



Assessment of Radical Scavenging and Sickling Inhibitory Activities of the Bark of *Disthemonanthus Benthamianus* (Caesalpiniaceae)

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Abstract

Among the medicinal plants used in the treatment of sickle cell disease in south-east of Côte d'Ivoire is *Disthemonanthus benthamianus*. This paper focuses on assessing its antioxidant properties and its antisickling activity. The

test of inhibition of sodium metabisulfite-induced sickling of the erythrocytes was carried out based on this evaluation. The total phenolic compounds contents were 45 ± 0.64 and 39.84 ± 0.69 mg/g of dry weight for ethanolic extract and the decocted respectively. The DPPH reduction test showed that the decocted ($9 \mu\text{g/mL}$) had a lower IC_{50} than the ethanolic extract ($16 \mu\text{g/mL}$). There was no significant difference between the sickling inhibitory activities of the ethanolic extract (87.00%) and the decocted (81.66%). This study showed that both extracts had sickling inhibitory and antioxidant activities.

Keywords: *Disthemonanthus benthamianus*, phenolic compounds, antioxidant activity, antisickling activity, Côte d'Ivoire

Introduction

The use of medicinal plants in therapeutics throughout the world is currently experiencing an interest among the population despite the progress of modern medicine. More than 80% of the world population use medicinal plants to cope with health problems (WHO, 2008). One of the reasons is that many diseases are treated successfully by plants (Kamanyi *et al.*, 1995). Indeed, plants are source of natural bioactive molecules with anti-inflammatory (Gonzalez *et al.*, 2011), antimicrobial (Daglia, 2012), antiviral (Chavez *et al.*, 2006), antisickling (Sawadogo *et al.*, 2017; Kitadi *et al.*, 2015; Akakpo *et al.*, 2018), and antioxidant (Pandey & Rizvi, 2009; Diomandé *et al.*, 2018) properties. The cost of patient care is not always affordable for low-income populations. Therefore, many Ivorian people use medicinal plants to treat several ailments even chronic diseases such as sickle cell anemia (Béné *et al.*, 2016). Sickle cell disease or sickle cell anemia is a genetic disorder with autosomal recessive transmission. It results from the substitution of glutamic acid in position 6 by valine, affecting the 6th codon of the β -globin chain. In deoxygenated red blood cells, hydrophobic valine, unlike glutamic acid, causes a change in hemoglobin conformation that leads to its polymerization and to the sickle-formation (Chiabi & Haenggeli, 2004). The polymerized hemoglobin could undergo self-oxidizing, thereby inducing the formation of methemoglobin by the oxidation of Fe^{2+} to Fe^{3+} . The auto-oxidation of hemoglobin generates free radicals (Hebbel *et al.*, 1988). Sickle cells and oxidative stress are important characteristics of the disease and play a major role in the process of hemolytic anemia, vaso-occlusion, and organs damage which are the major clinical signs of sickle cell patients (Nur *et al.*, 2011). Given the diversity and severity of diseases induced by oxidative stress, various research teams have investigated new antioxidants compounds. In view of the oxidative stress presence in sickle cell disease, new plant sources of natural antioxidants are now being sought (Arruda *et al.*, 2013). In this work, we reported the results of the assessment of the *in vitro* antioxidant and

sickling inhibitory activities of the decocted and ethanolic extract of *Disthemonanthus benthamianus* bark.

Materials and Methods

Harvesting and Conditioning Plant Material

The plant material consisted of *Disthemonanthus benthamianus* stem bark. It was harvested from Indenié-Djouablin (6° 43' 47" North and 3° 29' 47" West), a region in eastern Côte d'Ivoire. The plant identification has been done at the National Floristic Center with the specimen number of 12473. The stem bark has been washed, cut, and then air dried at room temperature (25°C) in Biology and Health laboratory at the biosciences UFR of Félix Houphouët-Boigny University. After three weeks of drying, then the bark was grounded into powder using a Severin® brand grinder.

Disthemonanthus Benthamianus Stem Bark Extraction

Ethanolic Extract (EDB) Preparation: Hydroethanolic extract has been produced according to the protocol of (Zirihi et al., 2003). One hundred grams (100g) of the ground plant were soaked in one liter of hydroethanolic 70% solution. The mixture was homogenized 10 times for 2 minutes each time using a Severin® brand blender. The obtained homogenate was wrung out using a square of white cotton fabric and then filtered successively three times on hydrophilic cotton and once on Whatman paper (3 mm). The filtrate was evaporated at 45°C using a Venticell® oven. The resulting extract was named EDB.

