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The Green Leafy Vegetable Psophocarpus Scandens as Putative Source of Nutraceuticals in Sickle Cell Disease: The Scientific-Based Evidences

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Abstract: In the Democratic Republic of the Congo (DRC), medicinal plants represent the main product for both urban and rural populations for their health care needs due to the high costs of conventional medicine. These plant species contain bioactive compounds also called phytochemicals that are capable of modulating metabolic processes and resulting in the promotion of better health. Some of these plants act therefore as functional foods and could serve as sources of nutraceuticals. *Psophocarpus scandens*, an unconventional green leafy vegetable, is well known to have antioxidant activity which is one of the modes of action of Sickle cell disease (SCD) drugs. To justify this study, it was hypothesized that *P. scandens* possess antisickling properties. The aim of the present study was to evaluate the antisickling activity of organic acids rich extract from *P. scandens* using in vitro biological experiments (Emmel, Itano and hemolysis tests). The results revealed that P. scadens have antisickling properties in vitro (normalization rate: 80%). This bioactivity is expressed as the re-appearance of the normal and classical biconcave form of red blood cells (RBCs: from 0.00±0.00 to 3.55±0.28 µm) in hypoxic conditions by reducing the perimeter of sickle RBCs (from 35.26±1.21 to 19.80±1.15 µm) and increasing their surface (from 21.41±1.84 to 34.10±1.76 μm^2) ($p<0.05$). The bioactivity displayed could be due to triterpenoic acids which are able to reduce both the rate of hemolysis (hemolysis inhibition >50%) and the aggregation of deoxy-Hb S (hemoglobin S polymerization inhibition rate >80%) as experimentally demonstrated. Literature search revealed that this green leafy vegetable contains polyphenolic compounds (glucosylated flavonoids and phenolic acids) with antioxidant properties and all essential amino-acids. Thus, the daily consumption of *P. scandens* fortified with the leaves powder of *Moringa oleirera* could considerably reduce in vivo oxidative stress and hemolysis associated with clinical manifestation of SCD. Based on current knowledge this is a first report on the antisickling activity of P. scandens. It is desirable that the bio-guided fractionation of organic acids extract could be carried out in order to isolate and elucidate the structure of bioactive pure compound(s).

Keywords: Sickle Cell Disease, Functional Foods, Nutraceuticals, Psophocarpus Scandens, Evidences-Based Medicine

1. Introduction

Tropical medicinal plant species are known for their richness in biologically active secondary metabolites of

therapeutic relevance [1]. The principal advantages claimed for therapeutic uses of botanicals against various ailments are their safety besides being economical, effective and their easy availability. According to these advantages, medicinal plants are widely used by traditional healers in their day to day practice [1, 2].

In the Democratic Republic of the Congo (DRC), medicinal plants represent the main product for both urban and rural populations for their health care needs due to the high costs of conventional medicine [3-5]. These plant species contain bioactive compounds also called phytochemicals that are capable of modulating metabolic processes and resulting in the promotion of better health. Some of these plants act therefore as functional foods and could serve as sources of nutraceuticals [6].

The use of edible medicinal plants is an interesting approach since these plants can be integrated into the daily diet of patients suffering from Sickle cell disease (SCD). Ten medicinal foods namely *Cajanus cajan, Sorghum bicolor, Ipomea batatas, Moringa oleifera, Adansania digitata, Ocimum basilicum, Vigna unguiculata, Gnetum africana, Grewia coriacea and Uvariodendron molundense* were scientifically validated as having antisickling activity in vitro. This activity is mainly due to anthocyanins and organic acids and their derivatives [7-9].

