Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2016; 5(4): 181-187
Received: 22-05-2016
Accepted: 23-06-2016

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Chemical composition and bioactivity of *Canarium schweinfurthii* stem bark extracts from DR Congo against Sickle cell disease and associated bacteria

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Abstract
The aim of the present study was to evaluate the Chemical composition and bioactivity of *Canarium schweinfurthii* stem bark extracts against Sickle cell disease and associated bacteria. The antisickling and antibacterial activities were carried out using Emmel and micro-dilution methods respectively. The results revealed that the stem bark of *Canarium schweinfurthii* contains various secondary metabolites such as the anthocyanins, flavonoids, tannins, quinones, saponins, alkaloids, steroids, terpenoids and leuco-anthocyanins. The n-hexane (non-polar solvent) extract displayed poor yield than the extracts obtained in the polar solvents which have a high yield. Flavonoids were most concentrated in the methanol solvent whereas the anthocyanins were concentrated in acidified methanol while the tannins were concentrated in n-hexane (condensed tannins). The extraction yield of organic/triterpenoic acids (betulinic acid rich extract) was 0.75%. All tested extracts displayed antisickling activity. Organic acid extract was the most interesting with an ED_{50} equal to 12.5 µg/mL. While, the antibacterial activity of different plant extracts tested was weak toward tested bacterial strains (CMI>250 µg/mL). The antibacterial activity can be improved by bio-guided fractionation of the ethyl acetate or methanol soluble fraction.
This study provides for the first time a scientific basis for the *in vitro* antisickling activity of *C. schweinfurthii*.

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

Introduction
Sickle cell disease (SCD) is a life-long blood disorder characterized by red blood cells that assume an abnormal, rigid and sickle shape. It is a genetic 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 [1, 2]. In recent years bone marrow transplantation and gene therapy have been proposed as an efficient way of treating SCD. However the cost implications, availability of necessary expertise, problem of finding suitable donors, inadequate transfusion and transfusion related infections constituted a major setback to this approach in developing countries. Currently, drugs such as hydroxyurea and erythropoietin are used as disease modifier agents. Cost and side effects of these drugs limit their clinical use [3].
In Democratic Republic of the Congo (DRC), it was reported that 12% of the hospitalized children are sicklers and that the annual cost of the treatment of this hemoglobinopathy is higher than 1.000,00 USD per patient. [4] This cost is 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 medicinal plants for the treatment of SCD and associated bacteria. [5-10]. *Canarium schweinfurthii* Engl. (family Burseraceae) is traditionally used in African Traditional Medicine as insecticide or against dysentery, gonorrhea, coughs, chest pains, pulmonary affections/*Mycobacterium tuberculosis*, stomach complaints, food poisoning, purgative and emetic, roundworm infections and other intestinal parasites, emollient, stimulant, diuretic, skin-affections, eczema, leprosy, ulcers; diabetes mellitus; colic, stomach pains, pains after child birth, gale; fever, constipation, malaria, sexually transmitted infection and rheumatism. [11, 12] A recent extensive survey of literature revealed that *C. schweinfurthii* is a good source of health promoting secondary metabolites such as phenolic and terpenoic acids

Among others that could have many wonderful applications [13]. The plant is also belongs to the great apes (GAs) feeding and is potentially non-toxic to man and could provide new sources of anti-sickle cell hemolytic compounds. [14] Great apes constitute a good model for the understanding of infectious disease patho-physiology and in designing new therapeutic strategies. As for humans, GAs have coevolved with malaria parasite in endemic regions. These animals adopt a particular feeding when they are displaying symptoms by selecting specific plants for controlling malaria parasite infection while this one cause hemolytic anemia of human red blood cells (like does the polymerization of hemoglobin S in sickle erythrocytes). [15, 16]
Since *C. schweinfurthii* is reported to contain phenolic and terpenoic acids [17-19], it can therefore, be hypothesized by chemotaxonomy approach that this plant could possess hemolytic inhibitory effects on sickle red blood, thus justifying the present study. The aim of this study is to evaluate the chemical composition and bioactivity of stem bark extracts from *Canarium schweinfurthii* against Sickle cell disease and associated bacteria**.**

2. Materials and Methods

2.1. Plant material collection and identification

The tested plant material (stem barks) used in the present study was collected by Professor Koto-te-Nyiwa Ngbolua in Abumombazi city (Nord Ubangi Province, Democratic Republic of the Congo) during a field work in July-August 2014 and was authenticated by Mr B.L. Nlandu of the INERA (Institut National d'Etudes et Recherches Agronomiques). Voucher specimen N0 Ngb011NU is on deposit at the Laboratory of Molecular bio-prospection (Department of Biology, Faculty of Science, and University of Kinshasa).