Plant Decoction (DDB): According to the method of Konkon *et al.* (2008), one hundred grams (100g) of ground plant were brought to a boil for 20 minutes in 2L of distilled water, the mixture was cooled at room temperature (25°C), filtered three times on cotton, and once on Whatman filter paper 3. The resulting filtrate was then dried at 50°C in the oven. The dried powder was named DDB.

Antioxidant Properties Evaluation

Total Polyphenols and flavonoids phytochemical screening were carried out in pharmacognosy laboratory at Pharmaceutical and Biological Sciences UFR of Félix Houphouët-Boigny University.

Determination of Total Phenol Content

The total phenolic compounds content has been determined using Folin–Ciocalteu assay method (Singleton *et al.*, 1999). One (1) mL of Folin–Ciocalteu reagent was added to the extract, 1 mg/mL in a test tube. After 3 minutes, 1 mL of 20% sodium carbonate solution (w/v) was added to the test tube and completed to 10 mL with distilled water. After 30 minutes, the

absorbance was read at 745 nm against a methanol blank on a spectrophotometer Jenway 7315. A standard range, based on 0.1 mg/mL of Gallic acid stock solution, allowed the determination of phenols concentration in the sample. The result was expressed in mg GAE/g.

Determination of Flavonoids Content

The total flavonoid content was determined from the calibration curve made of 0.1 mg/mL stock solution of Quercetin, using direct quantification by aluminum chloride method (Meda *et al.*, 2005). Zero point 5 (0.5) mL of distilled water, 0.5 mL of aluminum chloride, 0.5 mL of potassium acetate, and 2 mL of distilled water were added to 0.5 mL of EDB or DDB. After 30 minutes, the absorbance was read at 415 nm against methanol as blank. A standard range established from a 0.1 mg/mL Quercetin stock solution was used to determine the amount of flavonoids in the EDB or DDB. The result was expressed in mg QE/g:

Free Radical Scavenging Assay

DPPH (2, 2 diphenyl-1-picrylhydrazyl) is generally the most widely used substrate for rapid and direct assessment of antioxidant activity due to its stability in free radical form and simplicity of analysis. It absorbs in the visible at the wavelength of 517 nm. The experimental protocol used to study DPPH free radical scavenging activity was that described by Parejo *et al.* (2000) with some slight modifications.

Reducing Power Assay

The reducing power of plant extracts was carried out as described by Yildirim *et al.* (2001). It was measured by the increase in the absorbance at 700 nm of the Perl's Prussian blue due to the Fe^{3+} Fe^{2+} transformation.

Qualitative Research of Aromatic Amino Acids

Aromatic amino acids were sought through the xanthoprotein reaction. To 3 mL of extract, 1 mL of concentrated nitric acid was added. The tube was then boiled for 2 minutes. The appearance of a yellow coloration indicates a positive reaction which highlights the presence of aromatic nuclei (benzene ring); amino acids of the aromatic series: tyrosine, tryptophan, and phenylalanine. The test was performed according to Fofana (2004).

Sickling Inhibitory Activity Assessment

Collection and Conditioning of Blood Samples

An agreement was obtained from the ethics committee and an informed consent was approved by each voluntary sickle cell patients selected at Yopougon University Hospital in the Clinical Hematology Department. To

be included in the study, the blood should come from homozygous sickle cell patients. The voluntary patients shouldn't have undergone blood transfusion for at least two months prior to the blood test, regardless of age and gender.

Venous blood was collected in tube (EDTA). These samples were placed in a cooler and conveyed at 4 °C to Biology of Immunity Pole of Pasteur Institute of Côte d'Ivoire (IPCI).

Patients who had been transfused before the two months prior to blood collection, homozygous patients with foetal hemoglobin levels greater than 5g/dL, and patients in crisis were not considered.

