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ORIGINAL ARTICLE

Antisickling and antibacterial activities of *Uvariopsis congensis* Robyns & Ghesq. (Annonanceae)

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ABSTRACT

The aim of the present study was to evaluate the chemical composition and bioactivity of Uvariopsis congensis Robyns & Ghesq. stem bark extract against Sickle cell disease and associated bacteria. This plant species ingested by wild chimpanzees as medicinal food could inhibit the human hemoglobin S polymerization inducing hemolysis of sickle erythrocytes (as it does by inhibiting the Plasmodium falciparum inducing hemolysis of infected erythrocytes in great apes). The antisickling and antibacterial activities were assessed according respectively to Emmel and micro-dilution methods. The results revealed that Uvariopsis congensis Robyns & Ghesq. stem bark extract contains various secondary metabolites such as alkaloids, saponins, total polyphenols (87.942±0.536 µg GAE/g), flavonoids (0.623±0.630 µg QE/g), tannins (0889±0.002 µg TAE/g), anthocyanis (0.150±0.017 µg M-3GE/g), leuco-anthocyanins, quinones, terpenes and steroids. Ethanolic extract displayed interesting antisickling activity which was confirmed by the appearance of radius, the increase of the cell area and the decrease of it perimeter ($p<0.05$). The antisickling activity of Uvariopsis congensis Robyns & Ghesq. stem bark extract is dose dependent. The minimal concentration of normalization (MCN) and the dose of extract for which 50% of the sickled erythrocytes are reversed (ED50) were 100 µg/mL and 0.6 µg/ml respectively. The minimum inhibitory concentrations (MIC) were 250 µg/mL and 500 µg/mL respectively for Escherichia coli ATCC 27195 and Staphylococcus aureus ATCC 33591. The bioactivity of this plant species could be improved by liquid-liquid extraction and chromatographic bio-guided fractionation of ethanolic extract. This study validates for the first time the in vitro antisickling activity of Uvariopsis congensis Robyns & Ghesq. stem bark extract. The animal self-medication constitutes thus an innovative method which could lead to the discovery of new drugs for the treatment of human ailments like Sickle cell disease.

Keywords: *Sickle cell disease, Congo basin, great apes plant foods, zoopharmacognosy, novel drugs*

Introduction

Infectious diseases are recognized to affect the behavior and reproductive fitness of all animals.[1] As response, the animal kingdom developed anti-parasitic behaviors through plant-animal co-evolution relationships in which some animal phylum utilize the chemical defenses of plants to protect themselves from their own parasites.[2]

In African tropical forests like Congo basin, Great apes (GAs) were reported as a good model for the understanding of malaria infection patho-physiology. Indeed, Humans and great apes (bonobos, chimpanzees, gorillas, etc.) share a common gut anatomy. Although, some diseases that cause countless deaths in humans (like malaria) are ineffective or have minor non disturbing effects in GAs. These animals adopt a self medicative behavior when they are displaying malaria symptoms by selecting specific plants for controlling parasite infection while this one cause hemolytic anemia of human red blood cells.[3, 4]

In this regards, we recently hypothesized that these plant items could therefore protect human sickle erythrocyte against hemolysis (by inhibiting the polymerization of sickle hemoglobin and radical oxygen species formation within sickle erythrocyte) as it does for Plasmodium falciparum infected erythrocytes in great apes.[5, 6] So, some of such plant species occasionally ingested by GAs for their supposed medicinal properties were reported to contain natural product compounds that prevent the erythrocytes hemolysis through the inhibition of sickle erythrocyte sickling and this bioactivity was scientifically validated.[6-11]

Uvariopsis congensis is a shrub or small tree. The flowers are solitary and borne in the axils of the leaves and on the older wood. The leaf is smaller, long by 2 to 6 cm, broad and is elliptic instead of oblong to oblanceolate and entirely glabrous. The flowers and fruits are very shortly

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pedunculate; the petals ovate and sub-acute (instead of rotund). The fruits are pinkish red in color, about 1·5 cm diameter and constricted between the seeds.[12]

The plant species was reported to be eaten by wild chimpanzees (Pan troglodytes schweinfurthii).[13] The active compounds (acetogenins) isolated from *Uvariopsis congensis* Robyns & Ghesq. stem bark extract showed highly significant medicinal properties like antibacterial, antimalarial, and/or antileishmania.[14] However, no study has yet been reported on the antisickling activity of *Uvariopsis congensis* Robyns & Ghesq. stem bark extract. The plant could be potentially non-toxic to man and could provide new sources of anti-sickling compounds.

