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Antisickling properties, thermal and photochemical degradations of anthocyanin extracts from *Annona senegalensis* (Annonaceae)

P.T. MPIANA[1*], E.N. DIANZENZA[1], K.N. NGBOLUA [2], D.S.T. TSHIBANGU [1], B.M. MBALA[1], S.O. MIHIGO[1], E.K. ATIBU[1], M.K. KAKULE [3] and M.T. BOKOTA[4]

[1]*Département de Chimie, Faculté des Sciences, B.P. 190, Université de Kinshasa, Kinshasa XI, D.R. Congo.*
[2]*Département de Biologie, Faculté des Sciences, B.P. 190, Université de Kinshasa, Kinshasa XI, D.R.Congo.*
[3]*Département de Physique, Faculté des Sciences, B.P. 190, Université de Kinshasa, Kinshasa XI, D.R.Congo.*
[4]*Département de Chimie, Faculté des Sciences, Université de Kisangani, Kisangani, D.R. Congo.*
[*]*Corresponding author, E-mail: ptmpiana@yahoo.fr, Tel: +243 81 811 60 19*

ABSTRACT

Anthocyanin crude extracts and fractions of *Annona senegalensis* leaves were screened for their antisickling properties using a validated *in vitro* bioassay model of sickle cell anaemia. Preparative thin layer chromatography of acetylated anthocyanin crude extracts carried out with chloroform as solvent provided two active fractions named Ac_1 and Ac_2. Fraction Ac_2 was quantitatively isolated and submitted to physicochemical investigations. Thermo-degradation kinetics of the above fraction Ac_2 at 80 °C and 100 °C revealed first order reaction with the rate constants k (s^{-1}) of 8.10 10^{-4} and 11.0 10^{-4}, respectively. Anthocyanin crude extracts and the separated fractions showed *in vitro* antisickling activity. This activity justifies the use of this plant by congolese traditional healers; thus anthocyanins could be the major active principles. However, these natural pigments are unstable towards UV-vis light irradiations and heat. The conservation of this plant should then be performed in the absence of heat and/or light.

Keywords: Sickle cell anemia, *Annona senegalensis*, anthocyanins, light irradiation, thermodegradation.

INTRODUCTION

The Democratic Republic of Congo (DRC) is reputed for the extraordinary richness of its flora and boasts a wide variety of medicinal plant species which represent an enormous reservoir of new molecules with potential therapeutic values (Debroux et al., 2007). All over Africa and particularly in DRC, the majority of people rely on traditional medicine for their health care needs; this is due to the fact that the conventional drugs costs are unaffordable (Ngbolua et al., 2011). Drepanocytosis or Sickle Cell Anemia (SCA) is one of the illnesses for which the African population relies on medicinal plants for their treatment. SCA is a genetic disease which is endemic in some populations originating from sub-Saharan Africa, Asia and in some Latin and North African-Americans and African-Europeans (Kaplan and Delpech, 1993; Fattorusso and Ritter, 1994; Voet and Voet, 1998; Iyamu et al., 2002; Moody et al., 2003). It is a hemoglobinopathy due to a substitution

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in position 6 within the β chain of hemoglobin (Hb) whereby glutamic acid, a polar amino acid, is replaced by valine, a non polar amino acid.

This substitution decreases the affinity of Hb for oxygen. At low oxygen tension and when red blood cells (RBCs) are dehydrated, the mutant hemoglobin (Sickle hemoglobin or S hemoglobin) polymerizes inside the RBCs into a gel or further into fibers leading to a drastic decrease in the red cell deformability. Polymerization and precipitation of S hemoglobin within RBCs cause the change of erythrocytes shape from their normal globular form into one resembling a sickle. Sickling of RBCs is the cause of precocious hemolysis of erythrocytes and various complications of sickle cell disease patients (Antonini and Brunori, 1971; Sofowora, 1975; Lehninger, 1994; Mpiana et al., 1996).

In Africa, SCA is a public health problem as 20-25% of the sub-Saharan population suffers from or transmits this disease. Two percent of the population of the DRC suffers from SCA. About 80% of children suffering from SCA do not receive medical care and die before the age of five years (Mpiana et al., 2007a).

