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Research Article

Ethno-Pharmacological Survey, In Vitro Anti-Sickling and Free Radical Scavenging Activities of Carapa Procera DC. Stem Bark (Meliaceae)

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Abstract

Drepanocytosis is a genetic and neglected disease, endemic in negroids population. One of the main characteristics of this pathology is the production of a large amount of free radicals, leading to a severe oxidative stress and the consumption of NO by free oxygen radicals, and/or by cell-free plasma heme. The consequences of this defect are hemolytic anemia and tissue damage brought about by the blockage of blood vessels by the sickled cells. The present study evaluated the antisickling and radical scavenging activities of extracts from *Carapa procera* stem bark using Emmel's test and the DPPH assay. *Carapa procera* was selected through an ethno-pharmacological. The results showed that methanolic, ethyl acetate and dichloromethane soluble fractions, anthocyanins and organic acids exhibited a significant antisickling as revealed by the observed normal biconcave form of sickle erythrocyte (normalization rate > 70%) in hypoxic conditions. Methanolic extract exhibited a good radical scavenging activity (ED_{50} = 1.698 ± 0.079 µg/mL). The chemical screening performed on the plant revealed the presence of anthocyanins and organic acids which were then extracted. Total anthocyanins and organic acids revealed interesting antisickling and antioxidant properties that could justify the integration of *Carapa procera* in Congolese pharmacopoeia for the management of sickle cell disease. Bioactive extracts from this plant species could increase nitric oxide by scavenging free oxygen radicals. For the best our knowledge, *Carapa procera* has not been yet previously reported as antisickling plant in the traditional medicine database of Democratic Republic of the Congo.

Keywords: *Sickle cell disease, ethnopharmacology, Calotropis, anthocyanins, Sulfanilic Acids*

Introduction

Drepanocytosis also known as Sickle cell disease (SCD) is a life-long blood disorder characterized by erythrocytes that assume an abnormal, rigid, sickle shape. It is a genetically inherited disease in which a single base substitution in the gene encoding the human β-globin subunit results in replacement of β6 glutamic acid by valine, leading to the devastating clinical manifestations of SCD [1, 2]. This substitution causes drastic reduction in the solubility of sickle cell hemoglobin (Hb S) when deoxygenated. Under these conditions, the Hb S molecules polymerize to form long crystalline intracellular mass of fibers which cause the deformation of the biconcave disc shaped erythrocyte into a sickle shape. The consequences of this defect are hemolytic anemia and tissue damage brought about by the blockage of blood vessels by the sickled cells. The complications can be severe and include retarded growth, periodic attacks of pain and progressive organ dysfunction leading in most cases to a much reduced life expectancy [3]. Each year about 300,000 children are born with pathological hemoglobin of which 70% are affected by SCD. Most of them die before the age of five years when they do not receive regular medical care. In Democratic Republic of the Congo, almost 2% of the population is sicklers [3-5].

The first-line clinical management of SCD includes medullar transplantation, repeated blood transfusion to stabilize the patient's hemoglobin level, and the use of chemical agents which interfere with the mechanism and/or kinetics of the sickling process. Unfortunately, all current proposed therapies are quite expensive and have attendant risk factors in terms of clinical use [6-8]. Therefore, there is a need for more definite and effective treatments for the disease. Herbal extracts have been used in African folk medicine for decade in the management of various ailments [9-12].

Recently our research team shows that many medicinal plants used in traditional medicine in DRC to manage SCD had *in vitro* antisickling activity and that this activity is mainly due to anthocyanins [13-20]. As several other flavonoids, anthocyanins are natural products with a range of biological activities including free radical scavengers and show antioxidant activity [21, 22]. Our previous studies have also identified organic acids as antisickling agents [15, 23]. *Carapa procera* (Syn: *Carapa guineensis* Sweet ex A. Juss.;

Carapa gummiflua C. DC.; *Carapa touloucouna* Guillem. Ex Perr.; *Granatum surinamensis* (Miq.) Kuntze) is a plant from Meliaceae family. During an ethno-botanical survey, it was reported that *Carapa procera* (known under the vernacular name of "Ngenzo", Ngbandi name) is traditionally used by indigenous peoples to treat malaria (bark decoction), worms (decoction of leaves and roots), coughs and respiratory ailments (bark decoction). Since Meliaceae family are reported to display antisickling properties, it can therefore, be hypothesized by chemotaxonomy that *C. procera* could inhibit the sickling of red blood cells and the radical oxygen species formation within sickle erythrocyte.