Sickle cell disease (SCD) is a hemoglobinopathy which naturally occurring in black people. At the genetic level, SCD is characterized by a single base substitution in the gene encoding the human β-globin subunit and results in replacement of β6 glutamic acid by valine that causes an abnormal, rigid and sickle shape in hypoxia.[15, 16]

In Democratic Republic of the Congo (DRC), surveys reported that 12% of the hospitalized children are sicklers and the annual cost of the treatment of SCD is higher than 1.000,00 USD per patient.[17] It is therefore necessary to develop innovative strategy for identifying new source of antisickling agents through zoopharmacognosy.[6-11]

The aim of this study is to evaluate the chemical composition and bioactivity of *Uvariopsis congensis* Robyns & Ghesq. stem bark extract against SCD and associated bacteria, a plant species which has been selected through the animal source of knowledge (new paradigm) in order to discover new phytomedicine for human.

Materials and Methods

Plant identification and preparation of extracts

The plant material (stem barks) used in the present study was collected in Kisangani city (Tshopo Province, Democratic Republic of the Congo) during a field work in July-August 2015 and authenticated by Justin A. Asimonyio of the Biodiversity monitoring centre, University of Kisangani. Voucher specimen No JAA05T is on deposit at the Laboratory of Molecular bio-prospection (Department of Biology, Faculty of Science of the Kinshasa University).

Test extract preparation

The plant material (1 kg) was kept at room temperature (25 to 30 °C) for air drying (two weeks). The air-dried and powdered material (50 g) was extracted by repeated maceration with ethanol 90° (3×4 hrs, 1.5 L) at room temperature. After filtering the mixture, the aqueous-ethanol filtrates were pooled, dried over Na_2SO_4 and evaporated to dryness under reduced pressure using a rotary evaporator to yield crude ethanolic extract. Extract was stored at 4 °C.

Phytochemical screening

The dried and powdered plant material (10 g) was repeatedly extracted by cold percolation with 95% ethanol (EtOH) and water (100 mL × 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, anthocyanis, leuco-anthocyanins, quinones, terpenes and steroids according to standard protocol.[18]

Detection of phenols (Ferric Chloride Test)

Total phenolics contents were determined according to the Folin-Ciocalteu method with slight modifications.[19] The extract (200 µL) was mixed with 1.5 mL of Folin-Ciocalteu reagent (previously diluted 10 times with double distilled water) and allowed to stand at room temperature for 5 min. 1.5 mL of sodium bicarbonate solution (60 g/L) was added to the mixture and after incubation for 90 min at room temperature, the absorbance was measured at 725 nm using a UV-Visible spectrophotometer (GENESYS 10S). Total phenolic were quantified by calibration curve obtained from measuring the absorbance of the known concentrations of gallic acid standard solutions (10-150 µg/mL in 80% methanol). The results were calculated as gallic acid equivalent (GAE) per one gram dry powder and reported as mean value ± standard deviation (SD) (the standard curve equation: $Y= 0{,}006\times-0{,}002$; $R^2 = 0{,}997$).

Detection of flavonoids

Total flavonoid content was measured by the aluminum chloride colorimetric method. 20 An aliquot (1 mL) of each extract was added to 10 mL volumetric flask containing 4 mL of double distilled water. Then 0.3 mL $NaNO_2$ 5% was added to the flask and after 5 min, 0.3 mL $AlCl_3$ (10%) was also added. At 6th min, 2 mL NaOH (1 M) was added and the total volume was made up

to 10 mL with double distilled water. The solution was mixed completely and the absorbance was measured versus prepared reagent blank at 510 nm. Total flavonoid content was expressed as mg quercetin equivalents (QE) per one gram dry powder. One mL of standard solution (quercetin: 5-100 µg/mL) was used to construct calibration curve (the standard curve equation: Y = 0.009× + 0.006; R2 = 0,999).

Detection of tannins

To 1 ml of the extract was added 7,5 ml of distilled water and 0,5 ml of Folin-Ciocalteu reagent and 1 ml of sodium carbonate (Na2CO3 35%). The absorbance was measured at the wavelength of 725 nm. The tannins content (expressed as tannic acid equivalent, TAE) was calculated using the following relation: Y = 0,443 × - 0,264; R2 = 0,720.[22]

Detection of anthocyanins

The samples were diluted with the mixture ethanol/water/HCl conc. (70:30:1; v/v/v) and the absorbance was measured at the wavelength of 540 nM. The anthocyanins content (expressed as malvidin-3-glucoside equivalent, M-3-GE) was calculated using the following relation: Anthocyanins =A540 ×(10/0.6)×d (with A540= maximum of absorption at 540 nm; d = dilution factor; 0.6 maximum of absorption of 10 mg/L of M-3-GE standard solution).[21].