Proposed therapies such as the allogenic medular transplantation remain very expensive for most African population. Some proposed antisickling drugs are toxic especially those requiring a long time of use (Mehanna, 2001). That is why African population recourse to medicinal plants in order to treat SCA (Newinger, 2000).

A review of literature on SCA chemotherapy revealed that a number of medicinal plants have been shown to contain antisickling principles *in vitro* (Bunn, 1997; Gentilini, 1986; Kuchel and Palston, 1993; Mpiana et al., 1996; Mpiana et al., 2010a, 2010b). Our previous findings also revealed that anthocyanins and triterpenoids are the major antisickling constituents of some Congolese and South African plants (Mpiana et al., 2007b, 2010c; Tshibangu et al., 2010). As a continuation of our work on Congolese plants, *Annona senegalensis* Pers. was selected for investigation on the basis of its reported use against SCA in traditional medicine.

The main objective of this study was to verify if antisickling activity of the above mentioned plant is due to anthocyanin extracts as reported for others congolese plants (Mpiana et al., 2007a, 2007b, 2010c). In addition, since most traditional healers expose medicinal plants to sun and use decoction as the way of preparation of their recipes, we undertook to evaluate the effect of light and heat on the stability of these natural pigments.

MATERIALS AND METHODS

Plant materials

Plant materials (leaves) used in this study were collected in Kinshasa (DRC). Botanical identification was made by Mr Nlandu of the INERA (Institut National d'Etudes et Recherches Agronomiques) of the Faculty of Science, University of Kinshasa. Voucher specimens (A. Leonard 5635) are kept at the "INERA" herbarium service.

Extraction, acetylation and phytochemical investigations

The dried powdered plant material (10 g) was extracted by cold percolation with 95 % EtOH and water (100 ml x 1) for 48 hrs. Extracts were filtered and the solvent was evaporated under reduced pressure using a rotary evaporator. A chemical screening was then done on the aqueous and ethanolic extracts of the leaves of *Annona senegalensis*. Several classes of compounds were screened, including alkaloids, polyphenols (tanins, flavonoids, anthocyanins and leucoanthocyanins), terpenoids and lipids. Extraction of anthocyanins was then done using 100 g of dried powdered plant material according to the universal procedures (Bruneton, 1999).

A quantity of 1.2 g of anthocyanin extracts was acetylated and 1.5 g of amorphous powder were obtained and

subjected to thin layer chromatography (TLC) over silica gel 60, F_{254} aluminium barking plates from Merck, Germany. Chloroform was used as eluting solvent and plates were visualized under ultra-violet light at 254 nm and 366 nm. Fractionation was carried out by preparative thin layer chromatography using silica gel.

The effects of radiations were evaluated by exposing anthocyanin solutions to a CAMAG UV lamp emitting a maximum wavelength of 366 nm, while the thermal degradation was achieved using an oven (Telco model) at different temperatures and during different times. The absorbance of solution was measured using a Perkin Elmer Lambda 2 spectrophotometer.

Blood samples and antisickling assay

Blood samples were obtained from known sickle cell patients attending the "Centre de Médecine Mixte et d'Anémie *SS*" and "Centre Hospitalier Monkole", both located in Kinshasa area, DRC. None of the patients had been recently transfused. Blood samples were first characterized by haemoglobin electrophoresis in order to confirm their *SS* nature. All antisickling experiments were carried out with freshly collected blood. Blood samples were stored at ± 4 °C in a refrigerator. Ethical clearance on the use of SS blood was strictly observed according to international rules (WHO, 2002).

The antisickling activity of anthocyanins was evaluated using a validated *in vitro* bioassay model of SCA as reported elsewhere (Mpiana et al., 2007a, 2007b, 2010c). Briefly, A drop of 2% sodium metabisulfite in physiologic saline solution (NaCl 0.9%) is put on a glass slide. A drop of sickle blood (dilution ½ with NaCl 0.9%) is added and the slide is hermetically covered by a cover glass and the edges are clogged by melt paraffin wax. The slide is kept for 24 hrs (incubation) in hypoxic conditions. After 24 hrs, RBCs became deoxygenated while adopting a sickled shape.

