JOURNAL OF ADVANCEMENT IN
MEDICAL AND LIFE SCIENCES

Journal homepage: http://scienceq.org/Journals/JALS.php

Research Article Open Access

Anti-sickling and antioxidant activities of anthocyanins extracts from Dissotis brazzae Cogn (Melastomataceae)

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Received: September 29, 2015, Accepted: November 3, 2015, Published: November 3, 2015.

ABSTRACT

Sickle cell disease is among the widespread blood disorder affecting mainly Sub-Saharan African population. Some plants are used in traditional medicine to manage this chronic and hereditary disease. *Dissotis brazzae* Cogn, a medicinal herb used in Congolese traditional medicine against sickle cell disease, was tested *in vitro* for its antisickling and antioxidant activities using the Emmel and DPPH radical tests. The results show high antisickling activity of leaves aqueous extract of this plant with a minimal normalization concentration of 11µg/mL and a maximal normalization rate of about 86%. Anthocyanins could be among active chemical groups. Indeed, anthocyanins extract from *Dissotis brazzae* leaves displayed a significant antisickling activity. Furthermore, the presence of anthocyanins decreases the Fe^{3+}/Fe^{2+} ratio in sickle red blood cells and shows an interesting radical scavenging activities with an IC50 of 43µg/mL. The antisickling activity combined to the antioxidant activity of *Dissotis brazzae* can justify the use of this plant in the management of sickle cell disease in Congolese traditional medicine.

Keyword: *Sickle cell disease, Dissotis brazzae, anti-sickling activity, antioxidant activity, normalization rate, Emmel test*

INTRODUCTION:

Sickle cell anemia or Sickle cell disease (SCD) is a genetic blood disorder found mainly in tropical regions. This neglected and painful disease is due to a genetic mutation conducting to the substitution of a polar amino acid (glutamic acid) by a no polar one (valine) on β^6 position of hemoglobin chains [1,2]. At low oxygen tension or hypoxic condition, the mutant hemoglobin polymerizes inside the red blood cells (RBCs) into a gel or further into fibers leading to a drastic decrease in the red cell deformability. Polymerization and precipitation of sickle hemoglobin (HbS) within the erythrocytes cause the change of shape from the normal biconcave form into the one resembling a sickle [2-5]

SCD promotes harmful pathological effects that include vaso-occlusion and ischemia-reperfusion injury. The distortion of the shape of RBCs has also a critical role in perturbing the structure and function of the membrane of RBCs, mediated in part by oxidant stress. In fact, there is increasing evidence points towards an oxidative stress response responsible for increased pathophysiology of secondary dysfunctions in sickle cell patients. In hemoglobin preparations obtained from erythrocytes of SCD patients, autoxidation of oxygenated hemoglobin S is 1.7 times faster than oxygenated hemoglobin A (HbA) and sickle red cells have been reported to generate about two fold greater extent of superoxide, hydrogen peroxide, hydroxyl radical and lipid oxidation products compared with HbA containing red cells . The Fe^{3+}/Fe^{2+} (methemoglobin/hemoglobin) ratio is higher in sickle cell blood than in normal blood. Furthermore, several reports have indicated lower levels of carotenoids, flavonoids, vitamins C and E and zinc (structural component of superoxide dismutase) in sickle cell anemia patients [2,6-8].

Gene therapy and bone marrow transplantation have been proposed as efficient way of treating sickle cell disease but the cost implications, the availability of expertise and other related problems constitute a major setback to this approach in Africa. A variety of antisickling agents acting at different levels of the sickling mechanism have also been proposed including hydroxyurea. But cost and side effects of these drugs limit their clinical use [2-5, 9-11].

Despite all the progress in synthetic chemistry and biotechnology, plants are still an indispensable source of medicinal preparations. According to the World Health Organization (WHO), 80% of the world's population use medicinal plants for some aspects of primary healthcare [9-12]. Indeed, natural substances are a source of potential new types of drugs that can be used against several diseases in general and sickle cell anemia in particular. Neuwinger [13] listed a number of plants used in African traditional medicine against SCD.

