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Antisickling Activity and Thermostability of Anthocyanins Extract from a Congolese Plant, *Hymenocardia acida* Tul. (Hymenocardiaceae)

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Abstract: Antisickling activity of anthocyanins extract from a Congolese plant (*Hymenocardia acida* Tul.) was evaluated using Emmel test. Chromatographic separations using chloroform-benzene (2:1) provided three fractions A_1, A_2 and A_3 with the most polar [A_1 (TLC, R_f = 0.21)] exhibiting the highest activity. Thermal kinetic degradation of this fraction at 100 and 120°C produced a first order rate constants $k = 2.64\times10^{-4}$ and 4.08×10^{-4}, respectively. Structural elucidation of isolated compounds is in progress.

Key words: Sickle cell anaemia, drepanocytes, degradation kinetics, thermodegradation

INTRODUCTION

Drepanocytosis or sickling anaemia is among tropical diseases responsible of great mortality (Gentilini, 1986). Initially existing in tropical and Mediterranean regions where it predominated, this sickness is nowadays spread all over the world by means of migration.

Over 50 millions people are actually affected throughout the world (Diop *et al*., 2000; Gulbis *et al*., 2005). African continent remains the most affected by this disease with the highest prevalence in its West- and Central parts. In Nigeria more than 3% of the population is affected (Ibrahim *et al*., 2007), while R. D. Congo has approximately 2% of its population affected by this disease (Mpiana *et al*., 2007a). About 80% of children suffering from drepanocytosis that do not receive regular medical care, die before the age of five years (Mpiana *et al*., 2007a, b; Gini, 1985).

Drepanocytosis, also known as sickle cell anemia, is a genetic disease due to a mutation in position 6 within the β chain of hemoglobin whereby glutamic acid, a polar amino acid, is replaced by valine, a non-polar amino acid. This mutation decreases the affinity of hemoglobin for oxygen. At low oxygen tension, the mutant hemoglobin (sickle hemoglobin or S hemoglobin) polymerizes inside the red blood cells into a gel or further into fibers leading to a drastic decrease in the red cell deformability. Polymerization and precipitation of S hemoglobin within the erythrocytes cause the change of the shape of erythrocytes from their normal lobular form into one resembling a sickle. Sickling of blood cells modifies their flexibility, believed to be responsible of vaso-occlusive problems of sickle cell anemia (SS) subjects (Gentilini, 1986; Voet and Voet, 1998; Mehanna, 2001).

Most of the proposed therapies for sickle cell anemia appear to be unsatisfactory. Bone marrow transplantation is expensive for African poor population, foetal haemoglobin synthesis stimulants such as hydroxyl urea are toxic and repeated transfusions constitute high Human Immunodeficiency Virus (HIV/AIDS) infection risks (Mpiana *et al*., 2007c; Mehanna, 2001; Akinsule *et al*., 2005). Therefore, phytotherapy appears to be a promising alternative therapy because a plant-based remedy will be more affordable by the African population and because the great biodiversity of the tropical forests offer a high potentiality to find lead molecules that could be used in the fight against drepanocytosis, the same way as quinine and artemisinin have contributed to the treatment of malaria.

Several investigations have been conducted on medicinal plants and some antisickling molecules were isolated by Ekeke and Shode (1990), Fall *et al*. (1999), Mehanna (2001), Iyamu *et al*. (2002), Moody *et al*. (2003), Elekwa *et al*. (2005), Elujoba *et al*. (2005) and Mpiana *et al*. (2007a-c, 2008a, b). Plants used by Congolese traditional healers for the treatment of drepanocytosis were surveyed. *Hymenocardia acida* Tul., one of the plants previously reported for their antisickling properties, is known for its numerous useful utilisations in traditional medicine in the R. D. Congo

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(Ibrahim *et al*., 2007; Mpiana *et al*., 2007a; Kabangu, 1990; Neuwinger, 2000). In the recent reports on this plant and on other Congolese plants, it was suspected that the antisickling activity is due to anthocyanins (Mpiana *et al*., 2007b, c, 2008a, b). These plant colouring materials are unstable towards physical and chemical factors such as temperature and solar radiation (Kahkönen *et al*., 2003a). Nevertheless, these plants are exposed to the sun light by vendors and traditional healers.