Sickle-Formation Inhibition Test

The test was performed according to Emmel's method (1933) which was slightly modified (Mpiana et al., 2014). The blood sample was centrifuged for 5 minutes at 3000 rounds/min and the supernatant was removed with a Pasteur pipette. One (1) mL of the washed red blood cells was suspended in 1mL of physiological buffer (NaCl 0.9%). EDB and DDB solutions of 5 and 10 mg/mL were prepared with NaCl 0.9%. A volume of 50 µL was homogenized with 50 µL of washed blood in a test tube. A volume of 50 µL of sodium meta-bisulfite (2%, w/v) was added to the mixture to create the sickle-formation conditions. The tube was sealed with paraffin. Negative and positive controls were prepared. The negative control was prepared by mixing 50 µL of washed blood with 50 µL of physiological buffer and 50 µL of sodium meta-bisulfite (2%, w/v). The positive control was prepared by mixing 50 µL of washed blood with 50 µL of a phenylalanine solution at 10mg/mL and 50 µL of sodium meta-bisulfite (2%, w/v). The tubes were put in dark room for 120 minutes. Erythrocytes morphological analysis and counting of sickle cells were made by an observation X 40 under a microscope. The sickling Inhibitory activity was expressed in percentage of sickle cells formed in the presence of the plant extracts compared to the number of sickle cells present in the negative control. This activity is determined by the formula noted below:

$$AA = \frac{Po - P1}{Po} \times 100$$

AA: antisickling activity; Po: sickle cells rate in the control; P1: sickle cells rate in plant extract's presence. The experimental results were expressed as means ± standard error of means (SEM) of three replicates. Where applicable, the data were subjected to one way analysis of variance (ANOVA) and differences between samples were determined by Tukey and Duncan's Multiple Comparison test using Graph Pad Prism 7.0 program.

Results

Total Phenolic and Flavonoids Compounds Contents Determination

The quantitative analysis of EDB and DDB phenolic compounds was determined from the Gallic acid calibration curve, equation $Y = 8.1544X$ and $R^2 = 0.9732$. The total flavonoids were determined from the Quercetin calibration curves, equation $Y = 1.36x - 0.0181$ and $R^2 = 0.9976$. The polyphenol concentration of EDB (45.11 ± 0.64 mg GAE/g of dry weight) was significantly ($p < 0.05$) higher than that of DDB (39.54 ± 0.69 mg GAE /g of dry weight). The total flavonoids content in EDB and DDB follow the same trend as the polyphenols concentration. The content in EDB (15.52 ± 0.25 mg QE/g of dry weight) is higher than that of DDB (15.64 ± 1.44 mg QE/g of dry weight). The results were reported in Table I.

DPPH Radical Scavenging Activity

The results obtained during the determination of the different extracts by the DPPH radical were shown in Table I. The IC_{50} values obtained were expressed in $\mu\text{g/mL}$: 16 ± 0.01 and 9 ± 0.04 for EDB and DDB respectively. Ascorbic acid (6.05 ± 0.39 $\mu\text{g/mL}$) was used as the reference molecule in Figure 1.

Table I. Phenolic contents and scavenging activity of the aqueous and ethanolic extracts of *D. benthamianus*

Parameters Plants Extract	Polyphenols total (mg GAE/g)	Flavonoids (mg QE/g)	IC_{50} ($\mu\text{g/mL}$ of extract)	%Inhibition At 0.5 mg/mL
EDB	45.11 ± 0.64^a	15.52 ± 0.06^a	16 ± 0.01^b	80.85 ± 0.15
DDB	39.84 ± 0.18^a	15.64 ± 0.03^a	9 ± 0.04^a	85.29 ± 0.15
Ascorbic acid			6.05 ± 0.02	66.98 ± 0.06

EDB/DDB: Hydroethanolic extract / Decocted of *Disthemonanthus benthamianus* Baill

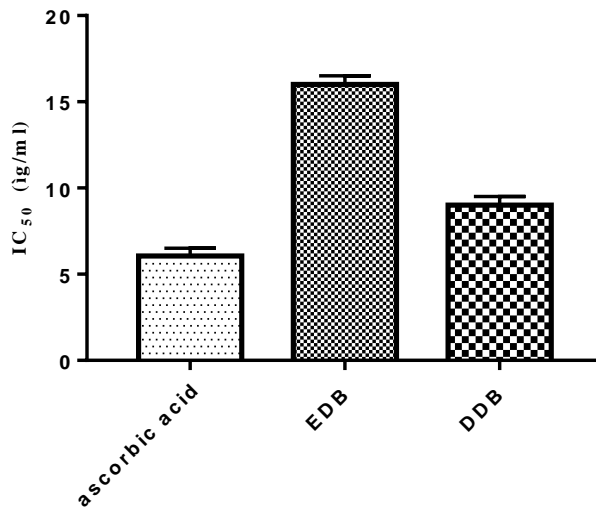


Figure 1: IC₅₀ of ascorbic acid, DDB and EDB on DPPH discoloration.