Bioassays

In vitro antisickling bioassay

Blood samples used to assess the antisickling activity of the selected plant extracts 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.

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) and statistical data analysis were processed using Microcal Origin 8.5 Pro package software as previously reported. [23-27]

Determination of antibacterial activity

The activity of the plant extract was tested against Staphylococcus aureus (S. aureus ATCC 33591) and Escherichia coli (E. coli ATCC 27195) strains. The tested strains were obtained from the American Type Culture Collection (ATCC, Rockville MD, USA)

Determination of Minimum inhibitory concentration (MIC)

The Minimum inhibitory concentration (MIC) was determined by broth micro-dilution method as reported in our previously research work.[28, 29] The inocula of used microorganisms were prepared from 24 hours old broth cultures. The absorbance was read at 600 nm and adjusted with sterile physiological solution (0.9% NaCl) to match that of a 0.5 McFarland standard solution (108 cells/mL). The prepared microbial suspension was diluted (1/100) to achieve 106 CFU/mL. Stock solutions of the plant extracts were prepared in Tween 80 (Fisher chemicals) (3 mg/300 µL) and diluted to 2.7 ml with Mueller Hinton Broth (MHB) (Conda, Madrid, Spain) to achieve a Tween 80 final concentration of 0.1%. This solution was transferred in 96-wells plates (200 µL/well) and two-fold serially diluted with MHB to give final concentrations ranging from 1000 to 3,906 µg/mL.

An aliquot (10 µL) of 106 CFU/mL overnight culture was added to wells of a sterile 96-well micro-plate titer. The positive control wells contained both MHB and bacteria suspension without plant extract while negative control wells contained MHB only. The MIC was determined as the lowest plant extract concentration at which no growth were observed after 24 hours. Resasurin (30 µL) in aqueous solution (0.01%) was used to evaluate the micro-organism viability.

Statistical analysis

The results of in vitro study are given as Mean ±Standard Deviation obtained from three independent experiments. The results of in vivo study were expressed also as Mean ± Standard Deviation and analyzed with Student's t-test for paired data using Origin version 8.5 Pro package software. All data were analyzed at a 95% confidence interval (α= 0.05).

Results and Discussion

Extract yields and chemical screening

The phytochemical analyses performed on *Uvariopsis congensis* Robyns & Ghesq. stem bark extract revealed the presence of alkaloids, saponins, total polyphenols (87.942±0.536 µg GAE/g), flavonoids (0.623±0.630 µg QE/g), tannins (0889±0.002 µg TAE/g), anthocyanis (0.150±0.017 µg M-3GE/g), leuco-anthocyanins, quinones, terpenes and steroids. Phenolic compounds such as anthocyanins[3, 9, 16, 23-27, 31-33] rosmarinic acid[34] and lunularic acid[35] and triterpenes like betulinic, maslinic, oleanolic[36] and ursolic acid[37] were reported to display antisickling activity *in vitro*.

Antisickling acctivity

The figure 1 give the morphology of untreated sickle erythrocytes (SE) and SE treated with *Uvariopsis congensis* Robyns & Ghesq. stem bark extract.

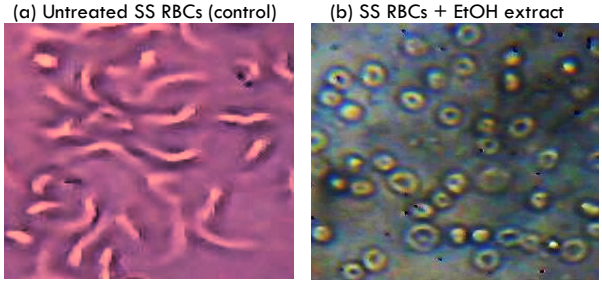

Figure 1. Morphology of untreated sickle erythrocytes
(a) SE treated with 50 µg/mL of *Uvariopsis congensis* (b) (X500), [NaCl 0.9%; Na2S2O5 2%].

Cell parameters	Untreated *SE*	*SE* + EtOH
Radius (µm)	0.00±0.00	3.5±0.4
Perimeter (µm)	36.0±1.5	19.2±1.1
Area (µm^2)	21.4±1.3	34.6±1.2

Table 1. Average values of radius, perimeter and surface of untreated and sickle erythrocytes (SE) treated with ethanolic extract of *Uvariopsis congensis*.

Figure 1a revealed that the control contains in majority sickle-shaped erythrocytes, confirming the homozygous (SS) nature of the blood. Mixed together with ethanolic extract (Figure 1b), the majority of erythrocytes are reversed normal-shape. This indicates that *Uvariopsis congensis* Robyns & Ghesq. stem bark extract have antisickling effect. Our previous research works revealed that this activity could be due to compounds such as anthocyanins, phenolic or triterpenoic acids.[3, 9, 16, 23-27, 30-37] The treated SE demonstrated a remarkable similarity to normal red blood cells.