The present study intends to verify the antisickling activity of the anthocyanins extract of *H. acida*, to separate it by chromatographic techniques, determine the most active fraction, study the effect of temperature on its stability using spectrophotometric methods and the kinetic degradation at a given temperature.

MATERIALS AND METHODS

Plant material: Plant materials (leaves) used in this study were collected between February and June 2008 from a *H. acida* Tul. growing at the Université de Kinshasa site, Kinshasa (R. D. Congo). Plants were authenticated by Mr. B.L. Nlandu of the INERA (Institut National d'Etudes et Recherches Agronomiques/Faculté des Sciences, Université de Kinshasa).

Extraction: The dried and powdered plant material (10 g) was repeatedly extracted by cold percolation with water (200 mL×1) for 48 h. Fractions were filtered and the solvent was evaporated under reduced pressure using a rotary evaporator. Extraction of anthocyanins was then done using 100 g of dried powdered plant material with distillated water and diethyl ether according to the universal procedures (Bruneton, 1999).

Biological material: Blood samples used to evaluate the antisickling activity of the plant extracts were taken from known drepanocitary adolescent patients attending the Centre de Médecine Mixte et d'Anémie SS and Centre Hospitalier Monkole, both located in Kinshasa area, R. D. Congo. In order to confirm their SS nature, the above-mentioned blood samples were first characterized by electrophoresis on cellulose acetate gel, as previously reported by Mpiana *et al*. (2007a). They were found to be SS blood and were then stored at ±4°C in a refrigerator.

Biological activity: Blood sample was placed in contact with plant extracts at different concentrations for 24 h (with the physiologic solution 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 by Mpiana *et al*. (2007a).

Fractionation: The thin layer chromatography was run on Merck plate with chloroform-benzene (2:1) mixture as eluting solvent. The resulting developed plates were visualized by ultra-violet light at 254 and 365 nm. Separation was realised by column chromatography using silica gel with the same solvent system.

Thermal degradation: Anthocyanins aqueous solutions were placed an oven at different temperatures for different periods time. Solution absorbances estimated spectrophotometrically.

Mathematical model and data analysis: Anthocyanin thermal degradation can be considered as a chemical reaction, thereby 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:

$$A \xrightarrow[\Delta]{k} B \quad (1)$$

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

$$\frac{dC_A}{dt} = kC_A \quad (2)$$

where, C_A and t are respectively the concentration of A and degradation time.

The integration of Eq. 2 gives:

$$C_A = C_A^o e^{-kt} \quad (3)$$

where, C^0_A is the initial concentration of A.

If A is the only compound that absorbed light at a chosen wavelength, the Lambert Beer equation for this case would be:

$$E = l\varepsilon C_A \quad (4)$$

and

$$E_0 = l\varepsilon C^0_A \quad (5)$$

where, E, E_0, l and ε are, respectively the absorbance at time t, the absorbance at time t = 0 sec, the optic pathway and the extinction coefficient.

The combination of Eq. 3, 4 and 5 gives:

$$E = E_0 e^{-kt} \quad (6)$$

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

$$E = l(\varepsilon_A C_A + \varepsilon_B C_B) \quad (7)$$

where, ε_A, ε_B, C_A and C_B are, respectively compounds extinction coefficients and concentrations.
Considering that:

$$C^0_A = C_A + C_B \quad (8)$$

and combining Eq. 3, 5, 7 and 8 provide

$$E = E_\infty + \varepsilon_A + \varepsilon_A E_0 e^{-kt} \quad (9)$$

Where:

$$E_\infty = l\varepsilon_A E_0 C^0_A$$

If $\varepsilon_A > \varepsilon_B$ Eq. 9 gives the same exponential decreasing trend as does the Eq. 6.