2.2. Extraction, chemical screening and preparation of increasing polarity extracts

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, anthocyanis, leuco-anthocyanins, quinones, terpenes and steroids according to standard protocol. [20] Fractions were filtered and concentrated to dryness under reduced pressure using a rotary evaporator. Organic/triterpenic acids were extracted as follow: the powdered stem bark of *C. schweinfurthii* (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. [21] To obtain the increasing polarity extracts, the powdered plant material was exhaustively extracted with n-hexane (1:10, p/v), ethyl acetate, and methanol and acidified methanol (HCl 1%). The resulting fractions were evaporated to dryness on an evaporator apparatus. All extracts were stored at +4 °C.

2.3. Quantification of secondary metabolites

2.3.1. Total phenolic

Total phenolics contents were determined according to the Folin-Ciocalteu method with slight modifications. [22] 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= 0,006x-0,002 ; R^2 = 0,997).

2.3.2. Flavonoids

Total flavonoid content was measured by the aluminum chloride colorimetric method. [23] 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 quecertin equivalents (QE) per one gram dry powder. One mL of standard solution (quecertin: 5-100 µg/mL) was used to construct calibration curve (the standard curve equation: Y = 0.009x + 0.006; R^2 = 0,999).

2.3.3. 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 =A_{540} *(10/0.6)*d (with A_{540}= maximum of absorption at 540 nm; d = dilution factor; 0.6 maximum of absorption of 10 mg/L of M-3-GE standard solution). [24]

2.3.4. 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 (Na_2CO_3 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 = 0,443x - 0,264; R^2 = 0,720. [25]

2.4. Biological testing

2.4.1. *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 andall 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 Haemoglobin 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. [26-30]

2.4.2. Determination of antibacterial activity

- **Microbial strains**

The activity of the plant samples was tested toward *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. [31, 32] 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 (10^8 cells/mL). The prepared microbial suspension was diluted (1/100) to achieve 10^6 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 10^6 CFU/mL overnight culture was added to wells of a sterile 96-well micro-plate titer. The positive control wells contained MHB+ 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.

3. Results and Discussion

3.1. Phytochemical study

The chemical screening performed on the aqueous and alcoholic extracts of *Canarium schweinfurthii* revealed the presence of alkaloids, saponins, total polyphenols, flavonoids, tannins, anthocyanis, leuco-anthocyanins, quinones, terpenes and steroids. Phenolic compounds such as anthocyanins [2, 3, 8-10, 14, 15, 26-30], rosmarinic acid [33] and lunularic acid [34] and triterpenes like betulinic, maslinic, oleanolic [35] and ursolic acid [36] were reported to display antisickling activity *in vitro*.

The table 1 gives the extraction yield and chemical composition of stem bark extracts from *Canarium schweinfurthii.*

Table 1: Extraction yield and chemical composition of stem bark extracts from *Canarium schweinfurthii*

Extract	Yield (%)	Secondary metabolites			
		Total phenol (µg GAE/g)	**Flavonoids (µg QE/g) (%ratio)**	**Anthocyanins (µg M-3-GE/g) (%ratio)**	**Tannins (µg TAE/g) (%ratio)**
n-hexane	0.22	32.390 ± 0.190	0.440±0.220 (1.35)	0.172±0.009 (0.53)	0.850±0.005 (2.60)
Ethyl acetate	0.48	146.660 ± 0.500	0.940±0.395 (0.64)	0.467±0.029 (0.31)	1.056±0.002 (0.70)
Methanol	1.36	92.220 ± 0.190	2.366±0.127 (2.565)	0.400±0.016 (0.43)	0.952±0.030 (1.00)
Acidified methanol	1.22	77.660 ± 0.835	1.366±0.353 (1.75)	0.550±0.016 (0.70)	0.864±0.025 (1.10)