In Democratic Republic of the Congo (DRC), surveys conducted by our research team around big cities revealed more than 115 medicinal plants used in traditional medicine in the management of SCD [10-12,14-27]. Some of them showed not only the anti-sickling activity *in vitro* but also antioxidant activity and, these activities were due mainly to anthocyanins and organic acids and their derivatives [14-17].

Dissotis brazzae Cogn called « mampa ya mfinda » (kikongo, DRC) is a medicinal plant of *Melastomataceae* family used in the western part of DRC mainly in Kikwit city to manage SCD.
The aim of this study is to test the antisickling activity of this plant *in vitro*, to extract anthocyanins and verify if they are, like in other medicinal plants used in the management of SCD in DRC, responsible of this activity. And, to determine their antioxidant activity.

MATERIALS AND METHODS

Plant material

The leaves of *Dissotis brazzae* Cogn were collected in the vicinity of the Kikwit city between February and June 2013. The plant identification was carried out by botanists of "Institut National d'Etudes et des Recherches Agronomiques (INERA)". Voucher specimen has been deposited at the Herbarium of INERA, situated at the department of Biology, Faculty of Science, University of Kinshasa in DRC

Anthocyanins extraction

Extraction of anthocyanins was done by maceration of 100 g of dried powdered plant material with acidified methanol (1% HCl) following an established protocol as previously reported [15-19, 22-25].

Biological material

Blood samples used to evaluate the antisickling activity of the plant extracts were taken from known sickle cell anemia patients attending the "Centre de Médecine Mixte et d'Anémie *SS*" located in Kinshasa, DRC. None of the patients had been transfused recently with Hb AA blood. All antisickling experiments were carried out with freshly collected blood. In order to confirm their sickle cells nature, the above-mentioned blood samples were first characterized by hemoglobin electrophoresis on cellulose acetate gel, and then stored at ± 4°C in a refrigerator.

Bioactivity evaluation

Emmel test

Blood sample was put in contact with plant extracts at different concentrations (with the physiologic solution as the dilution solvent) according to Emmel's test procedure as previously reported [15-19, 22-25]. The red blood cells images were treated with a computer assisted image analysis system (Motic Images 2000, version 1.3; Motic Chine Group Co LTD) and statistical data analysis and curves were processed using Microcal Origin 8.6 package software.

Determination of Fe^{3+} and Fe^{2+} profile

The Fe^{2+} and Fe^{3+} profile was determined according to the method of Mpiana *et al.* [11,17]. This profile was followed using UV-Vis spectroscopic method at 540 nm (for Hb or Fe^{2+}) and 630 nm (for metHb or Fe^{3+}) with time. Whole blood 0.02 mL was diluted in 5 mL of distilled water in which 0.02 mL of normal saline solution (NaCl 0.09%) or 0.02 mL of anthocyanin extracts were added. Solutions were incubated for 60 minutes.

Antioxidant Activity

The 1, 1-diphenyl-2- picrylhydrazyl (DPPH) free radical scavenging assay was performed as previously reported [21-24, 28]. Briefly, a 100 µM solution of DPPH radical in methanol was prepared. 3.5 mL of this solution was added to 0.5 mL solution of each extract in methanol at concentrations ranging from 0.1 to 1.0 mg/mL, thus obtaining the desired final concentrations in the reaction mixture. The mixture was shaken vigorously and the absorbances at 517 nm were recorded during 35 minutes considered as equilibrium time, using UV-vis 320/safas monaco Spectrophotometer, in order to determine the DPPH radical scavenging of reactions and to calculate parameters of antioxidant activity for ascorbic acid and extracts (Percent of reduction, the IC50 index and effectiveness antioxidant 1/IC50)[24,28].
Percentage of reduction was calculated according to the following equation:

$$I\% = \frac{A_{blanck} - A_{sample}}{A_{blanck}} x100$$

where: A_{blank}= Absorbance of blank (containing all reagents except the tested compound) and
A_{sample}= Absorbance of the tested compound.

