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Antisickling activity of three species of *Justicia* from Kisangani (D.R. Congo): *J. tenella, J. gendarussa* and *J. insularis*

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ABSTRACT

Some medicinal plants have these last years, shown an antisickling activity. What indicates a new therapeutic way to the range of the poor African populations which are affected by this hemoglobinopathy. *Justicia secunda* is among these plants and it is thus necessary to check whether the other species of *Justicia* are also active. Emmel and hypoxic induced sickle erythrocyte hemolysis bioassays were used to evaluate the effect of *Justicia gendarussa L.*, *Justicia insularis* T. Anderson and *Justicia tenella* (Nees) T. Anderson leaves and anthocyanins extracts on sickle cells. The results obtained indicate that all these three collected species from Kisangani and its surrounding, located at the North-East of the D.R. CONGO, showed an antisickling activity. The chemical screening performed on these plants showed in these three species the presence of polyphenols of which anthocyanins. The tests carried out with anthocyanins extracts showed a significant activity of these metabolites with a normalization rate of the form of the sickle cells of 87% for *Justicia gendarussa*, 92% for *Justicia insularis* and 80% for *Justicia tenella*. The minimal concentrations in anthocyanins necessary to have maximal normalization are respectively of 7.2 µg/mL for Justicia *insularis*, 7.6 µg/mL for Justicia *gendarussa* and 7.7 µg/mL for *Justicia tenella.* The results obtained for these three species of *Justicia* confirm those already obtained with species: *Justicia secunda*. That indicates similarity of these species in their phytochemical composition and biological activity.

Keywords: antisickling, *Justicia*, Emmel test, hemolysis test, normalization rate

INTRODUCTION

Several diseases cause million deaths in the world, and particularly in Africa. Among these, is the sickle cell disease (SCD) that affects more than 50 million people. Each year, nearly 100,000 children are born worldwide with this hemoglobinopathy (Buchanan, 2004; Girot and Bégué, 2003).

The replacement of a polar amino acid by a less polar one causes a polymerization of hemoglobin S in the red blood cells. This aggregation modifies the shape of blood cells and makes them fragile and less flexible; what causes many complications of sicklers (Girot and Bégué, 2003).

Several therapeutic options were tried in order to fight against SCD without

appropriate solution for poor African population. All of these therapeutic approaches are either expensive or toxic and are not accessible to the populations with low incomes (Girot and Bégué, 2003; Mehanna, 2001).

People in developing countries have resort to medicinal plants in order to treat SCD. This could be proposed as an alternative solution to treat this disease. Indeed, several studies showed that among plants quoted in traditional medicine as being able to treat SS anemia, a good number showed an *in vitro* antisickling activity (Akjie and Fung, 1992; Mpiana et al., 2007a, 2007b, 2007c, 2008, 2009a, 2009b, 2009c, 2009d, 2009e, 2010a, 2010b, 2010c).

Our research team listed indeed, a number of plants used in traditional medicine against drepanocytosis in D.R. Congo and tested their antisickling activity (Mpiana et al., 2007a, 2007b, 2007c, 2008, 2009a, 2009b, 2009c, 2009d, 2009e, 2010a, 2010b, 2010c). More recently (Mpiana et al., 2010b), a research on *Justicia secunda* Vahl (Acanthaceae) showed that anthocyanins extracts play a significant role in the stabilization of the cellular membrane and the inhibition of the polymerization of hemoglobin S.

The aim of this study is to evaluate the antisickling activity of three other species of *Justicia* genus: *Justicia gendarussa L.* , *Justicia insularis* T. Anderson and *Justicia tenella* (Nees) T. Anderson and to detect a probable similarity in their phytochemical composition and biological activities with *Justicia secunda* Vahl.

MATERIALS AND METHODS

Plant material

The tested plants were gathered in various sites in Kisangani and its surroundings. Their identification was carried out by M. Bola, Mabay and Kombozi in Herbarium of the Faculty of Science of the University of Kisangani. Voucher specimen are deposited at the INERA (Institut National d`Etudes et de Recherches Agronomiques) Herbarium in Yangambi on the numbers 14645 (*J. tenella*), 9419 (*J. Insularis*) and 13633 (*J. gendarussa*).

