extract of the bark of *Daniellia oliveri* therefore inhibited the sickling of red blood cells *in vitro* and could be considered as a preventive remedy for sickle cell attacks. In addition, it reversed sickle cells into normal shaped red blood cells and so could be considered in the development of curative treatments for the sickle cell crises. However, the extract did not increase haemoglobin and mean corpuscular volume. It did therefore not show any hematopoietic activity and could not be considered as a remedy for anaemia. In addition, the number of blood platelets did not increase indicating an absence of thrombopoietic

activity. It lowered the production of methaemoglobin in SS red blood cells indicating a decrease in oxidative stress in SS red blood cells. It could thus be used as an antioxidant for the prevention or treatment of attacks in people with sickle cell disease in combination with plants, which have antianeamic properties.

Keywords: Sickle cell disease; *Daniellia oliveri*; anaemia; Benin.

INTRODUCTION

Sickle cell disease is a genetic haematological disease very widespread in the world and was recognized as a public health problem with a very increasing prevalence of the major forms (Mashako *et al.,* 2019). It has resulted in polymerization of haemoglobin S, chronic haemolytic anaemia, a pro-inflammatory state, recurrent painful attacks, and increased susceptibility to infections with encapsulated bacteria. There is also a chronic inflammation, chronic haemolysis, an immune deficiency, a heterogeneous clinical phenotype and visceral damage. The pathogenic mechanism in sickle cell disease is mainly due to chronic inflammation associated with oxidative stress (Mukuku *et al.,* 2018; Abdala *et al.,* 2019; Wembonyama *et al.,* 2021). Every year, the disease is transmitted almost 300,000 children Africa and WHO stated that nearly 80% of them die before the age of 5 years for lack of diagnosis and adequate care (Amadou *et al.,* 2019; Wembonyama *et al.,* 2021). In sub-Saharan Africa, the frequency of carriers of the sickle cell trait was variable and can reach 40% within certain African populations. Sub-Saharan Africa is the most affected part of the continent. The frequency increased from West to East, North to South Africa. The sickle cell trait therefore went from 15% in Senegal to more than 40% in Central Africa (Mashako *et al.,* 2019). Its management in Africa was largely through traditional medicine. Medicinal plants were and remained the most widely used means, especially in rural areas, to solve human and animal health problems. In Benin, 5,000 plants species have been inventoried in forest ecosystems. Among these species, 814 were used by the Beninese to treat diseases. Traditional medicine was still relevant and was of growing health and economic importance in Benin (Koudoro *et al.,* 2018). Herbal medicine research became of great scientific concern in view of the continued growth in the use of traditional medicine (Niyah Njike *et al.,* 2005). WHO estimated that traditional medicine accounts for 80% to 90% of health care in Africa, and WHO was increasingly promoting herbal research used in herbal medicine (WHO, 2002). This work aims to verify the anti-sickle cell activity of aqueous extracts from the bark of *Daniellia oliveri* (Rolfe) Hutch. & Dalziel.

MATERIAL AND METHODS

Plant material and aqueous extraction: The bark of *Daniellia oliveri* was collected in Abomey in Benin. The identification and certification of the plant was made at the National Herbarium of the University of Abomey-Calavi on number YH267 / HNB. The bark was air dried at laboratory temperature (20 ° - 25 °) out of direct sunlight and moisture for three weeks. They were then powdered and stored in black sachets (Koudoro *et al.,* 2018; Tchogou *et al.,* 2021). The technique used to prepare the extracts was that of maceration. After filtration, the extracts were evaporated to dryness at 60 ° C using a Heidolph type rotary evaporator (Koudoro *et al.,* 2018; Tchogou *et al.,* 2021).

Human material: Blood samples from ten (10) SS sickle cell patients were taken at the Zou / Collines departmental hospital in Benin with the consent of the patients after approval by the Ethics Committee of the National

School of Applied Biosciences and Biotechnologies in Benin.

Animal material: The animal material comprised of six (6) strain albino Wistar female rats from the animal house of IBSA in Benin whose average weight is 143g. These rats were acclimatized to ambient rearing conditions in the animal facility of the Experimental and Clinical Biology Laboratory at the National School of Applied Biosciences and Biotechnologies in Benin. They had access to water and food light 12 hours a day and were in spacious cages. The cages were cleaned regularly and the water renewed very often. The behaviour of the animals was observed during the two weeks of acclimatization.