It is deduced from the table 1 that the n-hexane (non-polar solvent) extract has poor yield compared to the extracts obtained in the polar solvents which have a high yield. This reveals that the abundant metabolites in the stem bark of *C. schweinfurthii* are those which pass easily through the polar solvents (ethyl acetate and methanol). It should moreover be noted that the flavonoids are concentrated in the methanol whereas the anthocyanins are concentrated in acidified methanol while the tannins are concentrated in n-hexane. In this last case, they could be the condensed tannins. The extraction yield of organic/triterpenoic acids (betulinic acid rich extract) is 0.75%.

The figure 1 give the ultra-violet fingerprint of triterpenoic acids rich extract from the stem bark of *C. schweinfurthii*.

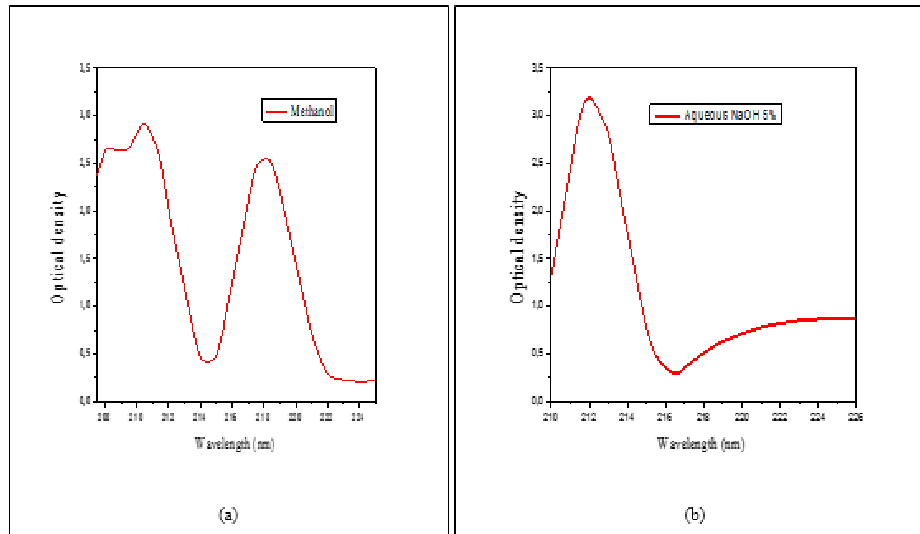

Fig 1: Ultra-violet spectrum/fingerprint of triterpenoic acids rich extract in methanol (a) and aqueous NaOH 5% (b).

The figure 2 give the morphology of untreated sickle erythrocytes or SS RBCs treated with *Canarium schweinfurthii* stem bark extracts.