An interesting study on the Congolese traditional foods as putative sources of antioxidants with health benefits in Konzo [6, 10], a toxico-nutritional neurological disease associated with oxidative damage due to free radicals (like SCD), revealed that the unconventional green leafy vegetable *Psophocarpus scandens* have antioxidant activity (one of the modes of action of antisickling drugs). It can therefore, be hypothesized that *P. scandens* possess antisickling properties, thus justifying this research work. The aim of the present study was to extract and evaluate the antisickling activity of organic acids rich extract from *P. scandens*. Indeed, SCD is a genetic disease due to the presence of hemoglobin S (Hb S) in the blood which could in hypoxia conduct to the formation of tactoids, the leading cause of erythrocyte sickling that plays a key role in the pathophysiology of SCD like vaso-occlusion (due to the loss of membrane elasticity) and hemolytic anemia. There is strong evidence that the polymerization of deoxy Hb S substantially lowers its oxygen affinity. In the sickling state, the erythrocytes of SCD patients contain 3-4 times more than the normal concentration of the calcium ions as a result of ATP depletion. Since there are little or no endocytic vesicles for calcium ion storage, most of the calcium ions are bound to membrane proteins, thereby resulting in dehydration and hemochrome formation with a resulting loss of erythrocytes deformability, cell-to-cell adherence and free radicals production. However, recent findings indicate that plant-derived antisickling agents may directly inhibit polymerization of hemoglobin, free radicals production or modify membrane stability. The membrane stability is evaluated in this study by measuring the erythrocytes hemolysis [11, 12].

2. Materials and Methods

2.1. Plant Material Collection and Authentication

The tested plant materials used in this study were collected in DRC in March 2013 and were authenticated at INERA (Institut National d'Etudes et Recherches Agronomiques). Voucher specimen is kept in INERA Herbarium (Faculty of Science, University of Kinshasa).

2.2. Extraction and Phytochemical Screening

The dried and powdered plant material (10 g) was repeatedly extracted by cold percolation with 95% ethanol (EtOH) and water (100 mL x 2) for 48 hours. Chemical screening was performed on the aqueous and organic extracts to investigate the presence of alkaloids, saponins, total polyphenols, flavonoids, tannins, anthocyanins, leuco-anthocyanins, quinones and triterpenoids according to standard protocol [13].

2.2.1. Detection of Phenols (Ferric Chloride Test)

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

2.2.2. Detection of Flavonoids

The ethanol extract (5 ml) was added to a concentrated sulphuric acid (1 ml) and 0.5 g of Mg. A pink or red coloration that disappear on standing (3 min) indicates the presence of flavonoids.

2.2.3. Detection of Anthocyanins

The presence of anthocyanins is revealed by a color change as a function of pH due to titration of the acidic aqueous solution with a solution of NaOH. If the solution turns a red color, the pH is less than 3, if against a blue color; the pH is between 4 and 6.

2.2.4. Detection of Tannins

Two methods were used to test for tannins. First, about 1 ml of the ethanol extract was added in 2 ml of water in a test tube. 2 to 3 drops of diluted ferric chloride solution was added and observed for green to blue-green (cathechic tannins) or a blue-black (gallic tannins) coloration. Second, 2 ml of the aqueous extract was added to 2 ml of water, a 1 to 2 drops of diluted ferric chloride solution was added. A dark green or blue green coloration indicates the presence of tannins.

2.2.5. Detection of Leucoanthocyanins

To 2 ml of aqueous extract was added few drop of Shinoda regeant in a test tube and then boiled. A red or purple coloration in the supernatant indicates the presence of leucoanthocyanins.

2.2.6. Detection of Saponins

To 1 ml of aqueous extract was added few volume of

distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth for 20 min.

2.2.7. Detection of Alkaloids

Five ml of the extract was added to 2 ml of HCl. To this acidic medium, 1 ml of Dragendroff's reagent was added. An orange or red precipitate produced immediately indicates the presence of alkaloids.

2.2.8. Detection of Free Quinones

To 1 ml of organic extract was added few drops of Borntrager reagent (NaOH 10% ou NH_4OH 10%) in a test tube. The solution was and then shaken vigorously. A sharp red or orange coloration indicates the presence of free quinones.

2.2.9. Detection of Triterpenoids

Ten (10) mg of the extract was dissolved in 1 ml of chloroform; 1 ml of acetic anhydride was added following the addition of 2 ml of Conc. H_2SO_4. The formation of reddish violet colour indicates the presence of triterpenoids.