Normalization, defined as the passage of the sickled shape into the circular and normal biconcave form after treatments of RBCs with anthocyanin extracts, was quantitatively evaluated by measuring various cellular parameters such as area, perimeter and the radius using Motic Images package software. A zoom 6 x CANNON-type digital camera was used to convert the photonic micrograph image into a digital image, which was then digitalized using a MOTIC image 2000 1.3.

Mathematical model for thermal and photochemical degradations data analysis

Considering the thermal degradation of anthocyanins as a chemical reaction, an anthocyanin molecule A decomposes irreversibly into one or several molecules assigned as molecule B. This transformation can be schematically represented by the following equation as previously reported (Mpiana et al., 2009a, 2009b, 2009c, 2009d):

$$A \xrightarrow[\Delta]{k} B \qquad [1]$$

The transformation is a first order decomposition for which rate equation is given by:

$$-\frac{dC_A}{dt} = kC_A \qquad [2]$$

Where, C_A is the concentration of A; k the rate constant and t the time of decomposition.
The integration of [2] gives:

$$C_A = C_A^o.e^{-kt} \qquad [3]$$

Where C_A^o is the initial concentration of A.
If A is the only compound that absorbs the light at a chosen wavelength, the Beer-Lambert relation for this case would be:

$$E = \varepsilon l C_A \qquad [4]$$

And

$$E_o = l\varepsilon C_A^o \qquad [5]$$

Where E, E_o, l and ε are respectively the absorbance at time t, the absorbance at time t=0 second, the optic pathway and the molar extinction coefficient.

The combination of equation [3], [4] and [5] gives:

$$E = E_0 e^{-kt} \qquad [6]$$

If the compound resulting from degradation process absorbs simultaneously with A at the same wavelength, the Beer-Lambert equation would be:

$$E = \ell(\varepsilon_A C_A + \varepsilon_B C_B) \qquad [7]$$

Where εA, εB, CA and CB are respectively compound molar extinction coefficient and concentrations.

Considering that

$$C_A^o = C_A + C_B \qquad [8]$$

And combining equations [3], [5], [7] and [8] provide

$$E = E_\infty + \frac{(\varepsilon_A - \varepsilon_B)Eo}{\varepsilon_A}.e^{-kt} \qquad [9]$$

If $\varepsilon_A \succ \varepsilon_B$ equation [9] gives the same exponential decreasing trend as does the equation [6].

The experimental result fitting with the two models (equations [6] and [9]) is carried out using Microsoft Origin 6.3 software package.

Statistical analysis

The Student's t-test was used to test the significance of the difference between the results for treated and untreated SS blood samples. Statistical significance was set at P=0.05. Data are expressed as means ± S.D. Data analysis was performed using Microcal Origin 6.1 package software. The *in vitro* bioassay was performed in triplicate and the number of observed erythrocytes was determined using Thomas' cell as previously reported (Mpiana et al., 2010c).

RESULTS

The chemical screening carried out on the aqueous and organic extracts of the leaves of *Annona senegalensis* Pers revealed the presence of anthocyanins, tannins, flavonoids, leucoanthocyanins and alkaloids.

Thin layer chromatography (TLC) using ethyl acetate/petroleum ether (2:1) mixture as eluting system revealed three spots with frontal ratio values (Rf) of 0.54, 0.68 and 0.94 respectively.

Thin layer chromatography using chloroform as eluent carried out on the acetylated total extracts of anthocyanins provided two spots (Ac_1 and Ac_2) with Rf respectively 0.1 and 0.87.