***In vitro* biological tests**

Identification of secondary metabolites: The metabolites were identified by colouring and precipitation reactions specific to each metabolite family (Houghton *et al.*, 1998; Dohou *et al.*, 2003; Agbangnan *et al.*, 2012; Koudoro *et al.*, 2015).

Haemoglobin electrophoresis: It is performed to determine the phenotypes of the blood samples taken and to ensure that they were of the SS phenotype. The principle is based on the difference in migration of haemoglobins in an electrophoretic field according to their electrical charges. It was carried out on cellulose acetate gel at pH 8.5 as previously reported (Mpiana *et al.*, 2012).

Anti-sickle cell activity

Emmel's test: The principle of the test was that in the absence of oxygen, haemoglobin S polymerizes giving rise to the formation of fibres that deform the blood cell and give it a sickle appearance. Between a slide and coverslip, a drop of blood was deposited with 2% sodium metabisulphite. The test was positive if after an hour of time, the red blood cells took the shape of a sickle or banana with pointed, often serrated ends (Mpiana *et al.*, 2013; Sènou *et al.*, 2017). Emmel's test was performed to assess the anti-sickle cell activity of the extracts. Volume-to-volume mixing was performed between the SS blood sample and physiological water on the one hand to constitute the control and between the blood sample and the extract on the other hand for the test. After 6 hours of incubation at room temperature in the laboratory, a drop of each mixture was mounted between slide and coverslip with a drop of 2% sodium metabisulphite, and then the edges were coated with candle wax to prevent drying. The preparations were then read under an optical microscope 4 hours later and the erythrocytes of different shapes observed were counted.

Blood samples of SS phenotypes were mixed with the extract at different concentrations (5, 10, 20 and 40 mg / ml) using physiological saline as a solvent.

Emmel reverse test: Blood samples of SS phenotypes were mixed with 2% sodium metabisulfite in equal volume and incubated for 2 hours at room temperature. To an aliquot of this mixture was added an equal amount of physiological water for the control or 40 mg / ml extract for the test. After 6 hours of incubation at room temperature, the preparations were read under an optical microscope after mounting between slide and coverslip.

Evaluation of the ratio of methaemoglobin production (Fe ++ / Fe +++): The principle was based on measuring the absorbance of methaemoglobin at 540 nm. The methaemoglobin profile was a bioindicator of intra-erythrocyte oxidative stress in sickle cell patients (Kambale *et al.*, 2013). Sickle cell erythrocytes were washed with 0.9% NaCl solution (5 volumes NaCl per 1 volume of well-homogenized whole blood). The mixture was homogenized by successive inversion, centrifuged at 3000 rpm for 10 minutes. The red blood cell pellet was haemolysed by adding a double volume in double-distilled water and then centrifuged at high speed. The haemoglobin S solution thus obtained was mixed with the plant extract (10 mg / ml) incubated for 2 hours. For the negative control,

the extract was replaced by physiological solution (0.9% NaCl). The absorbance of the solution was read at 540 ηm at time intervals (0, 30, 60, 90 and 120 min) using a UV-visible spectrophotometer: six tubes were available for this (in duplicate) T0 (reading at start time), T30 (reading after 30 min), T60 (reading after 60 min), T90 (reading after 90 min) and T120 (reading after 120 min). The change in optical density (delta OD) over time was calculated to quantify the formation or disappearance of methaemoglobin (adapted from Mpiana *et al.*, 2007a, b, c; Kambale *et al.*, 2013).

***In vivo* biological tests:** *In vivo*, the effect of the extract of *Daniellia oliveri* on the evolution of haemoglobin, mean corpuscular volume (MCV) and blood platelets was studied in female rats of the wistar strain. For this purpose, two groups of rats of three (3) rats each were formed. The first group being the control received physiological water by gavage and the second group received by gavage the extract of *Daniellia oliveri* at 200 mg / Kg of body weight daily for 28 days.

Blood tests: The blood of the rats of the two groups was taken at the beginning (D0) and on the twenty-eighth day (D28) by orbital puncture after ether anaesthesia. The haemoglobin level and mean corpuscular volume (MCV) and the number of platelets were determined by automated system (Sènou *et al.*, 2016; Agbogba *et al.*, 2019).

Statistical analysis: To assess the biological effect of the extract, Dunn's multi-comparison test and Mann Whitney test were used. The significance level was set at 5%. The graphs were drawn using Graphpad software.