2.3. Organic/Triterpenic Acids Extraction

The powdered material (40 g) were macerated with 100 mL of dichloromethane-methanol-NH_4OH (100:1:1; v/v/v) and then percolated with 300 mL of the same solvent mixture at room temperature. The extract was concentrated under reduced pressure until 100 mL (pH 10). The resulting solution was then mixed with 5% citric acid (v/v) to precipitate organic/triterpenic acids [14]. Fractions were filtered and concentrated to dryness under reduced pressure using a rotary evaporator.

2.4. In Vitro Antisickling Activities

2.4.1. Blood Samples Collection

Blood samples used to assess the antisickling activity were taken from known SCD patients attending the "Centre de Médecine Mixte et d'Anémie SS" located in Kinshasa, Democratic Republic of the Congo. None of the patients had been transfused recently with Hb AA blood and 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 to confirm their status and were then stored at +4 °C in a refrigerator.

An informed consent was obtained from all the patients participating in the study and all the research procedures have received the approval of Department of Biology Ethics Committee.

2.4.2. Emmel Test

An aliquot of Hb S-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). Duplicate analyses were run for each extract. The red blood cells (RBCs) were analyzed by a computer assisted image analysis software (Motic Images 2000, version 1.3; Motic China Group Co LTD).

2.4.3. Anti-Hemolytic Assay

Erythrocytes (RBCs) were washed twice in physiological saline (NaCl 0,9%, 1:5 v/v) by centrifugation at 3000 rpm for 10 min, re-suspended in phosphate buffer (150 mM, pH 7,4) containing 2% sodium metabisulfite and incubated in the absence (control) or presence of ethanolic extract of *P. scandens* (50 µg/mL)/organic acids extract of P. scandens (50 µg/ml of basified NaCl 0,9%) at 37°C for 60 min. At fixed time points, aliquots of the blood samples were removed and centrifuged at 3000 rpm at ambient temperature for 5 min. The absorbance of the supernatant was measured at 540 nm and was expressed as the degree of haemolysis. The rate of hemolysis inhibition (% HI) versus time was calculated from the absorbance of sickle erythrocyte (SS RBCs) suspension by the relation:

$$\%HI = 100\ X\ [\frac{\text{Absorbance of untreated SS RBCs suspension}}{\text{Absorbance of treated SS RBCs suspension2a}}]$$

The zero time corresponds to 30 min after pre-incubation of SS RBCs with plant extracts (treated SS RBCs) or NaCl 0,9% (untreated SS RBCs) in hypoxic conditions [11, 12].

2.4.4. Anti-Gelling Assay (Itano test)

Erythrocytes were washed twice in physiological saline solution (NaCl 0.9%) by centrifugation at 3000 rpm for 10 min, re-suspended in hypotonic medium. After that, the hemolysate of RBCs was centrifuged and an equivalent volume of 2% metabisulfite was added to supernatant. It was than incubated at ambient temperature for 45 min. At fixed time points aliquots (50 µL) of the 2% sodium metabisulfite pre-treated hemolysate were diluted with 500 µL of phosphate buffer (pH 7.5) containing $(NH_2)_2SO_4$ 30%, Saponine 1% and K_2HPO_4 1.2%. 50 µL of ethanolic or organic acids extract of P. scandens (50 µg/mL) were added to the test sample, mixed and incubated for 10 min. The equivalent volume of phosphate buffered saline (PBS) was added to the control sample instead of the drug. At predetermined time intervals aliquots of test or control samples were removed and centrifuged at 3500 rpm at ambient temperature for 5 min. The absorbance of the supernatant was measured at 700 nm. The solubility of the deoxygenated sickle cell hemoglobin was expressed as the decrease of the optical density at 700 nm (anti-gelling effect). The zero time corresponds to 30 min after pre-incubation of Hb S with plant extract (treated Hb S) or NaCl 0,9% (untreated HbS) in hypoxic conditions [11, 12].

2.5. Literature Search

A deep literature search was carried out in order to obtain information about the traditional uses, microscopic features, nutritional value, phytochemistry and bioactivities of *P. scandens* from various electronic databases namely PubMed, PubMed Central, Science Direct and Google scholar as previously reported [1, 4].