The present study was performed with the aim of evaluating the antisickling and free radical scavenging activities of different fractions, anthocyanins and organic acids extracts of *Carapa procera*. For the best of our knowledge, this plant has not yet been scientifically investigated for its antisickling properties.

Method

Ethno-botanical survey

Ethno-botanical information about the plant species selected for this study was obtained by interviewing traditional healers during field work which was conducted in the villages surrounding the Salonga National Park (Equateur, Democratic Republic of the Congo). Surveys were conducted from January to March 2011. A total of 10 traditional healers were interviewed. Informants were selected for their authentic knowledge on the utilization of medicinal plants. Lingala, the national language of Equateur was used during anthropological interviews. Traditional healers were interviewed on a voluntary basis. The study followed principles laid out in the Declaration of Helsinki as previously reported [24]. The questionnaires were divided into three sections: (i) personal information such as name, age, sex, marital status and studies level; (ii) traditional medicine practice (including knowledge of diseases and symptoms); (iii) plant vernacular names, plant part used, preparation methods, and administration route of remedies. Informed consent was obtained from both the provincial Government of Equateur to collect plant samples and to conduct non-commercial research on Congolese medicinal plants and the respondents to divulge information. A benefit-sharing agreement on mutually agreed terms was also established between University of Kinshasa and local community according to the principles laid out in the Nagoya protocol [25-27].

2. Selection and plant material collection

The tested plant material uses in this study were collected from *Carapa procera* by Professor K.N. Ngbolua during a field work in the Salonga National Park (Equateur province, Democratic Republic of the Congo) in March 2011 and were authenticated by Botanist Mathieu Bolaa and Mr B.L. Nlandu of the INERA (Institut National d'Etudes et Recherches Agronomiques). Voucher specimen is on deposit at the INERA Herbarium of the Faculty of Science (Université de Kinshasa). The plant species *Carapa procera* was selected based on its relative citation frequency and the informant consensus factor value.

3. Extraction and chemical screening

The dried and powdered plant material (stem bark, 10 g) was repeatedly extracted by cold percolation with 95% ethanol (EtOH) and water (100 mL x 2) for 48 hours. Chemical screening was done in aqueous and organic extract according to a well known protocol as previously reported [28]. Fractions were filtered and concentrated to dryness under reduced pressure using a rotary evaporator. Extraction of anthocyanins was then done using 100 g of dried powdered plant material with acidified methanol (1% HCl) following an established protocol [13-20]. Anthocyanins extract was then defatted by n- hexane. Organic acids were extracted according to the protocol of Ouattara et al. with minor modification [29]. Briefly, the powdered stem barks of *C. procera* (50 g) were macerated with 100 mL of methanol-H_2O (50/50) and then percolated with 400 mL of the same solvent at room temperature. The extract was concentrated under reduced pressure until 100 mL. The aqueous solution was basified to pH 9 with Na_2CO_3 and repeatedly extracted with ether. The aqueous solution was then acidified with 4% acetic acid. The resulting acidic (pH 3) solution was repeatedly extracted by ethyl acetate. The solution were dried over Na_2SO_4 and concentrated to give organic acids crude extract.

4. Preparation of methanol extract and increasing polarity extracts

Plant powder (100 g) was macerated in methanol 80% (1L x 2) for 48 hours. After filtering the mixture, the aqueous-methanolic filtrate was concentrated under reduced pressure using a rotary evaporator. The methanolic extract was suspended in distilled water and sequentially partitioned with n-hexane, dichoromethane, ethyl acetate, ethanol, and methanol (1:1, v/v) three times at room temperature. The resulting fractions were evaporated to dryness on an evaporator apparatus. All extracts were stored at +4 °C.

