



Antisickling and Antibacterial Activities of *Anthocleista schweinfurthii* Gilg. (Gentianaceae) from Non-human Primates Pharmacopoeia in Democratic Republic of the Congo

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Abstract : The aim of the present study was to evaluate the Chemical composition and bioactivity of *Anthocleista schweinfurthii* Gilg (Gentianaceae) fruits, leaves, root bark and stem bark extracts against Sick cell disease (SCD) and associated bacteria. The antisickling and antibacterial activities were carried out using Emmel and micro-dilution methods respectively. The results revealed that the fruits, leaves, root bark and stem bark extracts of *A. schweinfurthii* contains various secondary metabolites such as the alkaloids, saponins, total polyphenols, flavonoids, tannins, anthocyanins, leuco-anthocyanins and quinones. The ethyl acetate and methanol extracts displayed antisickling activity. While, the antibacterial activity of different plant extracts tested was weak toward tested bacterial strains (CMI $\geq 125\mu\text{g/mL}$). The antibacterial activity can be improved by bio-guided fractionation of the n-hexane, ethyl acetate or methanol soluble fraction. This study provides for the first time a scientific basis for the *in vitro* antisickling activity of *A. schweinfurthii*.

Keywords : Sick cell disease; antibacterial activity; Congo basin; great apes plant foods; zoopharmacognosy.

I. Introduction

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 β^6 glutamic acid by valine that causes an abnormal, rigid and sickle shape in hypoxia (Giot 2003, Mpiana et al. 2010). 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 (Tshilolo et al. 2009). It is therefore necessary to develop innovative strategy for identifying new sources of antisickling agents through zoopharmacognosy. Indeed, in African tropical forests like Congo basin, Great apes (GAs) were reported as a good model for the understanding of malaria infection patho-physiology. 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 (Ekutsu et al. 2016, Ngbolua et al. 2016a, Ngbolua et al. 2015).

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 (Ngbolua 2019a, Ngbolua et al. 2014a). 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 (Tshibangu et al. 2016, Ekutsu et al. 2016, Ngbolua et al. 2016a, Ngbolua et al. 2015).

The aim of this study is to evaluate the chemical composition and bioactivity of *Anthocleista schweinfurthii* Gilg. (Gentianaceae) fruits, leaves, root bark and stem bark extracts against SCD and associated bacteria, a plant species which has been selected through the animal source of knowledge (new paradigm) in order to develop new phytomedicine for SCD like Drepanoalpha (Ngbolua et al. 2019b). *A. schweinfurthii* is a shrub or tree of 3 to 30 m high whose trunk has 8 at 70 cm in diameter. Leaves are wide; their length reaches 45 cm and width 3.5 to 18 cm. The flowers have a white or cream color. They are long by 55 to 60 cm.

II. Methodology

2.1 Samples collection

The plant material (Fruits, leaves, root bark and stem bark) used in the present study was collected in the “MONASTERE L’ASSOMPTION” forest located in the Mont-Ngafula commune (4°25’ S latitude and 14°09’ E longitude). Its average altitude is 357 m above sea level.

2.2 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 methanol 500 mL at room temperature. After filtering the methanol filtrates were pooled and evaporated to dryness under reduced pressure using a rotary evaporator to yield crude ethanolic extract. Extract was stored at 4°C.

2.3 Phytochemical screening and organic acid extraction

The dried and powdered plant material (10 g) was repeatedly extracted by cold percolation with 95% methanol (MeOH) and water (100mL × 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, terpenes and steroids according to standard protocol (Bruneton 1999). The organic acids were extracted as previously reported (Tshibangu et al. 2016).

2.4 Blood and antisickling assay

Blood samples used to evaluate the antisickling activity of the plant extracts in this study were taken from known SCD 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 (Ngbolua et al. 2014b). They were found to be SS blood and were then stored at $\pm 4^{\circ}\text{C}$ 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. Sick cell blood was diluted with 150mM phosphate buffered saline (NaH_2PO_4 30mM, Na_2HPO_4 120mM, NaCl 150mM) 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 methanol 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 China Group Co LTD) and statistical data analysis were processed using Microcal Origin 6.1 package software.

