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In vitro Antisickling Activity of Anthocyanins from *Ocimum basilicum* L. (Lamiaceae)

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Abstract: Some Congolese plant extracts have recently shown an interesting antisickling activity. Four aqueous and ethanolic extracts from two plants *Cymbopogon citratus* (DC) Staff and *Ocimum basilicum* L. were evaluated for their antisickling activity. Only *O. basilicum* L. was found to be active. Anthocyanins crude extract of the leaves of *O. basilicum* exhibited attractive antisickling activity, thus, supporting the claims of the traditional healers and suggesting a possible correlation between the chemical composition of these plants and their uses in traditional medicine.

Key words: Congolese plants, aqueous extract, ethanolic extract, Emmel's test, antisickling activity, *Ocimum basilicum*, anthocyanins, *Cymbopogon citratus*

INTRODUCTION

Sickle Cell Disease (SCD) is a hereditary blood disorder resulting from the inheritance of two abnormal allelomorphic genes that control the formation of the β-chains of haemoglobins (Mehanna, 2002; Moody *et al*., 2003). In Africa, about 40% of the black population are carriers of the sickle cell gene. Although the severity of manifestations in SCD is severest in Africa, the disease is not limited to people of African origin. Saudi sicklers escape manifestations because of the inheritance of a different (longer) globin chain compared with the African sicklers (Cordeiro and Oniyangi, 2004; Moody *et al*., 2003).

The basic problem in sickle cell haemoglobinopathy is the production of an abnormal, less soluble, haemoglobin type leading to consequent crystallizing and distortion of the red cells. The sickle shaped red cells then increases the viscosity of the blood and the clumping of the red cells, as well as being destroyed in the reticulo-endothelial system starving the concerned organs of necessary blood and oxygen. This leads to the various manifestations such as painful, anaemia and other effects in almost all organs of the body. In an effort to increase the production of red cells, the marrow overworks and enlarges thus producing the abnormal physique associated with the disease. The crises are often precipitated by stress and may be produced in infections, pregnancy and situations of low oxygen tension such as flying in unpressurized aircraft and moving to a very high altitude prior to adjustment (Moody *et al*., 2003).

Various approaches have been adopted in an effort to find agents that inhibit the polymerization of Haemoglobin (Hb) and hence prevent or reduce the occurrence of crises in SCD (Iyamu *et al*., 2002; Akojie and Fung, 1992). Towards this goal, oxygen, carbon monoxide and sodium nitrite were used to reduce the amount of deoxy-Hb. The above approaches did not give the much-needed beneficial effects based on the reduction of painful crises as the criterion for successful treatment (Iyamu *et al*., 2002).

Bone marrow transplantation has in recent years been found to be an efficient but practically impossible method in developing countries in controlling the scourge. The cost implications, the availability of necessary expertise and the problems of finding suitable donors, however, constitute a major setback to this approach in third world countries with weak economies like Democratic Republic of Congo (DRC). While genetic counselling holds a prominent position in enlightening the population about this condition and have been found to be beneficial in guiding people with respect to the choice of a mate, its role in eradicating the condition is not feasible because of balanced polymorphism and the fact that providing the right counsel does not necessarily lead to the rational choice of a mate (Moody *et al*., 2003; Akinsulie *et al*., 2005).

Furthermore, several current research activities are focused on identifying new drugs that are capable of preventing the loss of water from Red Blood Cells (RBCs) or increasing the level of foetal haemoglobin, a variety of haemoglobin that prevents the sickling of RBCs. Clotrimazole, hydroxyurea and erythropoietin (a genetically engineered hormone that stimulates RBC production) were proposed in this regard (Rifai *et al*., 1995). Unfortunately, these drugs are known for their serious side effects, hence, limiting their clinical use (Mehanna, 2002; Eliot *et al*., 2006; Akinsulie *et al*., 2005).