RESULTS

***Daniellia oliveri* bark aqueous extract prevented the sickle cells formation:** Figure 1 showed the mean sickle cell count on Emmel's test. The mean sickle cell count was 0.85 ± 0.09 for control cases; 0.64 ± 0.13 for cases treated at 40 mg ; 0.83 ± 0.088 for cases treated at 20 mg ; 0.81 ± 0.112 for cases treated at 10 mg and 0.83 ± 0.068 for cases treated at 05 mg with *Daniellia oliveri* (Rolfe) Hutch. & Dalziel. The mean sickle cell count decreased significantly in those treated at 40 mg ($P < 0.05$) compared to controls.

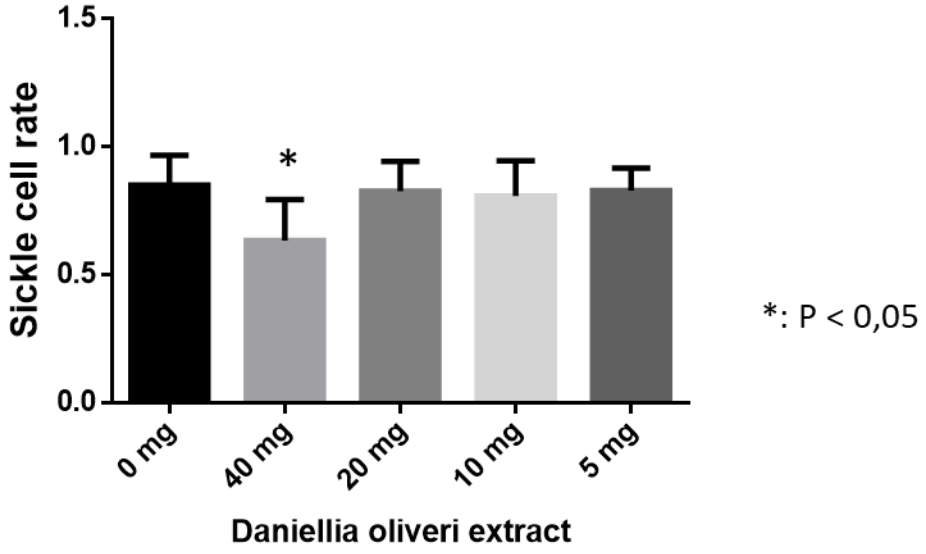

Figure 1: Mean sickle cell count in Emmel's test as a function of the extract concentration

The aqueous extract from the bark of *Daniellia oliveri* returned sickle cells to the normal biconcave form of red blood cells: Figure 2 showed the average sickle cell count on the Emmel reverse test. The mean sickle cell count was 0.76 ± 0.1092 for control cases and 0.55 ± 0.1048 for cases treated at 40 mg with *Daniellia oliveri* (Rolfe) Hutch. & Dalziel. The mean sickle cell count decreased significantly in those treated at 40 mg ($P < 0.05$) compared to controls.

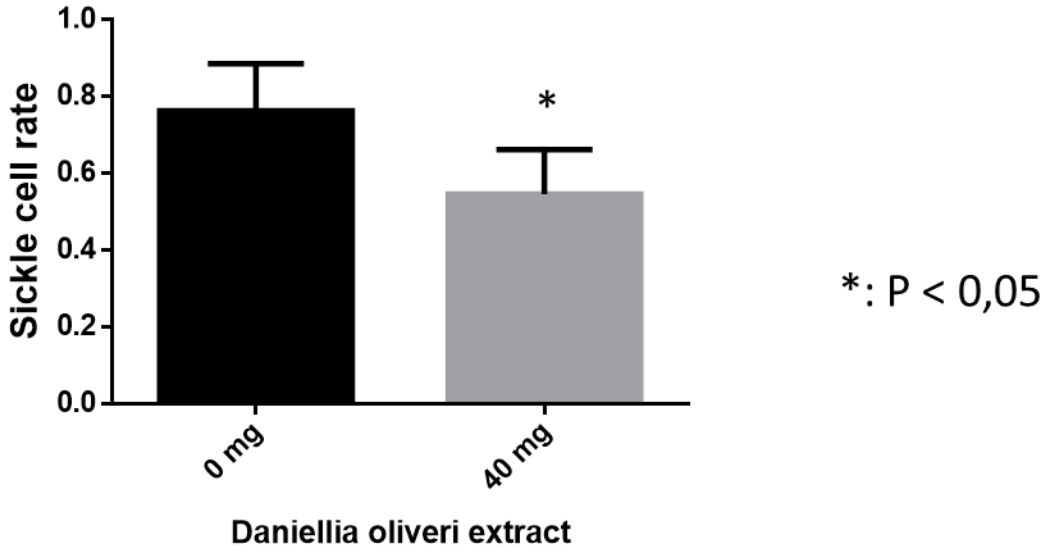

Figure 2: Mean sickle cell count on the Emmel reverse test

The aqueous extract of the bark of *Daniellia oliveri* reduces intra-erythrocyte oxidative stress: Figure 3 showed the change in methaemoglobin over time. The delta OD was 0.012 ± 0.004 for control cases and - 0.020 ± 0.004 for cases treated at 10 mg / ml with Daniellia oliveri. Delta OD was significantly reduced in those treated at 10 mg / ml compared to controls.