Reducing Power

The results in the form of activity curves show that all the curves have increasing slope. The reducing power of the plant extracts, as indicated by the absorbance at 700 nm, increased with increasing concentration of the samples (Figure 2). The reducing power of the plant extract versus plant extract concentration have a dose response relation. DDB shows higher reducing ability than EDB.

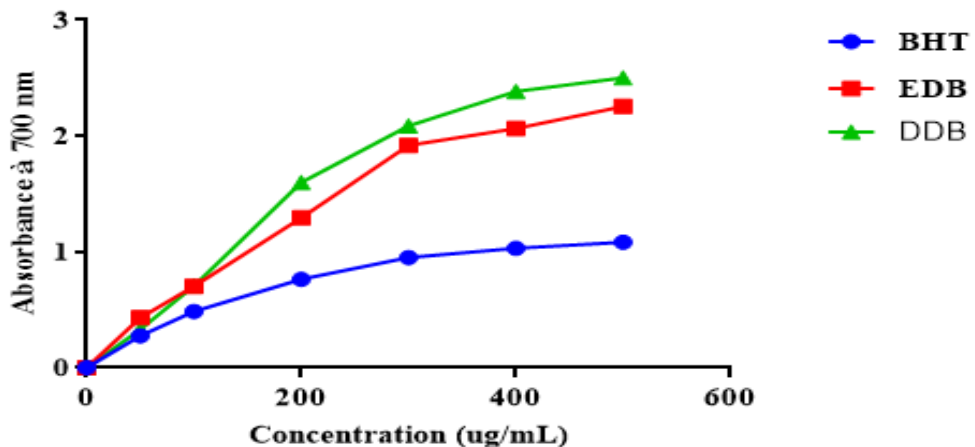


Figure 2. Reducing power of EDB, DDB and Butylated Hydroxytoluene

Aromatic Amino Acids Qualitative Research

The test carried out on EDB and DDB allowed detecting the presence of aromatic amino acids in both extracts. The amino acids present were characterized by the yellow coloration of the reaction (Table II).

Table II. Aromatic amino-acids presence

Extract	Aromatic amino acids
DDB	+
EDB	+

Sickling Inhibitory Activity

Figure 3 showed the microphotography of SS blood incubated in a tube containing 0.9% NaCl and sodium meta-bisulfite (2%, p/v) without plant extract. In this figure, all the erythrocytes had a sickle shape two hours later. Figures 4 to 7 showed SS blood incubated in 0.9% NaCl with sodium meta-bisulfite (2%, p/v) and plant extracts at 5 and 10 mg/mL. EDB and DDB have displayed an ameliorating sickling effect. The sickle cell inhibition activity was determined and recorded in Table III; EDB (87 % ± 1.33) had a higher inhibitory activity than DDB (81.66 % ± 1.12) at 5mg/mL.

Table III. DDB and EDB sickling inhibitory activity

Extracts	Concentration:	Concentration:
	5 mg/mL	10 mg/mL
EDB	87 %	73.39%
DDB	81.66%	72%
Phenylalanine	67.33 %	83.65 %