Figures 1 and 2 give the micrographies of the untreated and treated drepanocytes with the crude anthocyanin extracts. These morphological SS blood cells were observed in anaerobic conditions, i.e after deoxygenation of hemoglobin by 2% sodium metabisulfite solution. As it can be seen from the above images, the majority of non treated SS blood erythrocytes are sickled (Figure 1). In the presence of anthocyanin crude extracts of *A. senegalensis*, the shape of the erythrocytes are reversed and become normal, indicating the antisickling properties of anthocyanin extracts (Figure 2). Figures 3 and 4 illustrate modification of drepanocytes by acetylated anthocyanin extracts and Ac_2 fraction respectively. Figure 3 indicates that despite acetylating, anthocyanins remain active. Figure 4, indicates that when treated with the Ac_2 fraction, some erythrocytes preserve their sickle-shaped, suggesting that the antisickling effects of *A. senegalensis* anthocyanins may not be achieved by a single component. The calculated values of perimeter, surface and radius of untreated and treated sickle erythrocytes with the anthocyanin extracts are given in Table 1.

Table 1: Average values of the perimeters, surfaces and the radius of untreated and treated sickle erythrocytes with anthocyanin extracts.

Samples	Cellular perimeters (µm)	Cellular surfaces (μm^2)	Cellular radius (µm)
Control	34.03 ±2.70	21.41 ±2.01	-
Anthocyanin crude extracts	19.51 ±2.54	33.42±3.26	3.30 ±0.51
Acetylated anthocyanins extracts	20.01 ±2.13	32.18 ±3.29	3.10 ±0.53
Ac_2 (fraction)	21.01 ±2.24	32.23 ±3.25	3.23 ±0.52

Tabulated values are means ± S.D. of statistical treatments of ten determinations according to Student's t-test (applied with a probability threshold of 0.05 for 18 degrees of freedom (dof)), and enabled the determination of a significant difference between the average values of both the cellular perimeter and surface of the untreated and treated sickle erythrocytes. These results suggest that the effectiveness of the antisickling activity of *A. Senegalensis* could be related to the anthocyanin extracts.

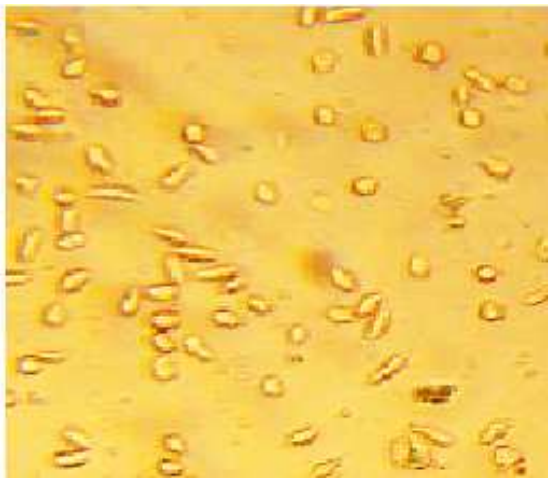

Figure 1: Morphology of untreated drepanocytes (negative control; X500).

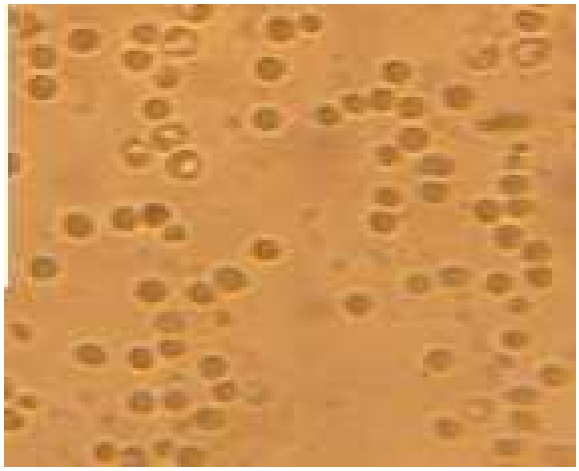

Figure 2: Morphology of drepanocytes treated with anthocyanin crude extracts (X500).