5. Biological material

Blood samples used to evaluate the antisickling activity of the plant extracts in this study 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, 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, as previously reported [5]. They were found to be *SS* blood and were then stored at ± 4 °C in a refrigerator. An informed consent was obtained from all the patients participating in the study. All the research procedures have received the approval of Department of Biology Ethics Committee.

5.1. Antisickling assay

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 ethyl acetate, methanolic or anthocyanins extracts and covered with a cover slip. Paraffin was applied to seal the edges of the cover completely to exclude air (Hypoxia). Duplicate analyses were run for each extract. The RBCs were analyzed 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; Motic Chine Group Co LTD) and statistical data analysis were processed using Microcal Origin 6.1 package software.

5.2. Free radical scavenging assay

The DPPH free radical (1,1-diphenyl-2- picrylhydrazyl) scavenging assay was carried out as previously reported [30]. The radical scavenging activity of extracts for DPPH free radical was measured on the principle that antioxidants reduce the DPPH radical to a yellow-coloured compound (diphenylpicrylhydrazin) and the extent of the reaction will depend on the hydrogen donating ability of the antioxidant. Briefly, a 100 µM solution of DPPH radical in methanol was prepared. 3,5 mL of this solution added to 0,5 mL solution of each extract in methanol at concentrations ranging from 0,1 to 1 mg/mL, thus obtaining the desired final concentrations in the reaction mixture. The mixture was shaken vigorously and incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using a spectrophotometer SP- 1105 Brand model. Methanol was used as a blank. The control solution consist of 0,5 mL of methanol and 3,5 mL of DPPH radical solution. The antiradical activity of a sample (calculated by the following formula) is given as percentage of reduced DPPH free radical: %I = [(OD control - OD sample)/OD control] ×100. The IC50 value (µg/mL) is the effective concentration at which DPPH radicals were scavenged by 50%. L-ascorbic acid was used as positive control. Duplicate analyses were run for each extract.

Results and Discussion

1. Ethno-Botanical Survey

During ethno-botanical survey, ten traditional healers were interviewed about medicinal plants used both in folk medicine and eaten by great apes. The most cited plant was Carapa procera with the use value and informant consensus factor of 0.42 and 0.27 respectively.

2. Extraction Yields

Extraction yields of *C. procera* stem bark are given in Figure 1.

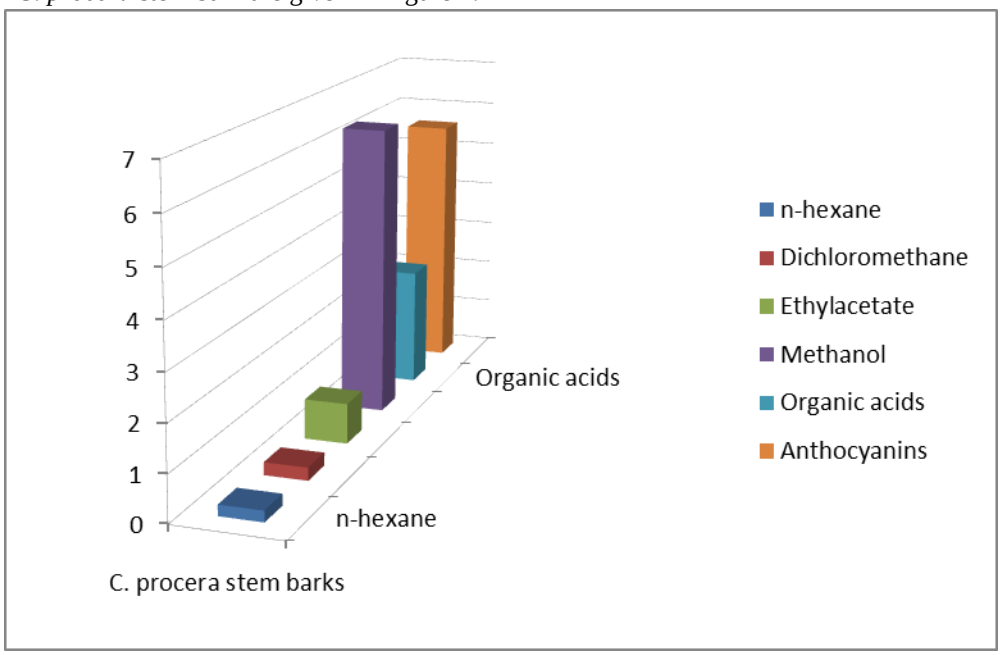

Figure 1: Extraction yields of *C. procera* stem bark.