RESULTS AND DISCUSSION

Anti-sickling activity of aqueous extract

Figures 1, 2 and 3 show respectively microscopic images of sickle cell blood alone in a NaCl 0.9% solution (negative control), in presence of betulinic acid (positive control) and in presence of aqueous extract of *Dissotis brazzae* Cogn leaves.

Figure 1: Morphology of erythrocytes of untreated sickle RBCs (negative control) (X500), [NaCl 0.9% ; $Na_2S_2O_4$ 2%].

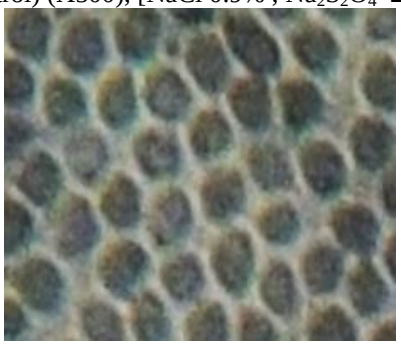

Figure 2: Morphology of erythrocytes of sickle RBCs in presence of betulinic acid (20 µg/mL) (positive control) (X500), [NaCl 0.9% ; $Na_2S_2O_4$ 2%].

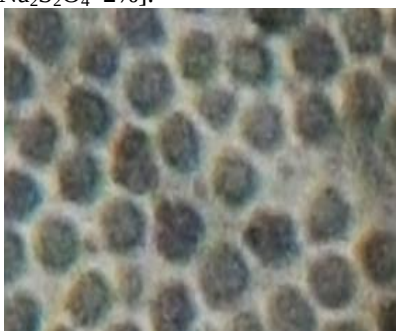

Figure 3: Morphology of erythrocytes of sickle RBCs in presence of aqueous extract of D. brazzae leaves (20µ/mL) (X500), [NaCl 0.9% ; Na2S2O4 2%].

Figure 1 showed that the majority of red blood cells are elongated (sickled), this confirms that the used blood is for sickle cell disease patient. Figure 2 shows that in presence of betulinic acid as positive control, in the same hypoxic conditions, RBCs have circular (biconcave) and normal shape. Figure 3 reveals that the form of RBCs are like that obtained in presence of positive control (betulinic acid) indicating the antisickling effect of aqueous extract of *D. brazzae* leaves. This *in vitro* activity confirms the use of aqueous decoction of this plant as medicinal herb in the management of sickle cell disease in Kikwit city.

In order to quantify this shape modification, perimeter, surface and radius of untreated and treated sickle RBCs with aqueous extract of *D. brazzae* leaves were calculated (Tab.1).

Table 1: Perimeter, surface and radius of untreated and treated sickle RBCs with aqueous extract of *D. brazzae* leaves

Samples	**Cellular Perimeter (µm)**	**Cellular Surface (μm^2)**	**Cellular Radius (µm)**
Negative control	25.0±1.1	33.4±2,6	-
Positive control	18.7±1.8	23.6±2.3	3.5±0.3
Extract of *D. brazzae*	19.6±2.0	25.4±2.1	3.6±0.5

Tabulated values (means ± S.D) show a significant difference between negative control values and that of *D. brazzae* extract ($p<0.05$) but not a significant difference between these latter with that of the positive control. This confirms the antisickling activity of *D. brazzae* aqueous leaves extracts.

The same activity was already observed for many others plants used in Congolese traditional medicines in the management of sickle disease [15-19, 22-25]. In order to compare the activity of *D. brazzae* to that of other plants, it is necessary to calculate the minimal concentration of normalization (MCN) i.e. the lowest concentration that achieves the maximum normalization rate.

Determination of minimal concentration of normalization

Figure 4 gives the evolution of the normalization rate of sickle cells form with the concentration of *Dissotis brazzae* leaves aqueous extracts.