Figure 3. Morphology of sickle cell in the presence of Na₂S₂O₄ 2%

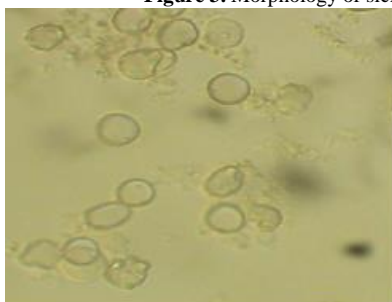


Figure 4 . Morphology of Sickle Cell Blood treated with 10 mg / mL of DDB

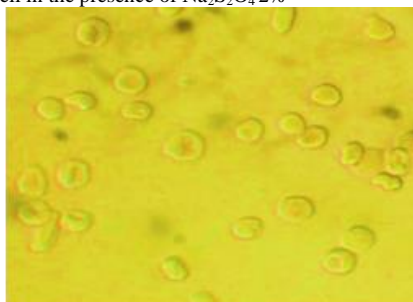


Figure 5 . Morphology of Sickle Cell Blood treated With 10 mg / mL of EDB

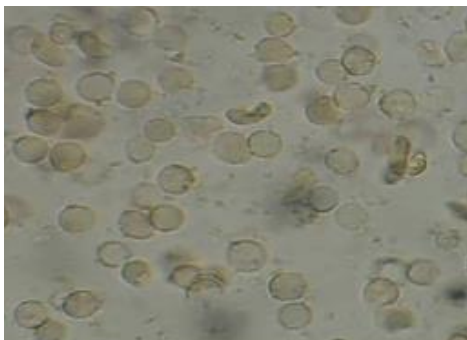


Figure 6 . Morphology of Sickle Cell Blood treated with 5 mg / mL of DDB

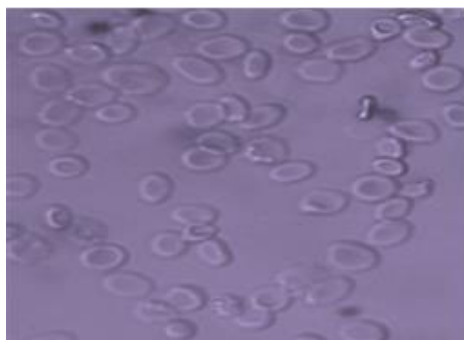


Figure 7 . Morphology of Sickle Cell Blood treated with 5 mg / mL of EDB

Discussion

The results of the total phenol assay were recorded in Table 1. The polyphenol compounds contents in EDB and DDB were 45 ± 0.01 and 39.85 ± 0.18 mg GAE /g of dry weight, respectively. Also, the total flavonoid contents were 15.88 ± 0.06 and 12.94 ± 0.03 mg GAE /g of dry weight, respectively. There was no significant difference between total EDB and DDB polyphenol levels. In addition, numerous other phytochemical examinations revealed the presence of tannins, flavonoids, alkaloids, steroids, and triterpenes in both extracts (Adebisi *et al.*, 2009; Bidié *et al.*, 2011; Kehindé *et al.*, 2012). The above-mentioned molecules could have some therapeutic effects in the treatment of diseases such as sickle cell (Longanga *et al.*, 2000). The presence of secondary metabolites in EDB and DDB could explain their antioxidant activity. Indeed, the results of the antioxidant activity test showed that both extracts were active. The DPPH radical scavenging activity of EDB and DDB was indicated by their IC_{50} value in Table I and Figure 1. EDB and DDB IC_{50} were 16 ± 0.01 and 9 ± 0.04 $\mu\text{g/mL}$ respectively. These two extracts had an antioxidant activity compared to ascorbic acid (6.05 ± 0.02 $\mu\text{g/mL}$). Kouassi *et al.* (2013) study showed that the IC_{50} of the ethanolic and aqueous extracts were 10.87 ± 0.18 $\mu\text{g/mL}$ and 551.7 ± 1.76 $\mu\text{g/mL}$, respectively. These authors worked on the leaves of *D. benthamianus*, while the bark was used in this present study. The antioxidant activities of the plant would be related to their total phenolic compounds content. Indeed, functional groups in phenolic chemicals could produce an electron or proton to neutralize free radicals (Chen & Ho, 1995).

According to Diatta *et al.* (2014), during oxidative stress in patients with sickle cell disease, lipid peroxidation products could produce oxidizing agents. These oxidative mediators could be responsible for the erythrocyte membrane structure damage. In a state of hypoxia, the oxidative stress occurring during sickle cell disease could increase methemoglobin level in red blood cells and weakened free radicals defense system (Nur *et al.*, 2011). Also, this increased methemoglobin level would decrease hemoglobin oxygen

affinity. The oxidized state of iron (Fe^{3+}) cannot bind the oxygen molecule (Sawadogo *et al.*, 2017; Kitadi *et al.*, 2015). The reducing power could allow EDB and DDB to change the iron from Fe^{3+} to Fe^{2+} . This reduced state could increase haemoglobin oxygen affinity. Reducing power is an important antioxidant action of phenolic compounds (Nabavi *et al.*, 2009a). EDB and DDB reducing power increased with increasing concentration (Figure 2). The reducing power test results showed that there was no significant difference between EDB (2.25 ± 0.33) and DDB (2.50 ± 0.38). This activity could be due to the presence of polyphenols compounds found in *D. benthamianus* extracts. Moreover, the work of Evenamede *et al.* (2017) highlighted a correlation between total phenol content and antiradical activity.