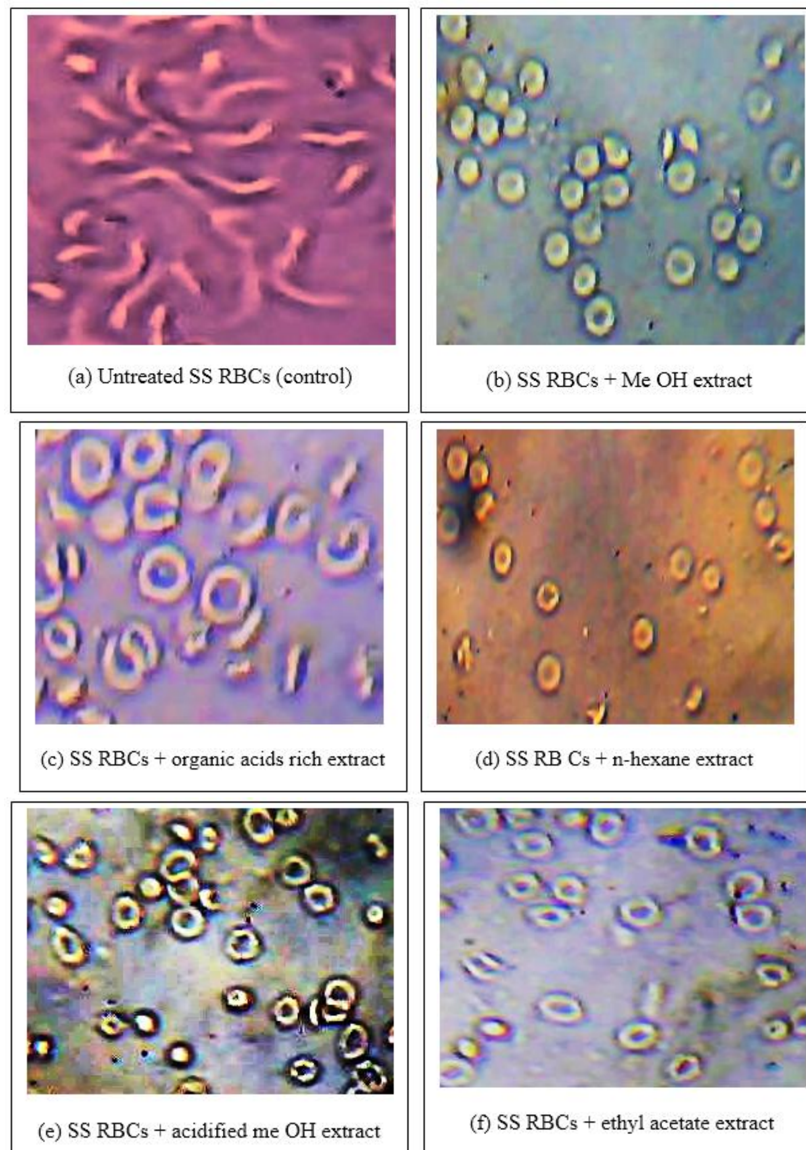

Fig 2: Morphology of untreated sickle erythrocytes (a) or SS RBCs treated with 50 µg/mL of *Canarium schweinfurthii* stem bark extracts (b-f) (X500), [NaCl 0.9%; $Na_2S_2O_5$ 2%].

Figure 2a shows that the control contains in majority sickle-shaped erythrocytes, confirming the SS nature of the blood. Mixed together with n-hexane, ethyl acetate, methanol, acidified methanol and organic acids extracts (Fig. 2, b-f), the majority of erythrocytes are reversed normal-shape. This indicates that *Canarium schweinfurthii* stem bark have antisickling effects. This activity could be due to compounds such as anthocyanins or phenolic or triterpenoic acids as previously reported. [2, 3, 8-10, 14, 15, 26-30, 33-36] The treated SS RBCs demonstrated a remarkable similarity to normal blood values.

Figure 3 shows the dose dependent antisickling activity of triterpenic acids rich extract from *Canarium schweinfurthii*.