Extraction and chemical screening

The dried and powdered plant material (leaves, 10 g) was repeatedly extracted by cold percolation with 95% EtOH and water (100 ml x 1) for 48 hrs. Fractions were filtered and concentrated to dryness under reduced pressure using a rotary evaporator. A chemical screening has been done on the three plants using an aqueous or organic fraction following an established protocol (Bruneton, 1999). Extraction of anthocyanins was then done by maceration of 100 g of dried powdered plant material in 200 ml of acidified methanol (0,01% v/v HCl) according to the universal procedures (Bruneton, 1999; Francis, 1989).

Biological material

The sodium citrate suspension of blood samples used in the evaluation of the antisickling activity of the plant extracts in this study were taken from known sickle cell children and adolescent patients attending the "Centre de Gracia Fondation", in Kisangani area, D.R. Congo. 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 SS nature, the above-mentioned blood samples were first characterized by hemoglobin 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 assays

In order to evaluate the antisickling activity of our plant extract samples, two *in vitro* antisickling assays were performed:

Emmel test (Coutejoie and Hartaing, 1992)

Sickle cell blood was diluted with 150 mM phosphate buffered saline (NaH_2PO_4 30 mM, Na_2HPO_4 120 mM, NaCl 150 mM) and mixed with an equivalent volume of 2% sodium metabisulfite. A drop from the

mixture was spotted on a microscope slide in the presence or absence of plant extracts and covered with a cover slip. Paraffin was applied to seal the edges of the cover completely to exclude air (Hypoxia). The red blood cells (RBC) images were treated with a computer assisted image analysis system (Motic Images 2000, version 1.3) and statistical data analysis and curves were processed using Microcal Origin 6.1 package software.

Hemolysis test (Iyamu et al, 2002; Mpiana et al., 2010 b)

The effect of the extracts on the capacity of the drepanocytes to hydrate itself while resisting to hemolysis, following a hypsometric shock is exploited as index of the biological activity of these drugs. So, washed SS erythrocytes were put in contact with sodium metabisulfite 2% in presence or absence of various extracts. At determined intervals of time, a diluted aliquot with NaCl 0.9% was and centrifuged (5 min to 4000 g). The absorbance of the treated or not treated supernatant solution of hemoglobin S was measured at 540 nm at various incubation times.

RESULTS

Phytochemical screening and anthocyanins extraction

The phytochemical screening revealed the presence of saponins, sterols, terpenes and anthocyanins in the three plants. Leuco-anthocyanins and tanins were present in *Justicia gendarussa* and *Justicia insularis*, while alkaloids were found only in *J. tenella*. The yield of extraction of the anthocyanins on leaves powders of the studied plants are respectively 5.14 g; 15.98 g and 8.47 g for *J. gendarussa*, *J. insularis* and *J. tenella*.

Antisickling activity

Effect of plants crude extracts on sickle cell morphology

Figures 1 and 2 show respectively micrographies of SS blood alone in a NaCl 0.9% solution (control) and the SS blood incubated with the aqueous total extract of *J. insularis.*

It should be noted that the extracts of two other species of *Justicia* also give the same type of micrographies as *J. insularis*.

As the anthocyanins extracted from *Justicia secunda* Valh showed antisickling activity (Mpiana et al., 2010b), we wanted to see whether the antisickling activity of three other species of *Justicia* is due to anthocyanins or not.

Effect of anthocyanins extracts on sickle cell morphology

Figures 3 to 5 show micrographies of the drepanocytes in the presence of anthocyanins extracted from these three species of *Justicia* genus.

It is necessary to point out that there is not yet a standard molecule which can be used as positive control for antisickling activity. Whatever, we can study the concentration effect of anthocyanins of these three plants on the rate of normalization of drepanocyte forms and determine the minimal concentration necessary to have a maximum of normalization, called minimal concentration of normalization (MCN) or the concentration that can normalize 50% of drepanocytes (ED_{50}) in order to compare their values to those of other plants.

Minimal concentration of normalization

Figure 6 gives the evolution of the rates of normalization of the form of the drepanocytes according to the concentration of anthocyanins extracted from the three species of *Justicia*.

These curves show that the normalization rate or the percentage of the drepanocytes which regain the normal shape increases with the concentration of anthocyanins for the three extracts. At a certain concentration, this rate reaches a maximum value which remains constant although the increase of the anthocyanins concentration. The values of MCN, ED_{50} and maximal rates of normalization calculated from these curves are shown in Table 1.

Hemolysis test

Resistance to the lysis of the erythrocytes of SS blood of various preparations with or without anthocyanins extracts, subjected to the hemolysis test is represented in Table 2. This table gives the absorbance of the supernatant solution of the SS red blood solution in the presence or in the absence of anthocyanins extracts.