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Alternative therapy with herbal remedies and medicinal plant products from local flora have long been used in folk medicine but only few of them have been scientifically validated, yet these would appear to present a more viable approach that can be more readily adapted to developing countries. Many traditional practitioners of herbal medicine in DRC claim to be able to cure SCD crises with the use of herbal preparations. Many of these claims are only just being subjected to scientific trials that have already led to attractive findings. As for us, our investigations have led to the identification of the class of compounds responsible of the sickling reversal activities of some of the Congolese plants (Mpiana *et al*., 2007).

The present study was performed with the aim of evaluating the antisickling activity of two more Congolese medicinal plants used in the management of various SCD-associated disorders in Kinshasa, DRC. The antisickling activity of the aqueous and ethanolic extracts of these plants was evaluated *in vitro* on blood from homozygous SCD patients (SS blood), using Emmel's test (Courtejoie and Hartaing, 1992) and this activity is expressed as the normalization of sickled cells. We have determined both the Minimal Concentration of Normalization (MCN) of sickle cell erythrocytes and the chemical class of natural products responsible of this activity, the anthocyanins.

MATERIALS AND METHODS

Plant material: The leaves of each of the species under investigation were collected from plants growing in Kinshasa, DRC and were authenticated by Mr. B.L. Nlandu of the INERA (Institut National d'Etudes et Recherches Agronomiques). Voucher specimens, (n°1468 for *O. basilicum* and n°2058 for *C. citratus*) are on deposit at the INERA Herbarium of the Faculty of Science (Université de Kinshasa).

Extraction: The dried and powdered plant material (leaves, 10 g) was repeatedly extracted by cold percolation with 95% EtOH and water (100 mL<×1) for 48 h. Fractions were filtered and concentrated to dryness under reduced pressure using a rotary evaporator. Extraction of anthocyanins and alkaloids was then done using 100 g of dried powdered plant material with distillated water and diethyl ether following an established protocol (Bruneton, 1999).

Biological material: The sodium citrate suspension of blood samples used to evaluate the antisickling activity of the plant extracts in this study were taken from known $Hb^{S}Hb^{S}$ adolescent patients attending the Centre de Médecine Mixte et d'Anémie SS and Centre Hospitalier Monkole, both located in Kinshasa area, DRC. All antisickling experiments were carried out with freshly collected blood. In order to confirm their SS nature, the above-mentioned blood samples were first characterized by electrophoresis on cellulose acetate gel at pH 8.5. They were found to be SS blood and were then stored at ±4°C in a refrigerator.

Antisickling assay: In order to evaluate the antisickling activity of our plant samples, an *in vitro* antisickling assay was performed, in which blood sample is put in contact with plants extracts at different concentrations (with the physiologic solution, NaCl 0.9%, as the dilution solvent) according to Emmel's test procedure (Courtejoie and Hartaing, 1992). In this study, Emmel's test was performed as previously reported (Mpiana *et al*., 2007).

The RBCs were analysed by measuring various parameters including the area, perimeter and the radius of each RBC using a computer assisted image analysis system (Motic Images 2000, version 1.3) and statistical data were processed using Microcal Origin 6.0). Each test was performed in triplicate and the number of observed erythrocytes was determined using Thomas' cell.

RESULTS AND DISCUSSION

Four extracts from two plants used in Congolese traditional medicine were tested for their sickling reversal activities. Plant parts used in this study are those used by traditional healers. The ethanolic and aqueous extracts of *C. citratus* were almost inactive (<10%); while ethanolic extract of *O. basilicum* presented high normalization rate (>50%) and the aqueous extract a very high normalization rate (>70%).