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

RESULTS AND DISCUSSION

Antisickling activity of anthocyanins total extract: Figure 1 and 2 shows the morphology of SS blood erythrocytes (control) and that of SS blood erythrocytes in the presence of anthocyanins total extract of *H. acida* T.

The above micrographs show that, the control contains the majority of sickle-shaped erythrocytes, confirming the SS nature of the blood (Fig. 1). Treatment with anthocyanins extract (Fig. 2) resulted in the reversal of the majority of erythrocytes to the normal shape. This observation show antisickling activity of anthocyanins total extract and accordingly may explain the usefulness of *H. acida* in traditional medicine (Ibrahim *et al*., 2007; Mapiana *et al*., 2007b, c, 2008a, b).

Anthocyanins have been shown to act as powerful antioxidants helping to protect living cells from free radicals formed during metabolic processes. There is nowadays, considerable interest in the possible health effects of anthocyanins in humans due to their reported positive effects on blood vessel walls (Kahkönen *et al*., 2003a, b; Mian *et al*., 1977; Wang *et al*., 1997). The observed biological activity of anthocyanins may also be probably due to their non covalent binding reaction to proteins (Charpentier *et al*., 1998; Wagner *et al*., 1984). Indeed, a possible interaction of anthocyanins with S haemoglobin may inhibit the polymerization of this haemoglobin in hypoxic conditions.

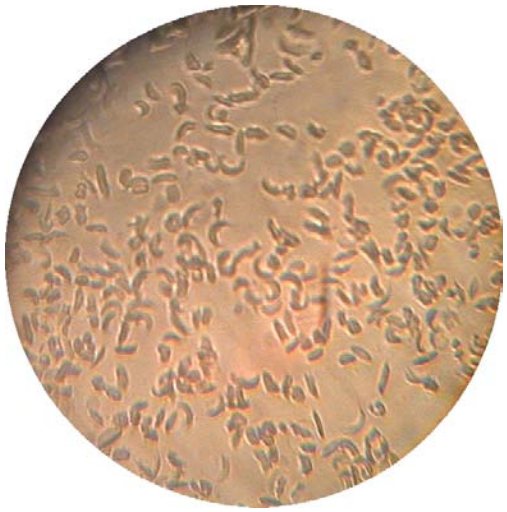

Fig. 1: Morphology of drepanocytes of non treated SS blood (control)

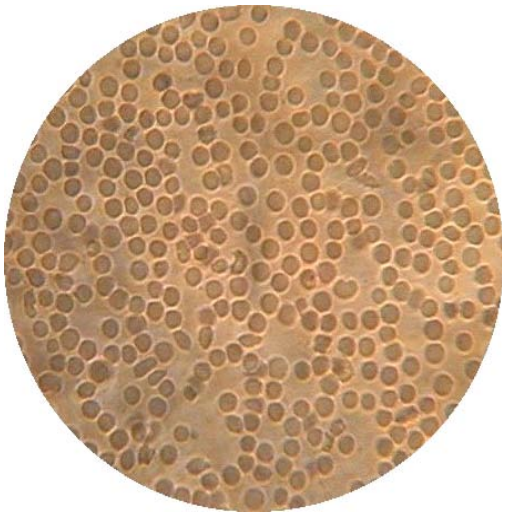

Fig. 2: Morphology of drepanocytes treated with anthocyanins extract

Antisickling activity of isolated anthocyanin fraction: Chloroform-benzene (2:1) mixture was used to separate anthocyanins total extract into three different fractions which R_f are 0.21, 0.37 and 0.62. These were coded fractions A_1, A_2 and A_3 after fractionation using column chromatography. Antisickling activity of these fractions was tested and fraction A_1 was found to be the most active (Fig. 3).