From the figure 1, it is can be noticed that the un-polar solvents have fewer yields than polar ones. This reveals that the abundant metabolites in the stem bark of *C. procera* Decne are those which pass easily through the polar solvents. The yield of extraction of the anthocyanins and organic acids on stem barks powders of the plant are respectively 5.59% (2.83g) and 2.58% (0.78 g).

3. Antisickling activity of different fractions from *Carapa procera* Decne stems bark

Figures 2 and 3(a-d) show respectively the micrographies of SS blood alone in a NaCl 0.9% solution (control, fig. 2) and the SS blood incubated with the n-hexane (fig. 3a), dichloromethane (fig. 3b) ethyl acetate (fig. 3c) and methanolic (Fig. 3d) soluble fractions of *Carapa procera* stem bark.

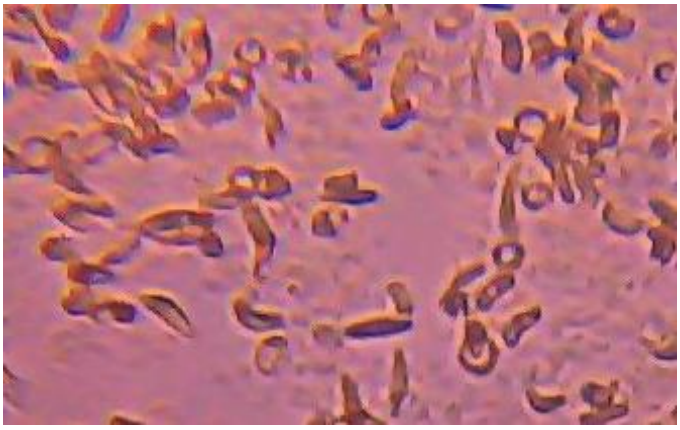

Figure 2: Morphology of drepanocytes of untreated SS blood (control) (x500) [NaCl 0.9%; Na2S2O5 2%,].

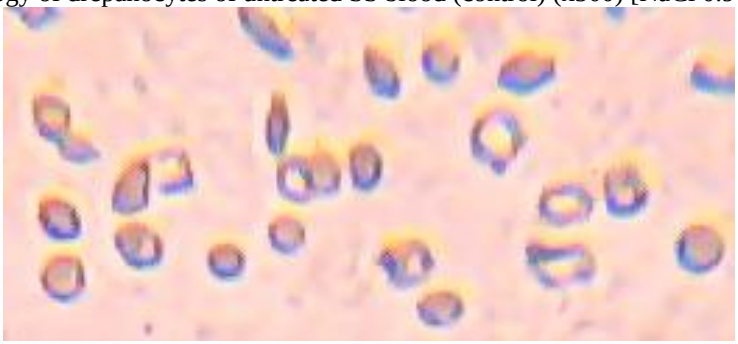

Figure 3a: Morphology of drepanocytes treated with 50 µg/ml of n-hexane soluble fraction of *Carapa procera* stem bark (X500), [NaCl 0.9%; $Na_2S_2O_5$ 2%,].