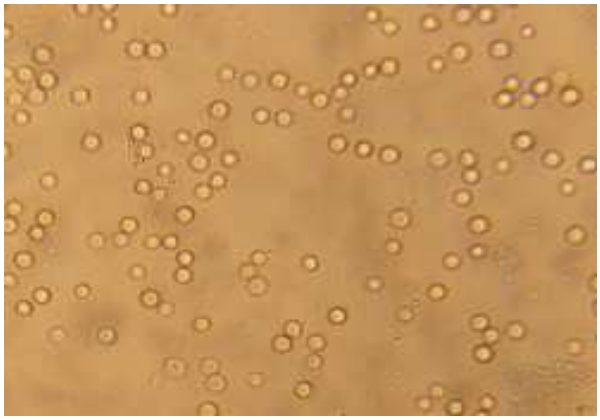

Figure 3: Morphology of drepanocytes treated with acetylated anthocyanin extracts (X500).

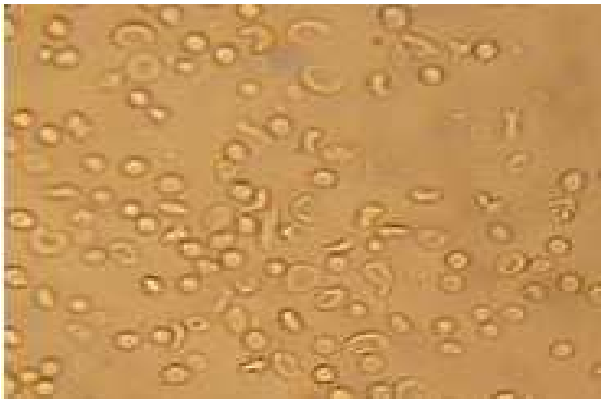

Figure 4: Morphology of drepanocytes treated with Ac_2 fraction (X500).

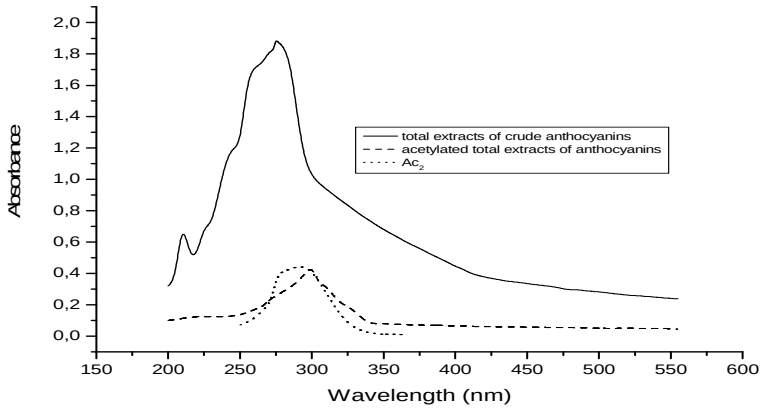

Figure 5: Spectra of the total extracts of crude anthocyanins, the acetylated total extract and the Ac_2 fraction.

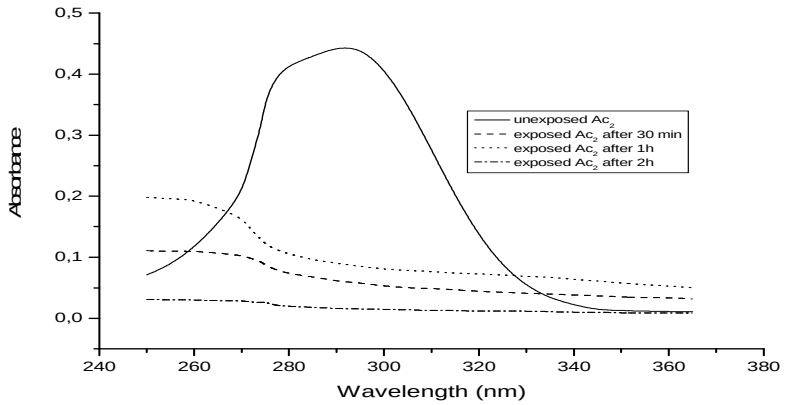

Figure 6: Spectra of Ac_2 fraction before and after exposure to UV light at 366 nm.