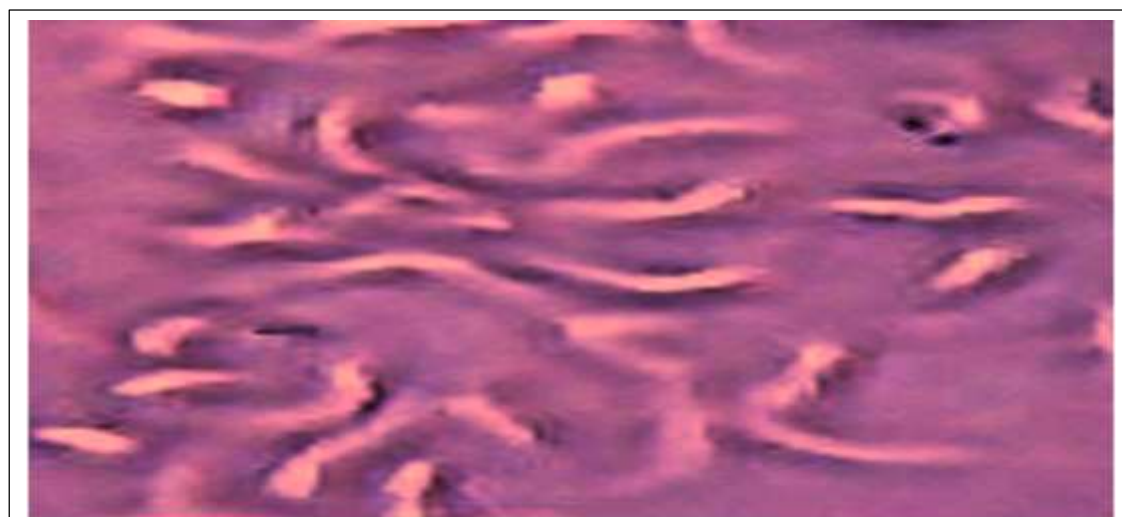
2.5 Determination of Minimum inhibitory concentration (MIC)

The antibacterial activity of *A. schweinfurthii* was assessed against selected bacteria strains by the micro-well dilution method and the minimum inhibitory concentration (MIC) values, which represent the lowest sample concentrations that completely inhibit the growth of microorganisms, were obtained using this method (Ngbolua et al. 2014c, Tshibangu et al. 2016). The 10 mg samples (Fruits, leaves, root bark and stem bark) were each dissolved in DMSO (250 μL) and diluted with Mueller–Hinton Broth (MHB) in order to reach concentrations of 2000 $\mu\text{g/mL}$ and a 5 mL solution (final volume, and 5% DMSO final concentration). These solutions were used as stock solutions. The inoculum of microorganisms was prepared from 24h old MHB cultures. The microbial suspensions were prepared by adding five colonies of each of the test bacteria to 2mL of with sterile physiological solution (0.9% NaCl) and adjusted with this sterile physiological solution to match that of a 0.5 McFarland standard solution (10^8 cells/mL). They were then diluted (1/100) to achieve 10^6 CFU/mL. The assay was carried out using sterile clear polystyrene 96-well microtiter plates (round bottom). The wells in the columns 2 to 8 and those in columns 11 and 12 were filled with 100 μL MHB (Mueller Hinton Broth). Briefly 200 μL of stock solution of each *A. schweinfurthii* sample were added to the wells in column 1 (A1 to H1), and two-fold serial dilutions were made from column 1 to column 8. Then 5 μL of the inoculum were dispensed to all the wells except those in column 12. The wells in columns 11 and 12 we used as positive and negative controls. The negative control wells (growth control) contained MHB and bacteria suspension without test sample (column 11) and the positive control wells contained only MHB (control of MHB sterility: column 12). The microplates (96 wells) were incubated at 37°C for 24 hours. After the incubation, 5 μL de colorant resazurin 1% (7-Hydroxy-3H-phenoxazin-3-one10-oxide) were added to each well and the microplates were then incubated for 5 hours. The minimum inhibitory concentration (MIC) was determined as the lowest *A. schweinfurthii* extracts dose at which no growth were observed after 24 and 48 hours.

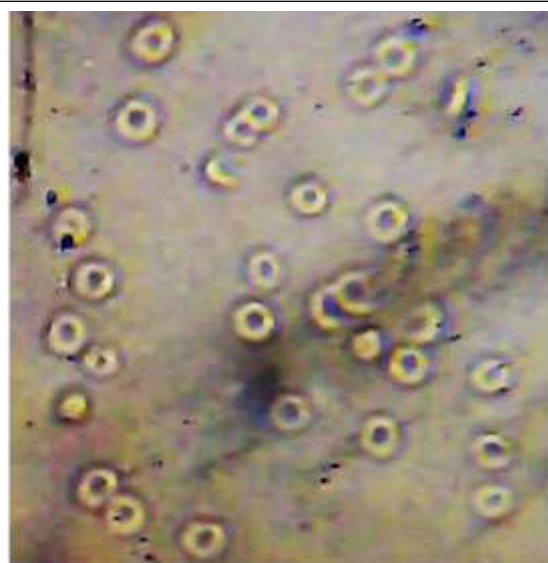
III. Discussion

The phytochemical analyses performed on *A. schweinfurthii* fruits, leaves, root bark and stem bark extracts revealed the presence of alkaloids, saponins, total polyphenols, flavonoids, tannins, anthocyanins, leuco-anthocyanins, quinones. The presence of various secondary metabolites in the plant could justify its medical use. Indeed, *A. schweinfurthii* is reported to

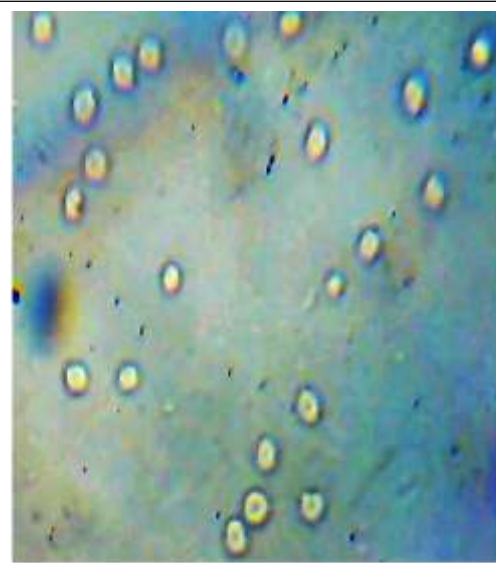
treat bacterial infections (Bruneton 1999). Compounds, which are significantly present in the plant, are well known for their large spectrum of pharmacological properties, including antimicrobial (alkaloids) and antioxidant (polyphenols) activities (Ngbolua et al. 2014c).