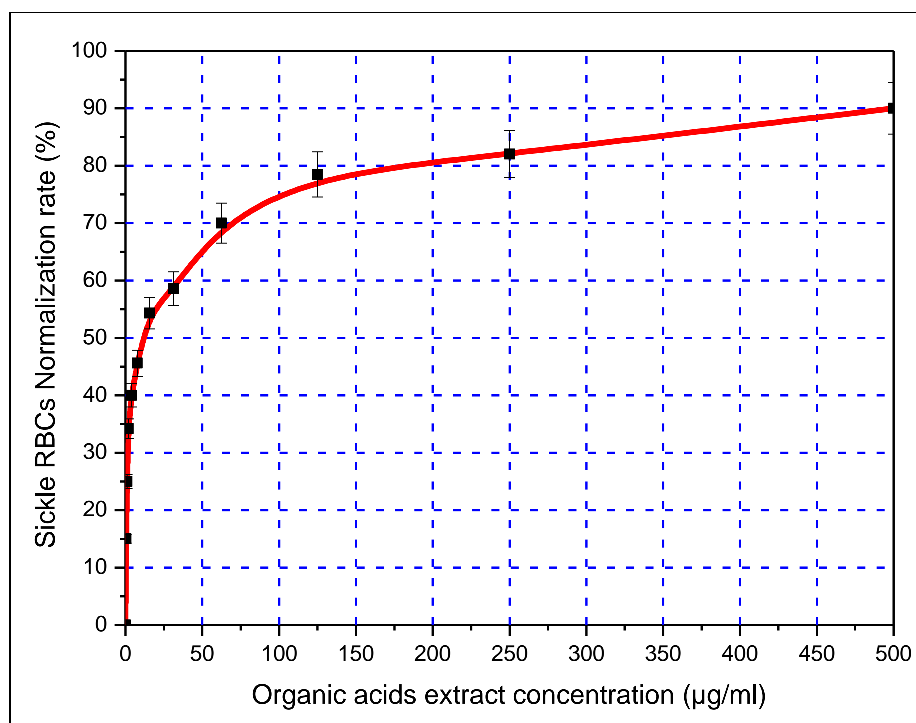

Fig 3: Evolution of normalization rate of sickle erythrocytes with triterpenic acids rich extract from *C. schweinfurthii.*

The curves show that, the normalization of sickle erythrocytes increases with the extract dose and reach a maximum and constant value at 125 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 78%. The ED_{50} (ie the dose of extract which may normalize up to 50% of the total cell used) was 12.5 µg/mL.

3.3. Antibacterial activity

Due to the high cost of modern therapy for SCD, plant extracts displaying at the same time antibacterial and antisickling activities could be useful in the management of this hereditary blood disorder. The antibacterial activity of *Canarium schweinfurthii* stem bark extracts against *E. coli* and *S. aureus* strains was evaluated and results are shown in Table 2.

Table 2: Antibacterial effect of plant extracts

Plant extracts	Concentration (µg/ml)									MIC (µg/ml)
	1000	500	250	125	62,5	31,25	15,625	7,813	3,906	
Escherichia coli ATCC 25922										
n-hexane	-	+	+	+	+	+	+	+	+	1000
Ethyl acetate	-	-	+	+	+	+	+	+	+	500
Methanol (MeOH)	-	-	+	+	+	+	+	+	+	500
Acidified MeOH	-	+	+	+	+	+	+	+	+	1000
Organic acids	-	+	+	+	+	+	+	+	+	1000
Staphylococcus aureus ATCC 1103										
n-hexane	-	+	+	+	+	+	+	+	+	1000
Ethyl acetate	-	-	+	+	+	+	+	+	+	500
MeOH	-	-	+	+	+	+	+	+	+	500
Acidified MeOH	-	-	+	+	+	+	+	+	+	500
Organic acids	-	-	+	+	+	+	+	+	+	500

It is deduced from this table that *S. aureus* is more sensitive to *Canarium schweinfurthii* (four extracts have a CMI=500 µg/mL) than *E. coli* (two extracts have a CMI=500 µg/mL).This antibacterial activity is however weak (CMI>250 µg/mL) [37] and would be due to the presence of phenolic and triterpenic compounds present in the tested extracts. This difference in the bioactivity would be due to the nature of their wall. [38] Indeed, contrary to *E coli*, *S. aureus* is a positive gram bacterium. Its wall is thick (several layers) and would be the pharmacological target of the biologically active compounds present in *Canarium schweinfurthii* whereas for *E coli*, the external

membrane would prevent the chemical compounds from penetrating in the bacterial cell. These results corroborate former work on the antimicrobial properties of the secondary metabolites of plant origin. [31, 32] It is well established that *S. aureus* and *E coli* constitute the principal bacteria responsible for septicemia and the osteomyelitis in SCD patients. [5] 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 of *Canarium schweinfurthii* is reported in the literature.

Conclusion

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

- The stem bark of *Canarium 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* (ED_{50}= 12.5 µg/mL.).
- The antibacterial activity of the plant extracts was weak toward tested bacterial strains (CMI>250 µg/mL).

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

Acknowledgments

The authors thank the "Académie de Recherche et d'Enseignement supérieur (ARES), Belgium" for Research grants PAH ARES/UNIKIN 2015 offered to Koto -te- Nyiwa Ngbolua, Pius T. Mpiana and Damien S.T. Tshibangu. The authors are also indebted to The World Academy of Sciences (TWAS) (Grant No. 15-156 RG/CHE/AF/AC_G–FR3240287018) for providing financial assistance.

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