Figure 3 shows that the erythrocytes have been normalized compared to the no treated control (Fig. 1). Therefore, this anthocyanins fraction may be responsible

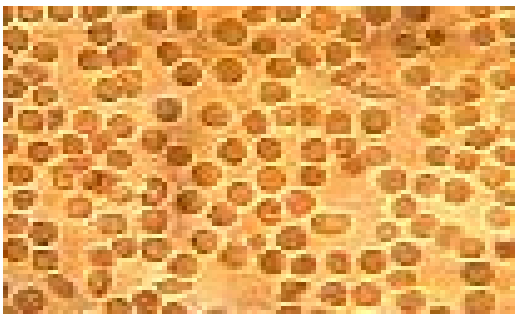

Fig. 3: Sickle cell erythrocytes treated with anthocyanins fraction A_1

for the antisickling activity of *H. acida*. Purification and structural identification of the active fraction is still in progress.

Thermostability of anthocyanin fraction A1: It is known that the anthocyanin compounds are unstable towards some physical and chemical factors such as temperature, UV-visible radiation, pH, etc. (Kahkönen *et al*., 2003a). Since, this plant is used in folk medicine either in infusion or decoction, it has been decided to evaluate the behaviour of purified anthocyanin at high temperature. Figure 4 is UV-visible spectra of fraction A_1 without exposure to heat and after being exposed to heat at 100°C for a period of 45 min and 24 h.

The spectrum of fraction A_1 shows an absorption peak at 268 nm assigned to π—π^* transition of flavylium ion which is the basic structure of anthocyanin (Kahkönen *et al*., 2003b).

Heating this fraction at 100°C drastically modified its absorption spectrum showing the degradation of the anthocyanin fraction when it is heated. A total modification was observed after only 45 min of heating.

It is interesting to follow the absorbance evolution of this anthocyanin fraction with the temperature at a constant exposure time.

The absorbance variation at λ = 268 nm of this anthocyanin fraction with the temperature at constant time is shown in Fig. 5.

Figure 5 shows a linear decrease in absorbance of the anthocyanin fraction with the increasing of temperature. This demonstrates the degradation of the isolated anthocyanins as temperature increases. The slope of the line is more important for 150 min of exposure than for 120 min, showing the influence of exposure time on the anthocyanins degradation.

The impact of exposure time on the isolated anthocyanin at constant temperature is the kinetics of thermodegradation.

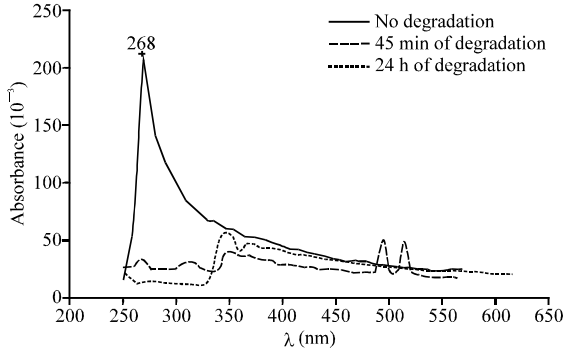

Fig. 4: Spectra of anthocyanin fraction A_1 before and after exposure to heat

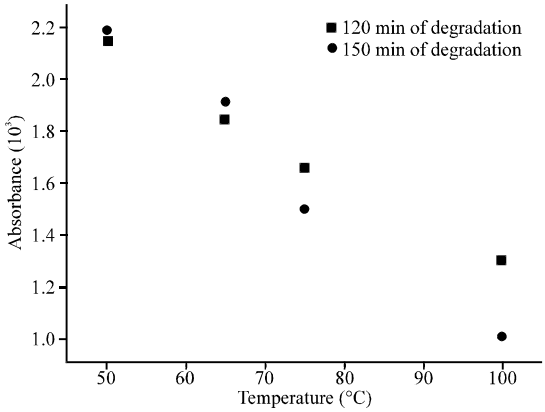

Fig. 5: Absorbance variation of anthocyanin A_1 fraction at wavelength 268 nm with different temperatures at constant time of exposure

Kinetics of thermodegradation of anthocyanin fraction: Figure 6 and 7 show the absorbance variation of isolated anthocyanin fraction with time at 100 and 120°C.

Figure 6 and 7 show considerable absorbance decrease with exposure time at the temperature of 100 or 120°C, respectively.