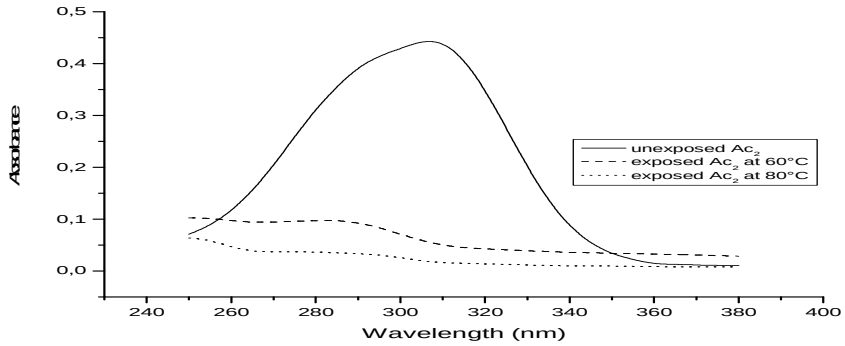

Figure 7: Spectra of Ac_2 before and after heat exposure at 60 °C and 80 °C during 1hour.

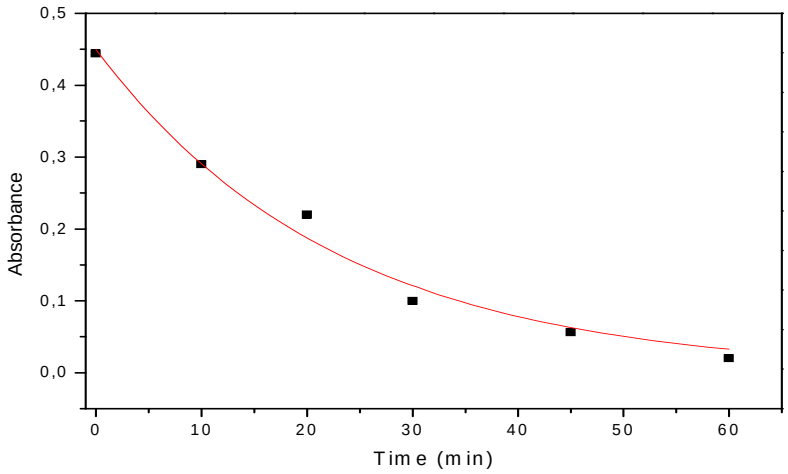

Figure 8: Absorbance evolution of Ac_2 fraction according to the thermal treatment time at 80°C.

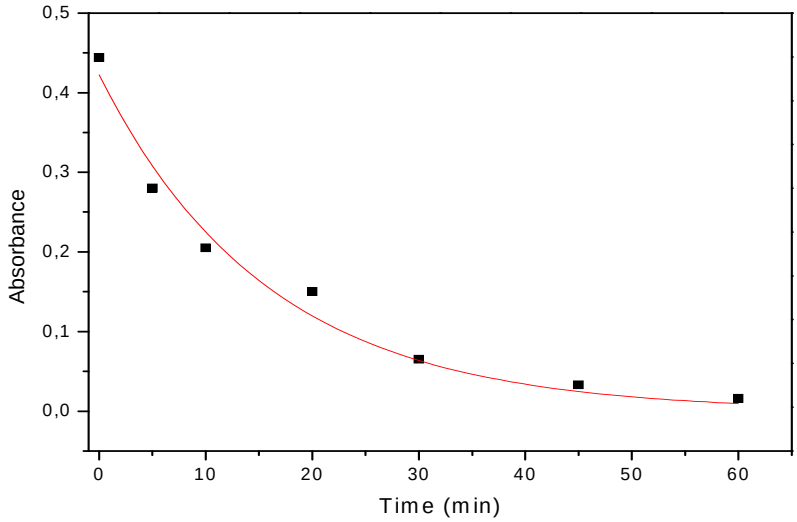

Figure 9: Absorbance evolution of Ac_2 fraction according to the thermal treatment time at 100°C.

The Figures 6 and 7 showed that the acetylated fraction of anthocyanins present an absorption band at about 310 nm. This band drastically decreases after UV light exposure (Figure 6). The same result is also observed when the Ac_2 fraction is exposed to the heat (Figure 7). Figures 8 and 9 show the thermo-kinetics curves of the Ac_2 compound at 305 nm.