As it can be seen in the Table 2, the absorbance of SS blood solution increases with time. This indicates that there is hemolysis of erythrocytes so hemoglobin is found more and more in solution. Indeed, during 60 min of incubation, the absorbance of the control solution passes from 0.38 to 1.00, means 163% of increase. But a contrary effect is noticed when anthocyanins extracts are added to SS blood. The absorbance of the supernatant solution decreases with time indicating the decrease of erythrocytes hemolysis with the time of incubation. This is the same for all used species of *Justicia*.

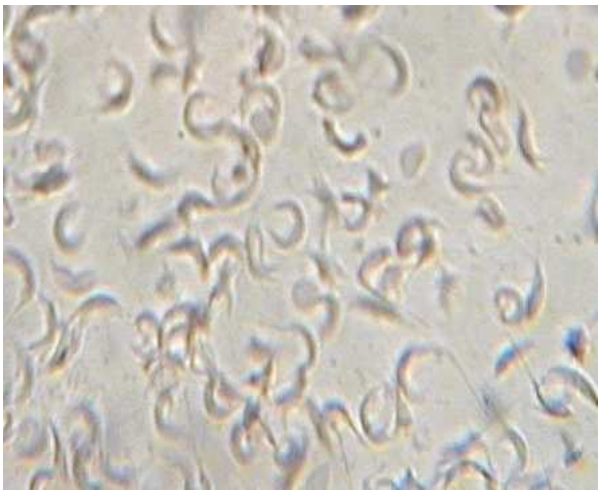

Figure1: Morphology of drepanocytes of untreated SS blood (control) (X500), [NaCl 0,9% ; $Na_2S_2O_4$ 2%].

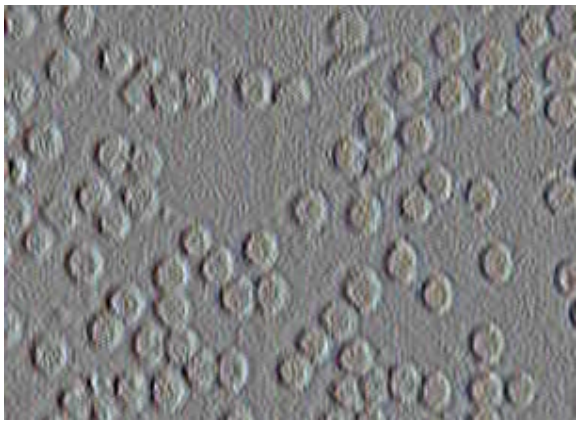

Figure 2 : Morphology of drepanocytes treated with 50 µg/mL of ethanolic extract of *Justicia insularis* (X500), [NaCl 0,9% ; $Na_2S_2O_4$ 2%,].

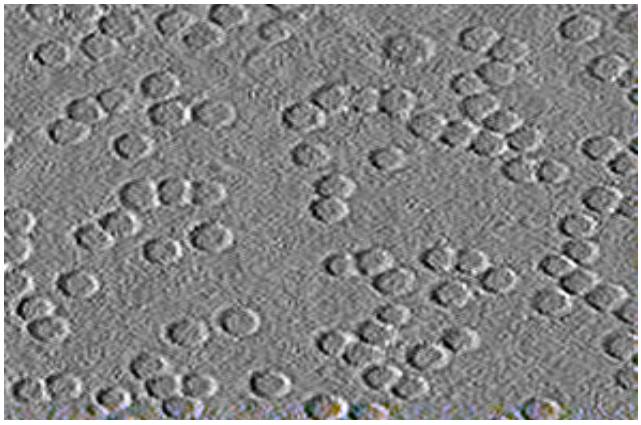

Figure 3: Morphology of drepanocytes treated with anthocyanin extracts (10 µg/mL) of *Justicia insularis*, [NaCl 0,9% ; $Na_2S_2O_4$ 2%, X500].

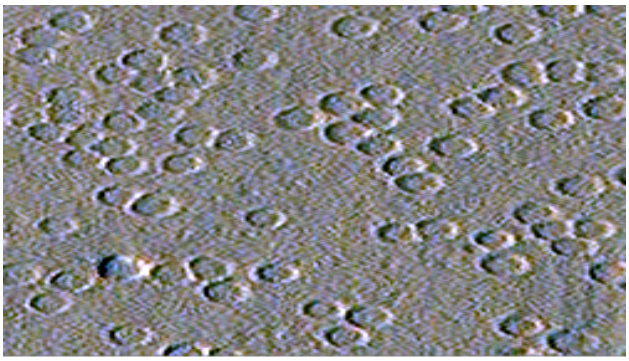

Figure 4 : Morphology of drepanocytes treated with anthocyanin extracts (10 µg/mL) of *Justicia tenella* , [NaCl 0,9% ; $Na_2S_2O_4$ 2%, X500].