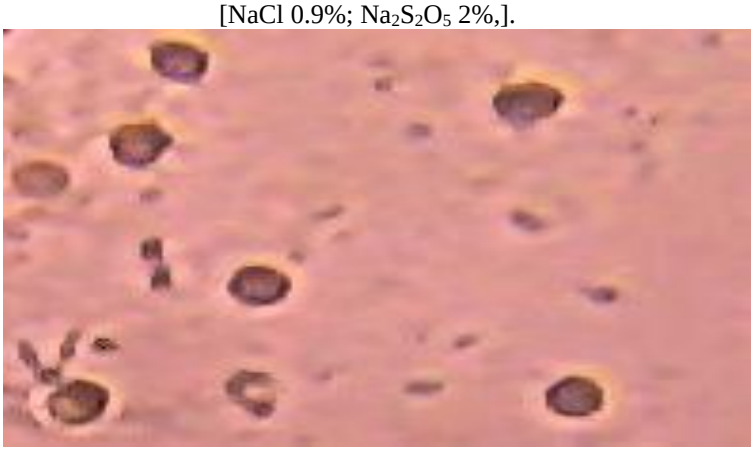

Figure 3b: Morphology of drepanocytes treated with 50 µg/ml of dichloromethane soluble fraction of *Carapa procera* stem bark (X500), [NaCl 0.9%; $Na_2S_2O_5$ 2%,].

Figure 3c: Morphology of drepanocytes treated with 50 µg/ml of ethyl acetate soluble fraction of *Carapa procera* stem bark (X500), [NaCl 0.9%; $Na_2S_2O_5$ 2%,].

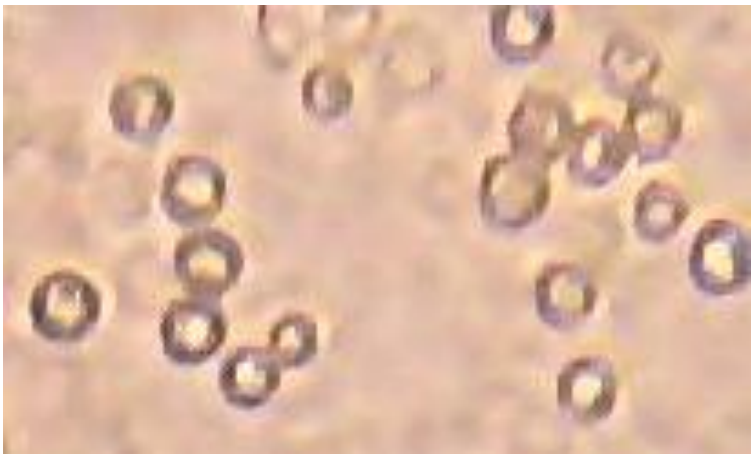

Figure 3d: Morphology of drepanocytes treated with 50 µg/ml of methanolic soluble fraction of *Carapa procera* stem bark (X500), [NaCl 0.9%; $Na_2S_2O_5$ 2%,].

Figure 2 shows that the control contains in majority sickle-shaped erythrocytes, confirming the SS nature of the blood. Mixed together with both n-hexane, dichloromethane, ethyl acetate and methanolic soluble fractions (Fig. 3, a-d), the majority of erythrocytes are reversed normal-shape. Normalization of sickle red cells is much better with dichloromethane, ethyl acetate and methanolic fractions than with n-hexane soluble fraction. This indicates that *Carapa procera* stem bark have antisickling effects, thus justifying the use of this plant in Congolese traditional medicine. A similar result was already obtained for some medicinal plant species used for the management of SCD by Congolese traditional healers. This activity could be due to compounds easily extracted by the used polar solvents such as anthocyanins or to phenolic or triterpenoic acids as previously reported [13-20, 23].
The treated SS RBCs demonstrated a remarkable similarity to normal blood values. The maximal normalization rate or minimal concentration of normalization (MCN) of potential fractions showing 70% of normalized red cells were determined.
Figure 4 shows the dose dependent antisickling activity of dichloromethane, ethyl acetate and methanolic soluble fractions of *Carapa procera*.