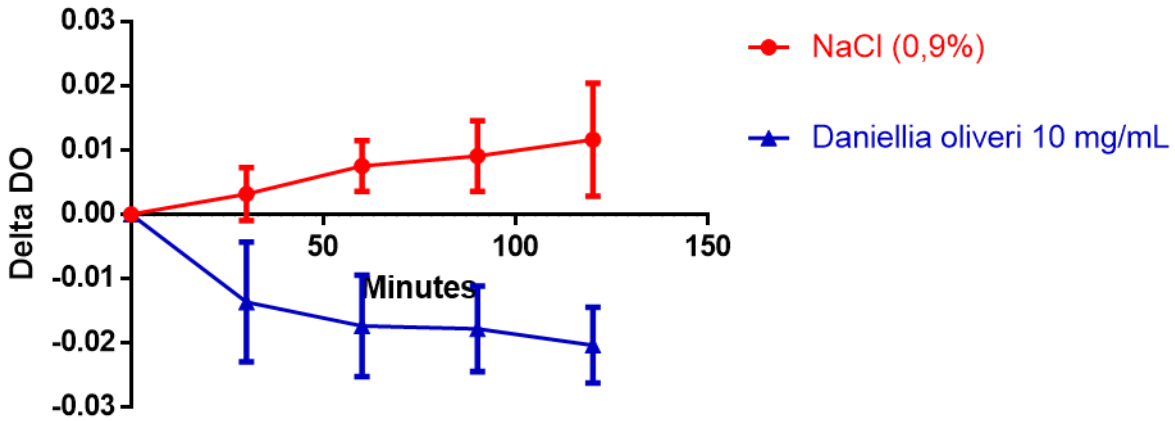

Figure 3: Change in methaemoglobin production in SS red blood cells over time

The aqueous extract from the bark of *Daniellia oliveri* did not stimulate blood cell production: Table 1 showed the changes in the mean haemoglobin level, mean blood cell volume and platelet count in rats. The mean haemoglobin level was 14.5 ± 0.291 g / dl in control rats and 14.6 ± 0.318 g / dl in rats treated with *Daniellia oliveri* (Rolfe) Hutch. & Dalziel. The mean haemoglobin level did not change significantly between control and treated rats, meaning that the extract did not stimulate haemoglobin synthesis. The mean blood volume was 56.7 ± 1.45 fL in control rats and 61.7 ± 1.45 fL in rats treated with *Daniellia oliveri* (Rolfe) Hutch. & Dalziel. The mean blood cell volume did not vary significantly between the control and the treated rats, meaning that the extract did not induce the release of immature red blood cells into the bloodstream. The mean blood platelet count was 603 ± 26.2 G / l in control rats and 545 ± 19.1 G / l in rats treated with *Daniellia oliveri* (Rolfe) Hutch. & Dalziel. The mean blood platelet count did not vary significantly between control and treated rats, suggesting that the extract did not stimulate blood platelet production.

Phytochemicals in the bark of *Daniellia oliveri:* Table 2 showed the phytochemical screening of the bark of Daniellia oliveri. The screening revealed the presence of flavonoids, catechic tannins, leucoanthocyanins, anthocyanins and reducing compounds in the bark of *Daniellia oliveri*. It also denoted the absence of gallic tannins, saponins, alkaloids, and mucilage.

Table 1: Change in rats Haemoglobin level, Mean corpuscular volume and blood platelet count.