Thermo-degradation kinetics can be studied by monitoring the absorbance at different times for a fixed wavelength as shown in Figures 8 and 9. The decrease of absorbance of Ac_2 fraction in these figures is fast at the beginning of the curve and slowing down with time. This decrease is more pronounced at 100 °C than at 80 °C, indicating that the thermo-degradation is faster at a high temperature. These figures show the experimental points and the calculated curves according to the first order reaction model by assuming that both the reagent and the product absorb at 305 nm (equation [9]).

DISCUSSION

Compared to Figure 1, Figure 2 shows that in the presence of anthocyanin crude extracts of *A. senegalensis*, RBCs adopt circular/biconcave and normal shape, indicating the antisickling effect of anthocyanins. This normalization could be due to the inhibition of the Hb S intra-erythrocytic polymerization, and this is similar to other results reported in the literature (Iyamu et al., 2002; Nwaoguike and Uwakue, 2005; Mpiana et al., 2010c; Sahu et al., 2012). It should be noted that in the antisickling bioassays there is no established standard molecule that can be used as a positive control (Mpiana et al., 2010d).

On the other hand, anthocyanins are known for their instability (Bruneton, 1999; Mpiana et al., 2009a, 2009b, 2009c, 2009d). Thus, we hypothesized that their acetylation could increase their stability. That is why the anthocyanin extracts was acetylated and the antisickling activity of the resulting product was tested.

The perimeter, surface and radius were calculated for untreated and treated sickle RBCs with the anthocyanin extracts in order to confirm the modification showed by their micrographies.

Table 1 shows that the average radius for the RBCs of the sickle cell blood could not be calculated because sickled RBCs of untreated blood are not circular. The average radius appeared after treatment of sickle RBCs by anthocyanin extracts, indicating the

re-appearance of the normal form of RBCs. This confirms previous results obtained with anthocyanin extracts from other Congolese plants (Mpiana et al., 2007b, 2009a, 2009b, 2009c, 2009d, 2010a, 2010b, 2010c).

The absorption band at 275 nm (Figure 5) are due to the π-π* transition of the flavylium ion which constitutes the basic structure of the anthocyanins. As already mentioned in the results section, the observed red shift may be due to the acetylation. Indeed, acetylation increases resonance in the molecule of anthocyanins and stabilizes the π* orbital, decreasing the difference in energy between the two orbitals, hence resulting in a displacement of the band towards the higher wavelengths (red shift). It should be noted that all these spectra present general characteristics of anthocyanins spectra in the UV domain (Kuchel and Palston, 1993; Mpiana et al., 2009a, 2009b, 2009c, 2009d).

These various spectra highlight the instability of the anthocyanins to UV light. The disappearance of the initially observed absorption band and the appearance of new bands around 290 nm after treatment indicate the sensitivity of the anthocyanins to the temperature. This justifies the need of preserving anthocyanin solutions at low temperatures and avoiding decoction or infusion recipes in traditional medicine. It can be noted that this behavior was observed for the anthocyanins extracted from other plants (Cissé et al., 2009; Mpiana et al., 2009a, 2009b, 2009c, 2010b).

The treatment of the experimental results by nonlinear regression according to equation [9] gave the values of the thermo-degradation rate constants at 80 °C, $k_{80} = 8.1\ 10^{-4}\ s^{-1}$ and 100 °C, $k_{100} = 11.0\ 10^{-4}\ s^{-1}$. These values show that the kinetics of degradation is rather slow and are of the same magnitude order than those of the anthocyanins isolated from *Ocimum basilicum* and *Hymenocardia acida* (Mpiana et al., 2009d, 2010a).

Conclusion

In vitro antisickling activity observed in this study justifies the use of *A. senegalensis* by Congolese traditional healers and anthocyanins could be the major biologically active principles. This study shows that these natural pigments are instable towards UV-vis light irradiations and heat. Traditional recipes from this plant should be conserved in the absence of heat and/or light.

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