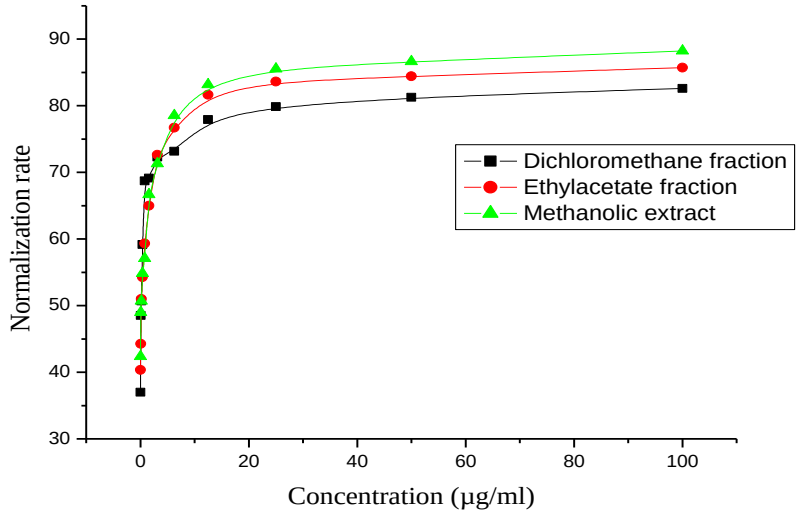

Figure 4: Evolution of normalization rate of drepanocytes with *C. procera* concentration extracts

The curves show that, the normalization of drepanocytes increases with the extract concentration and reach a maximum and constant value at 25 µg/mL. This minimal concentration corresponding to the maximal normalization rate is called minimal concentration of normalization (MCN). This corresponds to a normalization rate of 79.86% for the dichloromethane fraction, 83.62% for ethyl acetate fraction, and 85.52% for the methanolic extract. The antisickling activity is dose dependent. These results show that the methanolic extract and ethyl acetate fraction are more active than the dichloromethane.

4. Antisickling activity of anthocyanin and organic acids extracts from *Carapa procera* stem bark

Figure 5a and 5b give the optical micrograph phenotypes of SS blood treated with anthocyanins and organic acids crude extracts from *Carapa procera* stem bark.

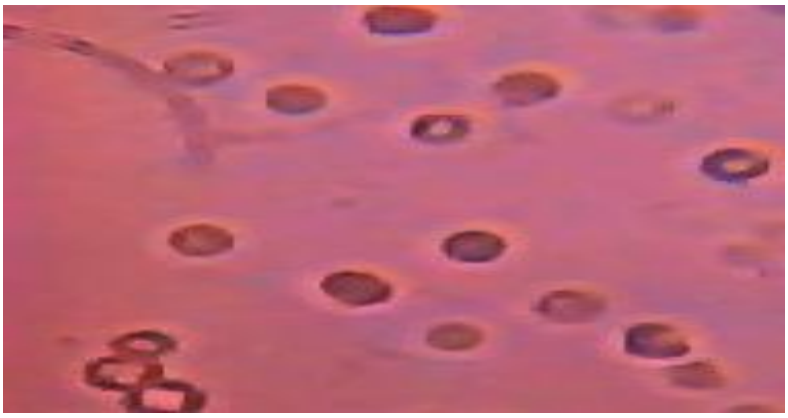

Figure 5a: Morphology of SS erythrocytes treated with anthocyanins extracts 10 µg/mL (x500) [NaCl 0.9%; $Na_2S_2O_5$ 2%,].

Figure 5b: Morphology of SS erythrocytes treated with organic acid extracts 10 µg/mL (x500) [NaCl 0.9%; $Na_2S_2O_5$ 2%,].

Figure 5a and 5b clearly show that in the presence of both anthocyanins and organic acids extracts, the majority of sickle-shaped erythrocytes in SS blood (Fig. 2) reversed into normal biconcave form. This indicates that anthocyanins and organic acids are the major antisickling agents of *Carapa procera* stem bark.

These results confirm those already given by our research team with anthocyanins and organic acids such as betulinic acid, maslinic acid and lunilaric acid from other plants used in traditional medicine for the management of sickle cell anaemia [15, 23]. In fact, it is known that anthocyanins have the ability to interact with proteins [28]. Interaction of these pigments with hemoglobin S could compete with the polymerization of this abnormal hemoglobin and prevent the sickling of sickle cells. In addition, anthocyanins (for which intestinal catabolism gives phenolic acids), also known for their antioxidant properties, could affect the Fe^{3+}/Fe^{2+} higher ratio in sickle cells and the stability of erythrocytes membranes by preventing the oxidation of membranes phospholipids [31]. As SCD is a chronic disease, using anthocyanins as medicinal foods or nutraceuticals would be a good approach instead of giving pharmaceutical products to sicklers during all their life.