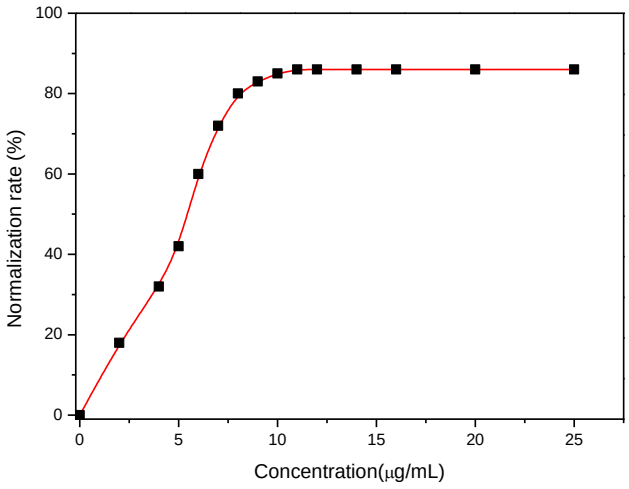

Figure 4: Evolution of normalization rate of sickle RBCs with D. brazzae leaves aqueous extracts

This curve shows that the normalization rate or the percentage of sickle cell which regain the

normal shape in hypoxic condition is dose dependant and increases with the concentration of *Dissotis brazzae* leaves aqueous extracts to achieve the maximum threshold above which the normalization rate remains constant regardless of the increase in concentration. The MCN value was 11 µg/mL (0.011gr/mL) corresponding to a maximal normalization rate (NRmax) of 86% with a concentration of extract for which 50% of the sickled erythrocytes are reversed normal shape (ED50) equal to 5.6 µg/mL.

This result shows that *D. brazzae* leaves aqueous extracts inhibit the sickling of drepanocytes induced by hypoxic condition and is very significant if compared with the results obtained with other plants. For instance, *Thomandersia hensii* has shown a MCN of 12.5mg/mL, *Centella asiatica* a MCN of 25 mg/mL, *Bombax pentadrum* (0.34 mg/ mL), *Ricinodendron heudelotii* (0.39mg/mL), *Ipomoea batatas* leaves (0.54mg/mL) and *Trema orentalis* (2.1mg/mL) [4,11,15,18,19].

Antisickling activity of athoncyanins

Previous works of our research team have shown that anthocyanins are among active chemical groups of medicinal plants used in the management of SCD in Congolese traditional medicine [22-25]. Thus, anthocyanin extracts from *D. brazzae* leaves were tested for antisickling activity. Figure 5 give of sickle RBCs treated with anthocyanins extract.

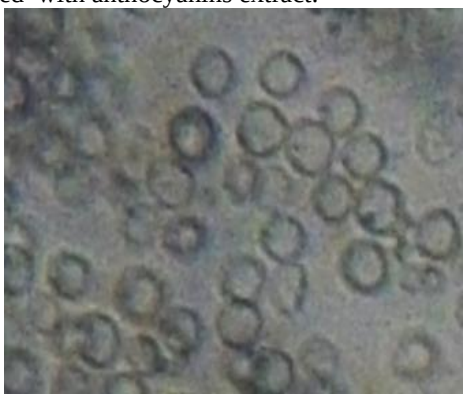

Figure 5: Morphology of erythrocytes of sickle RBCs in presence of anthocyanins extract of D. brazzae leaves (20 µg/mL) (X500), [NaCl 0.9% ; Na2S2O4 2%].

Figure 5 shows that in presence of anthocyanins extract of *D. brazzae* leaves, the majority of sickle-shaped erythrocytes in sickle cell blood (Fig. 1) reversed their shapes into the normal biconcave form resembling to positive control. This indicates that the anthocyanins extract of *D. brazzae* possess the capacity to prevent sickling process RBCs from sickle cell disease patients. These results confirm those already obtained by our research team with anthocyanins extracts from other plants used in Congolese traditional medicine to manage sickle cell disease [22-25].