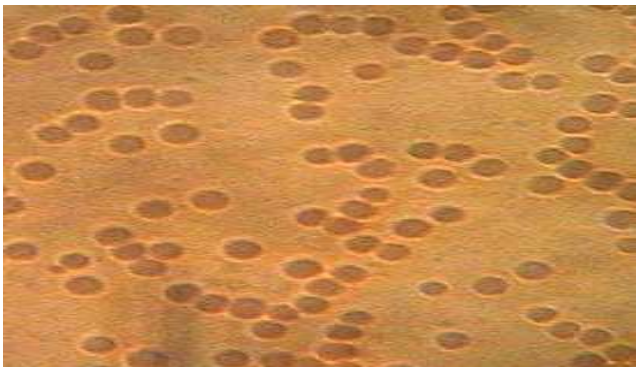

Figure 5: Morphology of drepanocytes treated with anthocyanin extracts (10 µg/mL) of *Justicia gendarussa* , [NaCl 0,9% ; $Na_2S_2O_4$ 2%, X500].

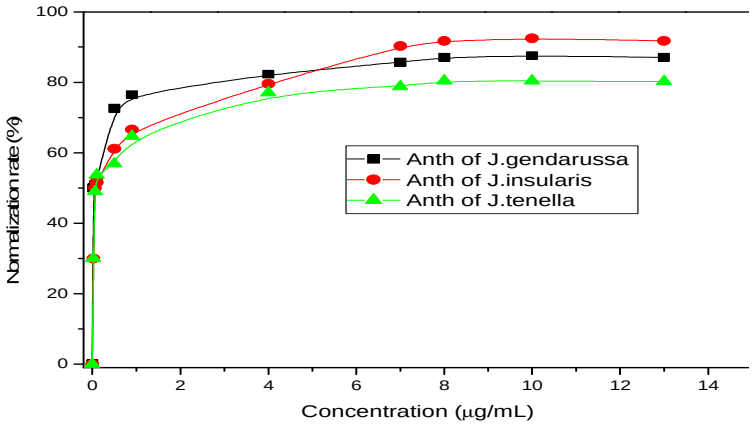

Figure 6 : Evolution of the normalization rate of the drepanocytes form with the anthocyanins concentration.

Table 1: Values of ED_{50}, MCN and maximal rates (NR_{max}) for the anthocyanins of the studied plants.

Plants	ED_{50} (µg/mL)	NR_{max} (%)	CMN (µg/mL)
Justicia gendarussa	0.44	87.1	7.6
Justicia insularis	0.38	92.1	7.2
Justicia tenella	0.51	80.4	7.7

Table 2: Anti-hemolysis effect of the anthocyanin extracts (1.4 mg/L) of *Justicia gendarussa, Justicia insularis and Justicia tenella* on drepanocytes.

Samples	Absorbance at 540 nm						
	0 min	10 min	20 min	30 min	40 min	50 min	60 min
Hb SS	0.38	0.40	0.48	0.50	0.59	0.60	1.00
Hb SS + JG	1.80	1.65	1.60	1.50	1.45	1.40	1.30
Hb SS + JI	1.21	1.00	0.93	0.89	0.85	0.80	0.71
Hb SS + JT	1.60	1.50	1.30	1.10	1.00	0.98	0.96

Hb SS: SS blood untreated with the anthocyanin extract (control)
Hb SS + JG: SS blood treated with the anthocyanin extract of *Justicia gendarussa*
Hb SS + JI: SS blood treated with the anthocyanin extract of *Justicia insularis*
Hb SS + JT: SS blood treated with the anthocyanin extract of *Justicia tenella*

DISCUSSION

As it was already shown by the chemical screening performed on *Justicia secunda* (Mpiana et al., 2010b), these three other species of *Justicia* also contain anthocyanins. The calculated values of the output in anthocyanins show that *J. insularis* (16.0%) presents the highest content of anthocyanins, while *J. gendarussa* (5.1%) presents the lowest. When compared to *Alchornea cordifolia* (1. 28%) and *Maesopsis eminii* (2. 36%), these three plants are richer in anthocyanins (Mpiana et al., 2007b; Mpiana et al., 2009e).