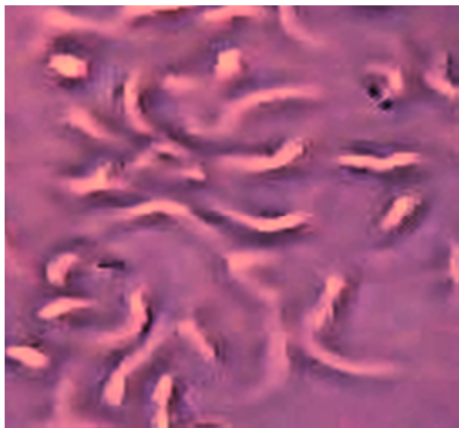
3. Results and Discussion

3.1. Laboratory Assays Results

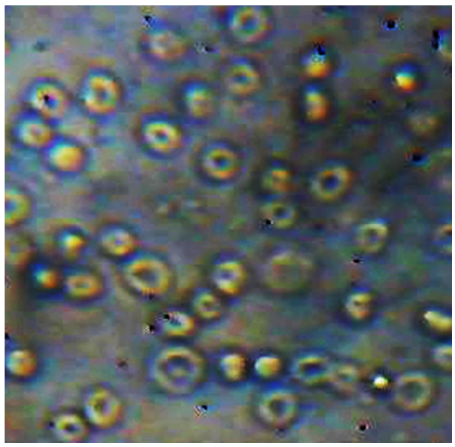
The chemical screening performed on the leaves of *P. scandens* revealed the presence of alkaloids, saponins, total polyphenols, flavonoids, tannins, anthocyanins, triterpenoids and free quinones.

Phenolic compounds such as anthocyanins [7, 11, 12, 14], rosmarinic acid [15] and lunularic acid [16] and triterpenes like betulinic, maslinic, oleanolic [17] and ursolic acid [18] were reported to display antisickling activity in vitro.

The figure 1 gives the morphology of untreated sickle erythrocytes (a) and SS RBCs treated with 50 µg/mL of P. scandens organic acids extract (b).



[A]



[B]

Figure 1. *Morphology of untreated sickle erythrocytes [A] or SS RBCs treated with 50 µg/mL of P. scandens organic acids extract [B] (X500), [NaCl 0.9%; $Na_2S_2O_5$ 2%].*

Figure 1a reveals that the control sample contains in majority sickle-shaped erythrocytes, confirming the SS homozygous status of the used blood. Mixed together with organic acid extract (Fig. 1b); the majority of erythrocytes are reversed normal-shape as revealed in the table 1. This indicates that organic acids extract of P. scadens have antisickling properties. The bioactivity displayed could be due to triterpenoic acids as previously reported [14].

Table 1. *Average values of radius, perimeter and surface of erythrocytes before and after treatment with P. scandens organic acids rich extract.*

Measured parameters	Untreated SS RBCs	SS RBCs (+P. scandens OA extract)
Radius (µm)	0.00±0.00	3.55±0.28
Perimeter (µm)	35.26±1.21	19.80±1.15
Surface (µm2)	21.41±1.84	34.10±1.76

(Legend: OA, organic acids)

As it can be seen in table 1, the used computer software package/program did not give the average radius for drepanocytes, as sickled cells of untreated SS blood are not circular. The average radius appeared after treatment of SS RBCs by *P. scandens* organic acids rich extract (50 µg/mL), conduct into the re-appearance of the normal and classical biconcave form of RBCs by reducing the perimeter of sickle RBCs and increasing their surface ($p<0.05$), thus confirming the antisickling effect of P. scandens.

Figure 2 shows the dose dependent antisickling activity of triterpenic (triterpenoic) acids rich/organic acids extract from *P. scadens*.