Before reaching a constant trend, the decrease is exponential at the initial stage of the reaction. This exponential evolution is characteristic of concentration variation with the reaction time as in a first order reaction kinetics.

Although the molecular mass of isolated anthocyanin molecule is not yet known, a fitting of experimental values using the equation of the absorbance variation as a function of exposure time was done. The fitting of experimental data with Eq. 6 where, only the degraded anthocyanin is supposed to absorb, does not give satisfactory results. However, the fitting using the Eq. 9 model where the initial anthocyanin molecule A and its

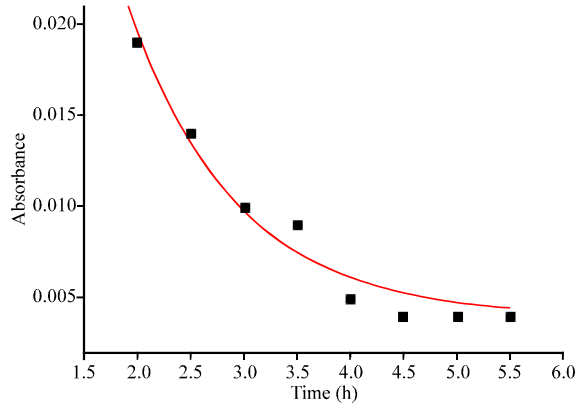

Fig. 6: Absorbance variation of isolated anthocyanin fraction with exposure time at 100°C (λ = 268 nm)

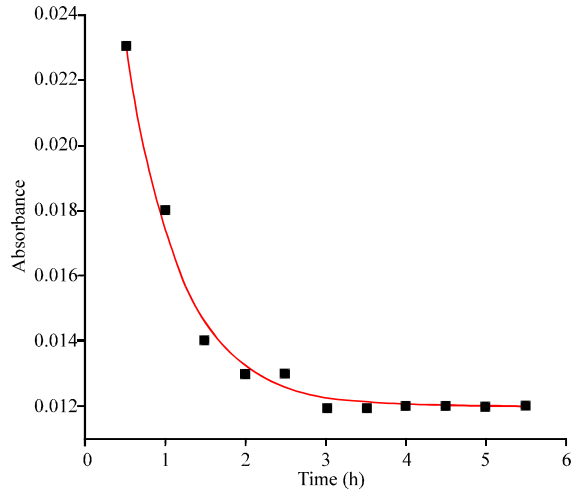

Fig. 7: Absorbance variation of isolated anthocyanin fraction with exposure time at 120°C (λ = 268 nm)

Table 1: Determined thermodegradation speed constants of isolated anthocyanin fraction and extinction coefficients at 268 nm

	Temperatures (°C)	
Parameters	100	120
ε_A (mol^{-1} l cm^{-1})	5.3×10^{3}	5.3×10^{3}
ε_B (mol^{-1} l cm^{-1})	5.2×10^{2}	5.2×10^{2}
k (s^{-1})	2.64×10^{-4}	4.08×10^{-4}

ε_A: Extinction coefficient of none degradated anthocyanins isolated fraction
ε_B: Extinction coefficient of degradated anthocyanins isolated fraction
k: thermodegradation speed constant of isolated anthocyanin fraction

degraded form B absorb at that wave length, agrees with experimental results. Obtained parameter values are provided in Table 1.

Table 1 elucidates that the speed constant at 120°C is greater that at 100°C with a ten fold factor which confirm the temperature effect in anthocyanin degradation. However, obtained kinetics constants are low.

CONCLUSION

The antisickling activity of *Hymenocardia acida* is due to anthocyanins. This confirms by earlier results that attribute this activity of some Congolese plants to anthocyanins. Anthocyanins were for the first time chromatographically separated, the most active fraction determined and the thermodegradation kinetics studied. The first order kinetics model permitted to determine speed constants for two temperatures of work. As the heat degrades anthocyanins, decoction and infusion of this plant are not recommended. Maceration would be the best preparation mode for traditional recipes.

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