Cymbopogon citratus (D.C.) appeared not to have any antisickling activity. This plant is often cited to be part of an association of plants used to treat SCD in Kinshasa, DRC and is perhaps useful for treating associated symptoms, such as pain, fever, or to enhance the immune system. Both aqueous and ethanolic extracts of *O. basilicum* have shown a sickling reversal activity, thus, supporting the claims of the traditional healers. This suggests a possible correlation between the chemical composition of this plant and its use in traditional medicine. *O. basilicum* L. aqueous extract was found to exhibit the highest antisickling activity and this prompted us to focus the continuation of our investigations on this extract.

Figure 1 and 2 shows the morphology of SS blood erythrocytes (standard) and that of SS blood erythrocytes in the presence of *O. basilicum* aqueous extract.

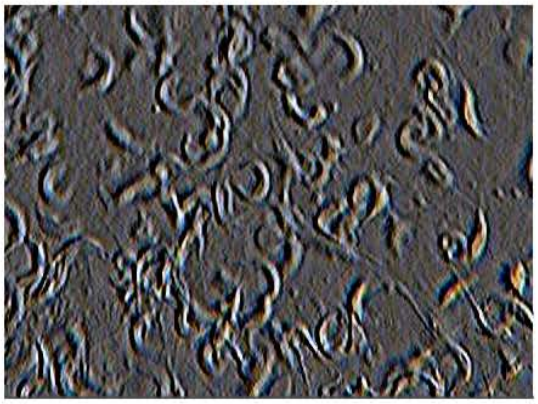

Fig. 1: Morphology of drepanocytes of non treated SS blood (standard), (Ordinary view 500 X)

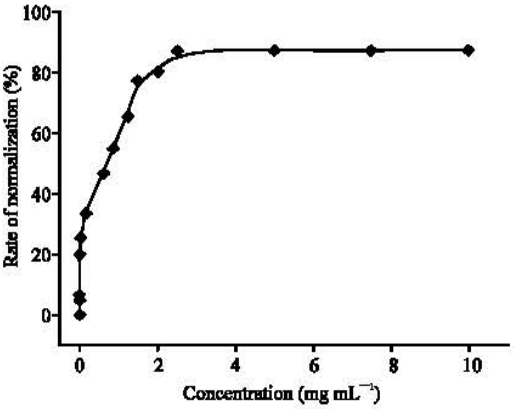

Fig. 3: Concentration-dependent antisickling effect of *O. basilicum* aqueous extract

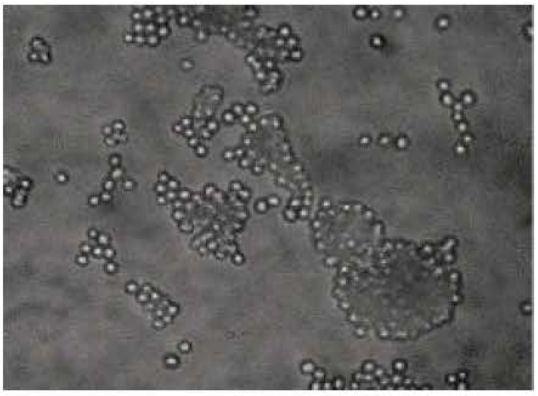

Fig. 2: Morphology of drepanocytes treated with aqueous extract of *O. basilicum* (2.5 mg mL^{-1}), (Ordinary view 500X)

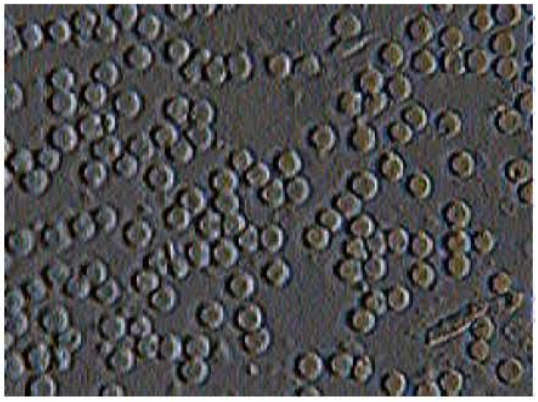

Fig. 4: Morphology of SS blood erythrocytes treated with anthocyanins extract of *O. basilicum* (ordinary view 500X)

As it can be seen from the above images, a normalization of erythrocytes of SS blood sample treated with *O. basilicum* extract indicates the influence of the extract on the sickliness of cells. Figure 3 shows the normalization of sickled cells with the plant aqueous extract concentration. This normalization increases with the extract concentration and reach a maximum and constant value at 2.5 mg mL^{-1} (MCN). This corresponds to a normalization rate of 87.00%.