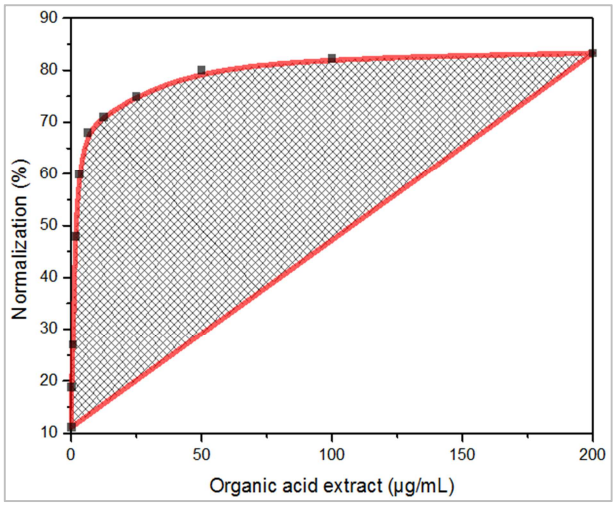


Figure 2. *Dose-dependent normalization rate of sickled erythrocytes (NaCl 0.9%; $Na_2S_2O_5$ 2%) (The curve was fitted with the help of Origin Pro 8.5 package software using B-spline: Fill area under the curve and inclusive broken by missing values).*

It can deduce from the figure 2 that the normalization rate of sickled cells in hypoxic conditions increases with the extract dose. At the dose of 50 µg/mL the rates of normalization were 80%. Thus, the antisickling activity of the tested plant extract is dose dependent. It is therefore suggested that bioactive extract could exert it

pharmacological effect by various mechanisms including the inhibition of free radicals formation, and the inhibition of erythrocyte hemolysis (figure 3) and the inhibition of hemoglobin polymerization (figure 4) as previously reported [11, 12, 14]. Thus, the daily consumption of *P. scandens* fortified with the leaves powder of *Moringa oleirera* could considerably reduce oxidative stress and hemolysis associated with clinical manifestation of SCD.

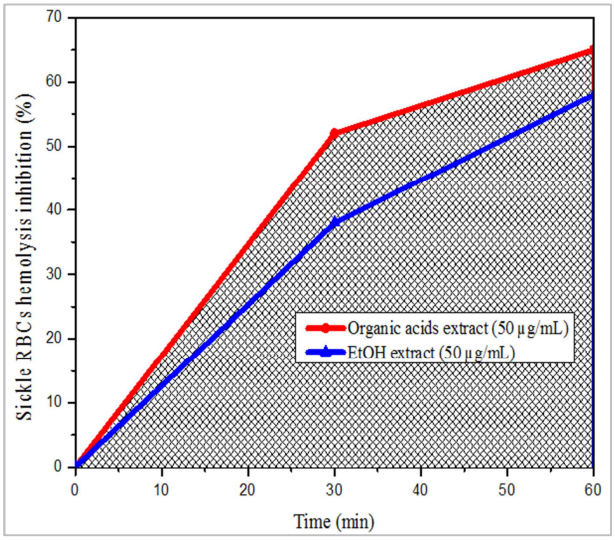


Figure 3. *In vitro effect of ethanolic extract (EtOH) and organic acids extract from P. scandens on the hemolysis of Sickle red blood cells (RBCs) (NaCl 0.9%; $Na_2S_2O_5$ 2%).*

Figure 3 reveals that after 60 min of incubation, the rate of RBCs hemolysis inhibition of the extracts of *P. scandens* is greater than 50%. The best result was obtained with organic acids extract.

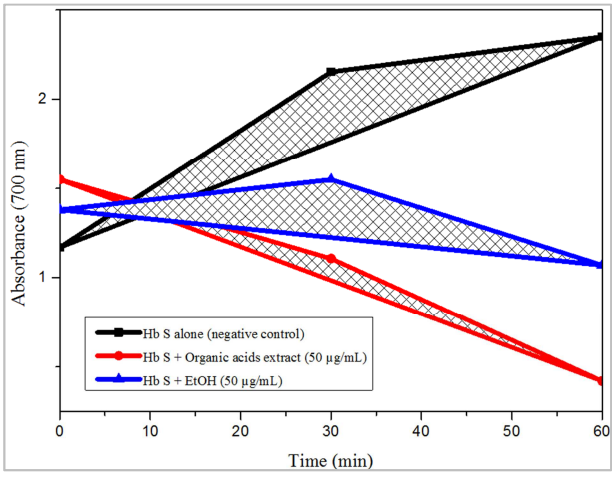


Figure 4. *In vitro effect of ethanolic and organic acids extracts of P. scandens on the aggregation of deoxy-Hb S (Phosphate buffered saline 150 mM; pH 7.4; 2% $Na_2S_2O_5$).*