Figure 6 shows the evolution of normalization rate of the anthocyanins and organic acids extracts on drepanocytes.

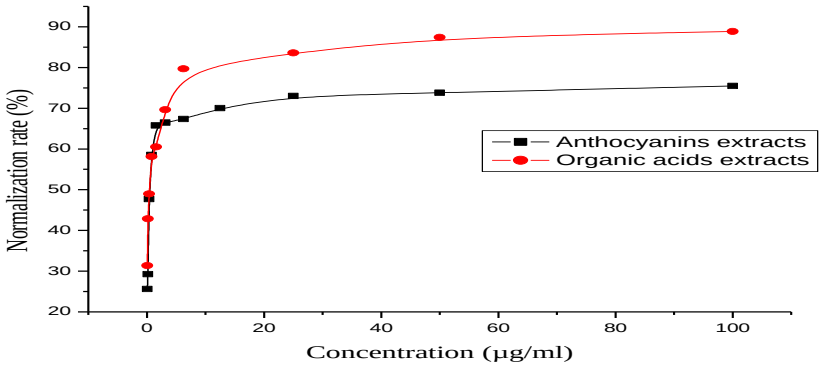

Figure 6: Evolution of normalization rate of drepanocytes with anthocyanin and organic extracts concentration of *C. procera*.

The normalization of sickled cells with the anthocyanins and organic acids extracts increase with the extract concentration and reached a maximum and constant value at 50 µg/mL (MCN). This corresponds to a normalization rate of 72.9% for anthocyanins extracts and 83.59% for organic acids extracts. Therefore, the antisickling activity of extracts is dose dependent.

5. Free radical scavenging activity

The radical scavenging activity of different fractions is given in Table 1.

Table 1: Radical scavenging activity of some fractions from *C. procera*.

Fraction	ED_{50} (µg/mL)	Free radical Scavenging Activity
L-Ascorbic Acid (Positive control)	0.562 ± 0.212	1.776
Methanolic extract	1.698 ± 0.079	0.592
Ethyl acetate fraction	No active	-
Dichloromethane fraction	No active	-
n-hexane fraction	No active	-
Anthocyanins extract	3.575 ± 1.35	0.279
Organic acids extract	8.569 ± 0.162	0.117

As it can be seen in Table 1, methanolic extract possess the lowest ED_{50}value, compared to the anthocyanins and organic acids extracts. Dichloromethane, Ethyl acetate fraction and n-hexane soluble fractions are no active.

Increasing evidence accumulated over the last decade indicates that reactive oxygen species (ROS) play a key role in the pathophysiology of various ischemic diseases including SCD. The oxidative stress in SCD is likely the result of intravascular sickling and transient vaso-occlusive event leading to the decrease of nitric oxide (NO) probably due to consumption of NO by free oxygen radicals, and/or by cell-free plasma heme as a result of hemolysis [32]. The results outlined in this paper, indicate the antisickling and scavenging effects of *Carapa procera*, as attractive potential candidate for SCD therapy for improving the quality life of sicklers. As reducing agent, *C. procera* could prevent *in vivo* oxidative reactions, often by scavenging ROS before they can damage cells.

Conclusion

The present study evaluated the phytochemical screening and the *in vitro* antioxidant and antisickling activity of *Carapa procera* stem bark. This plant species displayed promising antisickling and radical scavenging effects *in vitro*. The ability of methanolic, anthocyanins and organic acids extracts to display such pharmacological properties may represent a rational explanation for the use of this medicinal plant species as antisickling agentt. The combination of ethno-pharmacological and chemotaxonomy approaches as tool has permit us to detect antisickling activity in *Carapa procera*, a plant species no previously reported as antisickling plant in the Congolese pharmacopoeia. Further studies involving the chemical profiling of the active fractions are in progress.

Conflict of Interest

We declare that we have no conflict of interest.

Acknowledgments

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