Based on both the chemical screening results and the solubility of different chemical groups (Bruneton, 1999), we suppose that the antisickling activity of *O. basilicum* aqueous extract would be due to the presence of anthocyanins or alkaloids which are present in this plant. We then decided to focus the continuation of our investigation on these two chemical groups.

Figure 4 and 5 shows the morphology of SS blood erythrocytes (standard) and those of SS blood erythrocytes in the presence of *O. basilicum* anthocyanins and alkaloids extracts, respectively.

These morphological SS blood cells were observed in anaerobic conditions. As it can be seen from the above images, a normalization of erythrocytes of SS blood sample treated with *O. basilicum* anthocyanins extract indicates the influence of the extract on the sickliness of cells.

The antisickling activity due to anthocyanins extract is higher than that of the alkaloids extract. This normalization increases with the extract concentration and reach a maximum and constant values of 86.00% at 13 μg mL^{-1} for the anthocyanins (Fig. 6) and 20% of normalization for the alkaloid extracts, respectively.

The software used in this study did not give the average radius for the erythrocytes (Table 1), because sickle cells of non treated SS blood is not circular. The average radius appeared after treatment of SS blood cells with *O. basilicum* extract, indicating the re-appearance of the normal and classical form of RBC.

Statistical treatments [Two samples-independent Student-Test (Murray, 1985), applied with a probability

Fig. 5: Morphology of SS blood erythrocytes treated with alkaloids extract of *O. basilicum* (Ordinary view 500X)

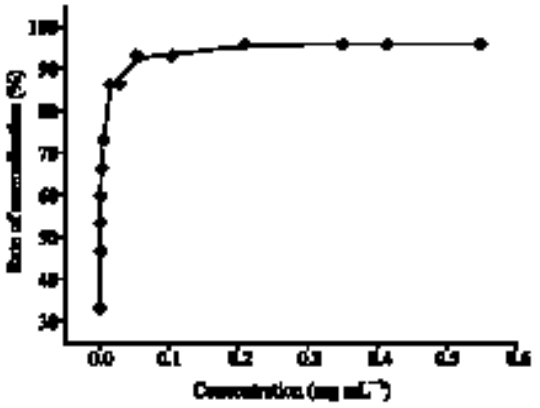

Fig. 6: Concentration-dependent antisickling effect of anthocyanins of *O. basilicum*

Table 1: Average values of radius, perimeter and surface of erythrocytes before and after treatment with a solution of 13 µg mL^{-1} of *O. basilicum* anthocyanins extract

Measured parameter	Untreated SS RBC	Treated SS RBC
Radius (µm)	-	3.23±0.43
Perimeter (µm)	29.60±3.64	20.32±2.69
Surface (µm^2)	13.49±1.42	33.00±4.13

threshold of 0.05, degree of freedom = 22] enabled the determination of a significant difference between the average values of both the perimeter and the surface of the untreated and treated erythrocytes on the images, thus confirming the modification of the erythrocytes form in presence of *O. basilicum* anthocyanins extract.

The ability of the extracts, in this study, to reverse the sickling of SS blood erythrocytes may represent a rational explanation for the use of these plants in managing sickle cell disease by Congolese traditional healers. To the best of our knowledge, these species have not yet been reported to exhibit antisickling effects.

Further studies, involving the isolation and characterization of secondary metabolites from this plant are in progress.

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