The figure 4 indicates that, in the untreated hemoglobin aqueous solution (control) the absorbance at wave length of 700 nm increase with the time as a result of the loss of hemoglobin solubility in hypoxic conditions created by 2% Sodium metabisulfite. However, after addition of plant extract, the absorbance decreases in the course of time. These results indicate that ethanolic and organic acids extracts of *P. scandens* inhibit the polymerization of hemoglobin S into tactoids (anti-gelling effect). The best result was obtained with organic acids extract.

3.2. Literature Search Results

P. scandens is a traditional food commonly known as kikalakasa in DRC where the plant is consumed all over the country. According to Mangala et al. [6], phytochemical studies (TLC and HPL-DAD) of extracts from P. scandens revealed the presence of polyphenolic compounds (glucosylated flavonoids and phenolic acids). P. scandens contains also flavonoids (40, 78 ± 1, 11 mg QE/g DW), tannins (102, 56 ± 0, 08 CE/g DW). The extracts from this plant species displayed moderate antioxidant activity in vitro (radical ATBS, IC_{50}: 53, 2 ± 0, 97 µg/mL; radical DPPH, IC_{50}: 62, 81 ± 0, 97 µg/mL).

Study on the botanical microscopic characters of the leaves powder of P. scandens revealed abundant prisms of calcium oxalate crystals, starch grains, fragments of lignified fibers, diacytic stomata, circular epidermal cells, spiral vessels and unicellular non glandular trichomes. These results indicate that the studied unconventional green leafy vegetable could provide benefits in protecting SCD patients against oxidative damage.

The assessment of the nutritional value of P. scandens revealed that this unconventional green leafy vegetable contains (in g/100 g FM): crude proteins (7, 03), fats (1, 28), carbohydrates (5, 40), crude fibers (2, 21), total ash (1, 38). This corresponds to the energy value of 61 kCal and the moisture was evaluated to 82, 7%. Biochemical analysis revealed also the presence of all essential amino-acids [19]. This tropical plant species could putatively be used as functional food to prevent erythrocytes sickling and oxidative damage associated with SCD. In DRC, 12% of the children hospitalized in the hospital are sickle cell disease patients and it is estimated that the annual cost of the treatment is higher than 1,000 USD per patient, a cost hard to bear for the majority of the population whose average income is lower than 2 USD per day and who for the needs for primary health care turns mainly to Traditional Medicine [9]. Thus, P. scandens can be integrated into the daily diet of SCD patients for a sustainable solution.

4. Conclusion and Suggestions

The aim of the present study was to evaluate the antisickling activity of organic acids rich extract of P. scandens. The results revealed that P. scadens have antisickling properties *in vitro* (normalization rate: 80%). This bioactivity is expressed as the re-appearance of the normal and classical biconcave form of RBCs in hypoxic conditions by reducing the perimeter of sickle RBCs and increasing their surface. The bioactivity displayed could be due to triterpenoic acids which are able in vitro to reduce both the rate of hemolysis and the aggregation of deoxy-Hb S. Thus, the daily consumption of P. scandens fortified with

the leaves powder of *Moringa oleirera* could considerably reduce *in vivo* oxidative stress and hemolysis associated with clinical manifestation of SCD. Based on current knowledge this is a first report on the antisickling activity of *P. scandens*. It is desirable that the bio-guided fractionation of organic acids extract could be carried out in order to isolate and elucidate the structure of bioactive pure compound(s) using the combination of chromatographic and spectroscopic techniques (LC/MS/NMR, FT-IR).

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