(a) Untreated SS RBCs (control)



(b) SS RBCs + organic acid extract



(c) SS RBCs + MeOH extract

Figure 1. Microscopy phenotype of sickle erythrocyte in different conditions (NaCl 0.9%, $\text{Na}_2\text{S}_2\text{O}_5$ 2%, X500)

Figure 1a shows that the control contains in majority sickle-shaped erythrocytes, confirming the SS nature of the blood. Mixed together with ethyl acetate and methanol extracts of the fruits (Figure 1b, c), the majority of erythrocytes are reversed normal-shape. This indicates that *A. schweinfurthii* have antisickling effects. This activity could be due to compounds such as anthocyanins or phenolic or triterpenoic acids as previously reported

(Tshibangu et al 2016 et Ngbolua et al 2014a). The treated SS RBCs demonstrated a remarkable similarity to normal red blood cells through the cellular parameters such as radius, area and the perimeter of RBCs. Indeed, the antisickling activity results in the resurgence of the radius (passage of the irregular shape lengthened to the circular shape), the increase of the cellular surface (from 299.5 to 314.2 μm^2) and the decrease of its perimeter (from 110.3 to 62.8 μm) as revealed by the results of computer analysis in table 1.

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

Paramètre mesuré	Untreated SS RBCs (Control)	Treated SS RBCs	Observation
Radius (μm)	0.0	10.0	Reappearance
Surface (μm^2)	299.5	314.2	Increase
Perimeter (μm)	110.3	62.8	Decrease

[Image size: width (460 pixels), height (435 pixels), objective lens (x100), accuracy (1), Resolution (460x345x24 bit), uncompressed size (464 kb), software package: Motic Images ver 1.3.] (In the analysis of images by this software, the normalized erythrocytes are considered as a circle while the drepanocytes have irregular geometric form).

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 plant extract (organic acids rich extract, 50 $\mu\text{g/mL}$), conduct into the re-appearance of the biconcave form of RBCs by reducing the perimeter of sickle RBCs and increasing their surface ($p < 0.05$).

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 *A. schweinfurthii* fruits extract was evaluated against *E. coli* and *S. aureus* strains. The n-hexane, ethyle acetate and methanol extracts are exhibited moderate antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* with MIC values of 125 $\mu\text{g/mL}$ and the n-hexane extract showed a weak antibacterial activity against *Escherichia coli* with MIC values of 500 $\mu\text{g/mL}$.

This antibacterial activity is however weak if compared to the MIC reference value of 100 $\mu\text{g/mL}$. Indeed, a value lower or equal to 100 indicates a great antibacterial activity. The difference in the bioactivity would be due to the nature of the bacterial wall (Tshibangu et al. 2016).

These results are in agreement with previous works on the antimicrobial activity of natural products of plant origin. It was reported that *S. aureus* and *E. coli* constitute both the principal bacteria responsible for septicemia and the osteomyelitis in SCD patients (Diagne et al. 2003). To this end, *A. schweinfurthii* is a better candidate for the development of

phytomedicine with broad spectrum of action for the management of SCD. These results corroborate former work on the antimicrobial properties of the secondary metabolites of plant origin (Ngbolua et al. 2016b). It is well established that *S. aureus* and *E. coli* constitute the principal bacteria responsible for septicemia and the osteomyelitis in SCD patients (Diagne et al. 2003). To this end, a plant species displaying at the same time antibacterial and antisickling activities is a better candidate for the development of phytomedicine with broad spectrum of action for the management of SCD. The weak antibacterial activity observed in this study can be improved by bio-guided fractionation of the ethyl acetate or methanol soluble fraction. To our knowledge, it is for the first time that the antisickling activity *A. schweinfurthii* is reported in the literature.

IV. Conclusion

The present study evaluated the chemical composition and the antisickling and antibacterial activities of *A. schweinfurthii*. The results revealed that:

- The fruits of *A. schweinfurthii* contains various secondary metabolites such as the anthocyanins, flavonoids, tannins, quinones, saponins, alkaloids, steroids, terpenoids and leuco-anthocyanins ;
- All tested extracts displayed interesting antisickling activity. Organic acid extract revealed the most interesting antisickling activity *in vitro* ;
- The antibacterial activity of the plant extracts was weak toward tested bacterial strains (CMI >125 µg/mL).

This study provided experimental evidence that supports further development of *A. schweinfurthii* extracts as a medicine for the management of SCD in endemic areas.

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