In fact, anthocyanins are known for their properties to interact with proteins [28, 29]. This interaction would compete with the aggregation process of desoxyhemoglobin S and then, could reduce the sickling process of erythrocytes as revealed by Emmel test in figure 5.

Another probable mode of anthocyanins' action is their antioxidant effect on sickle erythrocytes. This can prevent the oxidation of hemoglobin into methemoglobin and reduce the Fe^{3+}/ Fe^{2+} ratio or/and prevent erythrocyte membrane lipoxidation [30, 31].

Effect of anthocyanins extract on Fe^{3+}/Fe^{2+} ratio

Figures 6 and 7 give the evolution of Fe^{3+} and Fe^{2+} in absence and in presence of anthocyanins extract from *D. brazzae* leaves.

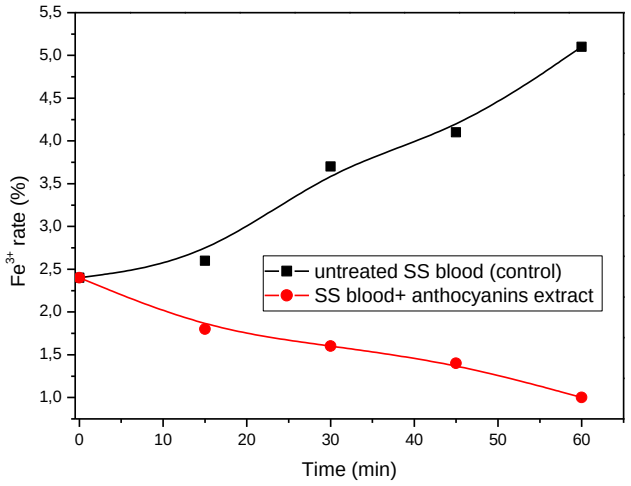

Figure 6: Evolution of Fe^{3+} in absence (control) and in presence of anthocyanins extract from *D. brazzae* leaves

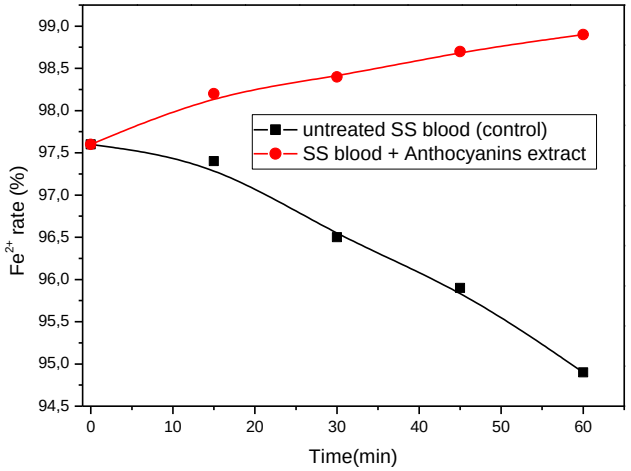

Figure 7: Evolution of Fe^{2+} in absence (control) and in presence of anthocyanins extract from *D. brazzae* leaves

Figures 6 shows that in the sickle RBCs alone (control) the Fe^{3+}rate increases with time, but when the anthocyanins extract is added, it is the opposite that is observed, the rate of Fe^{3+} decreases. It is the inverse for Fe^{2+} (Fig.7), its rate decreases for the control and increases with time when anthocyanins extract from *D. brazzae* leaves is added to the sickle RBCs.

This indicates that anthocyanins extract prevent the oxidation of Fe^{2+} into Fe^{3+} or the transformation of hemoglobin into methemoglobin. It therefore, decreases Fe^{3+}/ Fe^{2+} ratio in sickle cell blood. This would be a gain for sickle cell disease patients since the increase of methemoglobine (Fe^{3+}) decreases the capacity of hemoglobin to bind to oxygen so increases the deoxyhemoglobin form and therefore, the polymerization conducting to the sickling process [1-3].