By comparing micrographies of SS blood (control) and that of SS blood in the presence of plant extracts, one can notice that, the negative control contains the majority of

sickle-shaped erythrocytes; this confirms the SS nature of the blood (Fig. 1). When the SS blood is mixed with the crude aqueous extracts of *Justicia species* (Fig. 2), the majority of sickle-shaped erythrocytes are reversed into normal and biconcave shape. This shows that these three species of *Justicia* have an antisickling activity as it was recently shown in *Justicia secunda*, a specie used against anaemia in general and SS anaemia in particular, in Congolese traditional medicine (Mpiana et al., 2010b). It should be noted that although one of the studied species *J. gendarussa* is known for its medicinal properties, the literature search does not announce the use of one of these plants against sickle cell anaemia. It is thus interesting to seek in the same genera of the species used in traditional medicine, in order to quote if their phenotype and genotype similarities can influence biological activity. Indeed, one of the first plants to be tested for the antisickling activity was *Fagara zanthoxyloides* Lam. Other species of the same genus showed antisickling activity although it was not quoted in the literature as having this activity (Elekwa et al., 2005; Mpiana et al., 2008).

The micographies of SS blood in the presence of anthocyanins extracted from these three plants (Figs. 3-5), compared to the negative control (Fig. 1), showed that the majority of the drepanocytes reversed their shapes to the normal biconcave form. This confirms the activity of the anthocyanins on the normalization of the erhytrocytes form. The same results was observed for the anthocyanins extracts from several plants used in traditional medicine in D.R. Congo against sickle cell anaemia (Mpiana et al., 2007b, 2007c, 2008, 2009a, 2009b, 2009c, 2009d, 2009e, 2010a, 2010b, 2010c).

From the calculated MCN values of the anthocyanins of these three species of *Justicia* compared to those of other studied plants, it can be seen that these species of *Justicia* present higher MCN values than those of *Zizyphus mucronata* (0.9 µg/mL) (Mpiana et al., 2008) and of *Alchornea cordifolia* (0.097 µg/mL) (Mpiana 2007b); but lower than for *Ocimum basilicum* (13 µg/mL) (Mpiana et al., 2007c).

The TN_{max} values of anthocyanins of all the studied species of *Justicia* are higher than that of *Trema orientalis* (80.0%); while *Zizyphus mucronata* (88%) has a NR_{max} superior to that of *J. gendarussa* and *J. tenella*, but lower than that of *J. insularis* (Mpiana et al, 2007c, 2009b).

The analysis of the influence of anthocyanin extracts on the hemolysis of the drepanocytes (Table 2), indicate that the absorbances decreased with 28% for *J Gendarussa*, 41% for *J. insularis* and 40% for *J. tenella.* These results show that anthocyanins of these plants have an antihemolytic activity on the erythrocytes of SS blood. This fact was also noticed for *J. secunda* (Mpiana et al., 2010b).

Indeed, one of the major signs of the drepanocytosis is a chronic anemia. This anemia is due to a precocious hemolysis of SS blood erythrocytes. An antihemolytic activity is thus an essential quality of an antisickling agent.

The properties of anthocyanins to adsorb themselves on proteins would block the polymerization of the desoxyhemoglobin S in tactoids; this could reduce the sickling process and thus, induce the return to the normal biconcave form of the erythrocytes as the Emmel test reveals it. The sickling modifies the membrane flexibility, which would make it more fragile and would increase the precocious risk of hemolysis.

But it is as possible as the anthocyanins, according to their antioxydant or free radical scavenger effect, prevent hemoglobin from oxidizing in methemoglobin and inhibit the generation of free radicals. It is thus probable that the anthocyanin extracts exert this protective effect according to their reducing properties preventing that the lipids membrane, hemoglobin and the enzymatic equipment are destroyed or inactivated by oxidation (Misra and Fridovich, 1972; Hebbel et al., 1982; Mpiana et al., 2010b).

Conclusion

Research on *Justicia secunda* Vahl, in order to explain its use in management of SCD by Congolese traditional practitioners confirmed the activity of the anthocyanins using Emmel, Itano and osmotic fragility tests. In this work, three other species of *Justicia* genus showed also antisickling activity which could be due to the presence of anthocyanins in these plants. This indicates that there is a similarity between these species of a same genus not only on their phytochemical composition but also on their biological activity.

Studies on other species of *Justicia*, fractionation of anthocyanin extracts and structure elucidation of isolated compounds are in progress.

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