The table 1 gives the average values of cell parameters (radius, perimeter and area) of untreated and sickle erythrocytes treated with the extract of *Uvariopsis congensis*.

Table 1 indicates that untreated sickle cells are not circular, so it's not possible to calculate the radius due to their sickled shape in hypoxic conditions. While, in the presence of plant extract; drepanocyte recovered normal shape.

This phenomenon is confirmed by the appearance of radius, the increase of the treated cell area and the decrease of it perimeter if compared to untreated sickle erythrocyte ($p<0.05$). Indeed, the computer software package (Motic Images 2000 version 1.3) used in this study did not give the average radius for untreated sickle

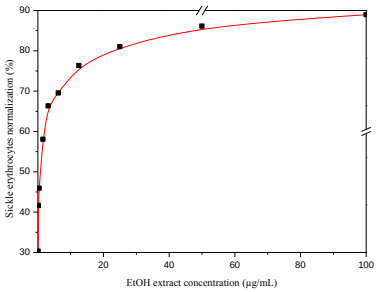

Figure 2. Dose-dependent normalization of sickle erythrocytes treated with extract from *Uvariopsis congensis*

cells, as sickled cells of untreated blood were not circular. The average radius appeared after treatment of sickle cells with ethanolic extract of *Uvariopsis congensis* is an indication of the bioactivity of this plant species.

The normalization rate of sickled cells increases with the extract concentration and reached a maximum and constant value at 100 µg/mL (minimal concentration of normalization, MCN). This corresponds to a normalization rate of 89% with an ED50 (dose of extract for which 50% of the sickled erythrocytes are reversed) equal to 0.6 µg/ml. Thus, the antisickling activity of *Uvariopsis congensis* is dose dependent (figure 2). The extracts from *Uvariopsis congensis*, because of their bioactive secondary metabolites richness like phenolic compounds (anthocyanins), could exert the antisickling effect through various mechanisms like the inhibition of free radicals formation, the inhibition of hemoglobin polymerization, and the inhibition of erythrocyte hemolysis.

Antibacterial activity

Due to the high cost of modern therapy for SCD, a medicinal plant species displaying at the same time antisickling and antibacterial activities could be useful in the management of this disease. The antibacterial activity of *Uvariopsis congensis* Robyns & Ghesq. stem bark extract was evaluated against E. coli and S. aureus strains.

Results revealed that the negative bacterium E. coli was more sensitive to plant extact (MIC=250 µg/mL) than S. aureus (MIC=500 µg/mL). This antibacterial activity is however weak if compared to the MIC reference value of 100 µg/mL. Indeed, a value lower or equal to 100 indicates a great antibacterial activity. 8 The difference in the bioactivity would be due to the nature of the bacterial wall. 38 These results are in agreement with previous works on the antimicrobial activity of natural products of plant origin. 28, 29 It was reported that S. aureus and E coli constitute both the principal bacteria responsible for septicemia and the osteomyelitis in SCD patients. 39 To this end, *Uvariopsis congensis* is a better candidate for the development of phytomedicine with broad spectrum of action for the management of SCD. The antibacterial activity observed in this study can be improved through liquid-liquid extraction and chromatographic bio-guided fractionation of the ethanolic extract. To our knowledge, it is for the first time that the antisickling activity of *Uvariopsis congensis* is reported in the literature.

Conclusions

The present study evaluated the chemical composition and the antisickling and antibacterial activities of *Uvariopsis congensis* Robyns & Ghesq. stem bark extract. The results revealed that: The stem bark of *Uvariopsis congensis* contains various secondary metabolites such as the anthocyanins, flavonoids, tannins, quinones, saponins, alkaloids, steroids, terpenoids and leuco-anthocyanins; Ethanolic extracts displayed very interesting antisickling activity in vitro (ED50= 0.6 µg/mL). These extracts could exert their antisickling effect through various mechanisms including the inhibition of free radicals formation, the inhibition of hemoglobin polymerization, and the inhibition of erythrocyte hemolysis. The minimum inhibitory concentrations (MIC) were 250 µg/mL and 500 µg/mL respectively for E. coli ATCC 27195 and S. aureus ATCC 33591.

This study provided experimental evidence that supports further development of *Uvariopsis congensis* extracts as a medicine for the management of SCD in the Congo basin. The animal self-medication constitutes thus an

innovative method which could lead to the discovery of new drugs for the treatment of human ailments.

Conflict of Interest

None

Acknowledgements

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