Rats parameters	Day 0	Day 28	P value
Haemoglobin level (g/dl)	14.5 ± 0.291	14.6 ± 0.318	0.8
Mean corpuscular volume (fl)	56,7 ± 1,45	61,7 ± 1,45	0.2
Mean blood platelet count (G/l)	603 ± 26,2	545 ± 19,1	0.2

Table 2: Phytochemical screening of *Daniellia oliveri*

Compounds	*Daniellia oliveri* (Rolfe) Hutch. & Dalziel
Flavonoids	+
Catechetical tannins	+
Gallic tannins	-
Leucoanthocyanins	+
Anthocyanins	+
Saponosides	-
Reducing compounds	+
Mucilages	-
Alkaloids	-

DISCUSSION

Sickle cell disease was a genetic disease in which the prevalence of major forms is very high. This constitutes a real public health problem. This work was part of a search for remedies that can relieve victims of this pathology. Beninese traditional therapists used *Daniellia oliveri* to relieve sickle cell crises. Thus, this work tested the effectiveness of the aqueous extract of the bark of this plant in preventing or correcting sickling of SS red blood cells, responsible for sickle cell crises. At a concentration of 40 mg / ml, the aqueous extract of *Daniellia oliveri* significantly inhibited the sickling of SS erythrocytes under conditions of hypoxia. It therefore prevented the polymerization of haemoglobin S and

therefore the sickling of red blood cells, responsible for sickle cell crises (Ngbolua *et al.,* 2013). This polymerization of haemoglobin into a tactoid led to reduced glycolytic and ionic flow and cell dehydration. The formation of these large polymer fibers led to a cascade of other cellular abnormalities that participate in the pathophysiological mechanism (Mpiana *et al.,* 2007; Elion *et al.,* 2010). At this same concentration, in addition to the preventive effect, the extract significantly induced the conversion of sickle cells into normal biconcave red blood cells. The aqueous extract of *Daniellia oliveri* could therefore depolymerize haemoglobins S in hypoxic conditions in order to restore them to their normal form in erythrocytes. This suggested that the extract has a curative power and therefore justified the use of this plant in the treatment of sickle cell crises by traditional Beninese therapists. This property was also mentioned in certain plants in Congo, which inverted sickle cells into normal biconcave erythrocytes (Kambale *et al.,* 2013; Ngbolua *et al.,* 2013). Sall (2016) found the same properties with *Maytenus senegalensis*, which is a plant from the Senegalese pharmacopoeia. To better understand the mechanism of action of the extract, we evaluated its effect on the production of methaemoglobin inside sickle cells. The aqueous extract of *Daniellia oliveri* lowered the production of methaemoglobin in SS red blood cells compared to untreated SS control red blood cells. Haemoglobin S was very unstable and oxidizes faster to methaemoglobin. This was reflected either by an increase over time in the level of methaemoglobin in solution or by a decrease in the level of haemoglobin in aqueous solution (Kambale *et al.,* 2013). Therefore, the plant therefore had an antioxidant effect, which would prevent intra-erythrocytic oxidation in SS red blood cells. The action of this plant could therefore be linked to its reduction in oxidative stress *in vitro*. This result was similar to that obtained by kambale (2013) who showed that *Uapaca heudelotii* Baill would prevent the oxidation of haemoglobin in vitro. To verify the action of the extract on the stimulation of haematopoiesis to compensate for sickle cell anaemia, it was tested in vivo by chronic gavage in Wistar rats. *Daniellia oliveri* extract did not significantly increase haemoglobin level, Mean Corpuscular Volume and blood platelet count in rats, specifying that its action did not involve stimulation of erythropoiesis or thrombopoiesis. It could not therefore be considered to compensate for sickle cell anaemia, at least under our experimental conditions. This explained why its use in traditional medicine was coupled with anti-anaemic plants such as *Cocos nucifera* L. (Arecaceae) and *Psorospermum febrifugum* whose roots aqueous extracts compensated for anaemia (Tchogou *et al.,* In 2016; Agbogba *et al.,* 2019). The phytochemical screening of the bark of *Daniellia oliveri* revealed the presence of several secondary metabolites including anthocyanins. This family of chemical compounds was believed to be involved in inhibiting the polymerization of haemoglobin S, thus preventing the sickling of erythrocytes. It would also act by stabilizing the erythrocyte membrane through its antioxidant properties (Mpiana *et al.,* 2008; Mpiana *et al.,* 2010; Ngbolua, 2012). The biological effects observed in the extract of the bark of *Daniellia oliveri* could be linked to anthocyanins, whether or not associated with other families of bioactive compounds.

CONCLUSION

Daniellia oliveri extract prevented and therefore reversed the sickling of red blood cells of SS phenotypes. It minimized intraerythrocytic oxidative stress without stimulating haematopoiesis. The action of *Daniellia oliveri* was believed to be due to the

abundant secondary metabolites it contained, including anthocyanins. It could therefore be considered in the preventive or curative treatment of sickle cell crises, after toxicity tests.

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