This antioxidant activity of *D. benthamianus* could be beneficial for the erythrocyte membrane. Indeed, the antioxidant activity of polyphenols is often exploited to prevent and treat diseases related to oxidative stress such as sickle cell disease. In fact, flavonoids (myricetin, quercetin, and rutin) are involved in inhibiting the damaging effects of reactive oxygen species produced during sickle cell disease (Railson *et al.*, 2013). Some studies have shown that tannins, saponins, flavonoids, sterols, and ployterpenes have shown anti-inflammatory, antibacterial, antiviral, and anti-pain activities (Zouhri *et al.*, 2016; Adeniyi *et al.*, 2011; Bajerova *et al.*, 2014; Gédéon *et al.*, 2017; Akakpo-Akue *et al.*, 2018). Indeed, these properties could neutralize free radicals by exchanging electrons or protons (Chen & Ho, 1995). Polyphenols could block the free radical reaction chain by hydrogen transferring (Meir *et al.*, 1995). They could also chelate metal ions (Ibrahim *et al.*, 2017).

These current results also show the presence of aromatic amino acids EDB and DDB through the displayed yellow color. Many studies have shown that certain amino acids would stimulate the production of glutathione, significantly reduce the formation of sickle cell, and have a reversal effect on sickle cell shape (Gibson *et al.*, 1998; N'Draman-Donou *et al.*, 2015; Nur *et al.*, 2012). The literature has shown that aromatic amino acids such as phenylalanine prevent polymerization of deoxygenated hemoglobin (Ogoda *et al.*, 2002). The presence of these amino acids in our plant extracts could participate in the fight against sickle cell disease by having a polymerization inhibition activity (Tharoux, 2008).

The chemicals exposed in EDB and DDB could also be responsible for their sickling inhibitory activity. Indeed, morphological analysis of red blood cells incubated in 0.9% NaCl and sodium meta-bisulfite (2%, w/v), without plant extracts showed two hours later in Figure 3, that all red blood cells had a sickle shape. The hypoxic environment created by the sodium meta-bisulfite (2%, w/v) led to the formation of the sickle cells. This proved that the blood cells had the HbSS genotype and came from a homozygous sickle cell patient.

In the presence of plant extracts, red blood cells treated with DDB showed in Figure 6 (5mg/mL) and 4 (10mg/mL) that all red blood cells have kept a rounded shape. Also, the erythrocytes treated with EDB had rounded and biconcave shape. Two hours after counting the sickle cells, the percentage of residual sickle cells compared to the negative control was determined. And the sickling inhibitory activity of EDB and DDB at 5 and 10 mg/mL was calculated in Table III. The result of the sickling inhibitory activity test showed that there was a dose-response relationship between plant extract concentration and inhibitory activity. Lower the concentration, better the activity. The sickling inhibitory activity of EDB at 5 and 10 mg/mL was 87% and 73.39% respectively. The same trend was observed for DDB. At 5 and 10 mg/mL, DDB extract had sickling inhibitory activity of 81.66% and 72% respectively. At 5 mg/mL, the activities were superior to those of phenylalanine which was 83%. At 10 mg/mL, EDB (73.39%) and DDB (72%) activities were lower than phenylalanine (83%). Higher concentrations of EDB and DDB may have reduced sickling inhibitory activity or a toxic effect on red blood cell HbSS. However, there was no significant difference between the sickling inhibitory activities of the ethanolic extract (87.00%) and the decocted (82.36%) at 5mg/mL.

The experimental results obtained during this *in vitro* study showed that both EDB and DDB extracts had an antifouling activity. This property could be due to the presence of polyphenols compounds in EDB and DDB.

Conclusion

The results of this study indicate that both the two *Disthemonanthus benthamianus* extracts are rich in phenolic compounds and flavonoids that have the ability to trap free radicals and reduce oxidants effect. The sickling inhibitory activity displayed by *Disthemonanthus benthamianus* extracts were correlated to phenolic compounds. These chemicals could be responsible for free radical scavenging and sickling inhibitory activity of *D. benthamianus*. Thus, these activities helps to explain the use of *Disthemonanthus benthamianus* to the traditional healers.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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