Antioxidant activity

Figure 8 shows the evolution of DPPH radical rate with time at different concentrations of anthocyanins extract from *D. brazzae* leaves.

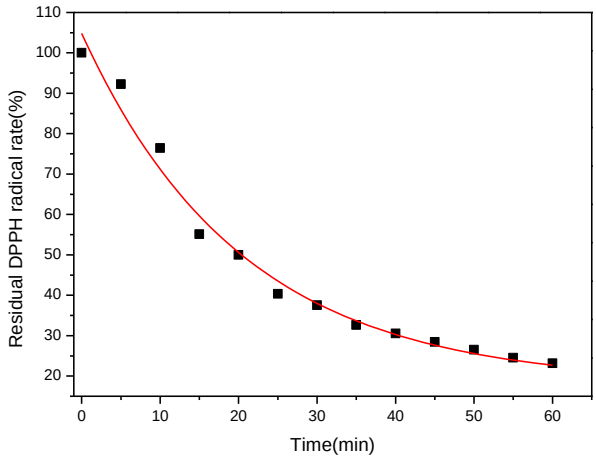

Figure 8: Evolution of DPPH radical rate with time in presence of anthocyanins extract from *D. brazzae* leaves.

This figure 8 shows that rate of residual DPPH radical decreases with time in presence of anthocyanins extract indicating the radical scavenging activity of the anthocyanins extract from *D. brazzae* leaves. The calculated IC50 and antiradical activity (ARA or 1/IC50) are given in table 2 and compared to ascorbic acid (control) values.

Table 2: Antioxidant activity of anthocyanins extract from D. brazzae leaves

sample	IC50 (µg mL^{-1})	ARA (1/IC50) (µg-1mL)
Ascorbic acid	22±5	0.045 ±0.010
D.brazzae anthocyanins extract	43±7	0.023 ±0.004

Anthocyanins extract from *D. brazzae* leaves show a good antioxidant activity, but this is lower than that of ascorbic acid used as positive control.

The evidence of oxidative stress and the role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in SCD accumulated over the last decade. Several molecular mechanisms have been proposed to contribute towards a high oxidative burden in sickle cell patients. Some of the mechanisms that disturb the redox state include, the excessive levels of free hemoglobin that catalyze the Fenton reaction, the recurrent ischemia-reperfusion injury promoting the activation of the xanthine-xanthine oxidase system and higher autoxidation of HbS generating superoxide anion radicals and hence hydrogen peroxide. Furthermore, a chronic proinflammatory response in sickle cell patients induced by constant recruitment of neutrophils and monocytes has been shown to play an important role in causing complications. Thus, ROS and RNS are not only potential markers of sickle cell disease severity but are also important targets for antioxidant therapies [1, 2, 6-8, 29-31].

The radical scavenging activity shown by anthocyanins extract from *D. brazzae* leaves, indicates that it contributes to the reduction of ROS and RNS and then, the oxidative stress in sickle

cell disease patients. This, combined to its antisickling activity can explain the use of this plant in Congolese traditional medicine.

CONCLUSION

The aim of this study was to check antisickling activity of *Dissotis brazzae in vitro*, to extract its anthocyanins and test their antisickling and radical scavenging activities. *Dissotis brazzae* aqueous leaves extract showed a high antisickling activity and this activity is due, very likely, to anthocyanins. These latter, showed also a good radical scavenging activity and decreased the Fe^{3+}/Fe^{2+} in sickle red blood cells. These activities can justify the health benefit of the use of this plant in traditional medicine for sickle cell disease patients. Isolation of bioactive molecules and test of organic acid is undergoing.

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Citation: P.T. Mpiana *et al*. (2015). Anti-sickling and antioxidant activities of anthocyanins extracts from Dissotis brazzae Cogn (Melastomataceae). J. of Advancement in Medical and Life Sciences. V3I4. DOI: 10.15297/JALS.V3I4.01