Original Research Article

Anti-sickling and antibacterial activities of Some Extracts from *Gardenia ternifolia* subsp. *jovis-tonantis* (Welw.) Verdc. (Rubiaceae) and *Uapaca heudelotii* Baill. (Phyllanthaceae).

ABSTRACT

Aims: To evaluate the antisickling and antibacterial activities of *Gardenia ternifolia* and *Uapaca heudelotii.*

Study design: Evaluation of the antisickling and antibacterial activities of anthocyanins and organic acids extracted from *Gardenia ternifolia* and *Uapaca heudelotii In vitro*

Place and Duration of Study: Faculty of Science, University of Kinshasa, between November 2012 and April 2013.

Methodology: The antisickling and antibacterial activities of anthocyanins and organic acids extracted from *Gardenia ternifolia* and *Uapaca heudelotii* were assessed using Emmel, and disc diffusion and micro-dilution methods respectively. The disc diffusion method was used to determine the antibacterial activity of extracts while the micro-dilution method was performed to determine their MIC and MBC.

Results: The present study revealed that anthocyanins and organic acids extracts from *G. ternifolia* and *U. heudelotii* possess antisickling and antibacterial activities. All tested extracts from *U. heudelotii* displayed interesting antisickling and antibacterial effects. At the extract dose of 6. 25 µg/mL, the calculated normalization rates were 70% (for anthocyanins extract of *U. heudelotii*), 80% (for organic acids extract of *U. heudelotii*), 68% (for anthocyanins extract of *G. ternifolia*) and 72% (for organic acids extract of *G. ternifolia*). The reference bacterial strains *S. aureus* were more sensitive to anthocyanins extract: MIC = 31.25 (*U. heudelotii*) and 62.5 µg/mL (*G. ternifolia*) than the *E. coli* strains: MIC = 62.5 µg/mL (*U. heudelotii*) and 125 µg/mL (*G. ternifolia*).

Conclusion: This study provides a scientific basis for the antimibacterial and antisickling activities of anthocyanins and organic acids extracts from *Gardenia ternifolia* and *Uapaca heudelotii*. Isolation and purification of different phytochemicals may further yield significant antibacterial and antisickling new leads compounds.

Keywords: Sickle cell disease, bacterial infections, Medicinal plants, drepanocyte normalization rate, MIC, Democratic Republic of the Congo.

Abbreviations: ACE: Anthocyanis extract, ATCC: American Type Culture Collection, DRC: Democratic Republic of the Congo, MBC: Minimum bactericidal concentration MHB: Mueller Hinton Broth, MIC: Minimum inhibitory concentration, OAE: organic acids extract, RBC: Red blood cell, SCD: Sickle Cell Disease

1. INTRODUCTION

Microbial infections, especially those due to bacteria are recurrent pathologies of the Sickle Cell Disease (SCD) and remain the leading cause of death in children patients (1, 2). Epidemiological data reveal that about 300,000 children are born each year in the world with pathological hemoglobin of which 70% are affected by SCD (3). Most of the patients die before the age of five years when they do not receive regular health care. This disease is endemic to tropical regions. In some African regions, carriers of S hemoglobin can reach 20% of the population with a prevalence of 25-30% in central Africa. In Democratic Republic of the Congo (DRC), more than one million of inhabitants or almost 2% of the population are sicklers.

Clinical management of SCD focuses mainly on prophylactic measures for alleviating the painful crises through administration of analgesics, antipyretics, oral antibiotics such as penicillin and the anticancer drug hydroxyurea as epigenetic modulator of fetal hemoglobin gene. Unfortunately, not only these treatments were not effective, they are also expensive for the poor African population and may present HIV / AIDS infection risk (4-6).
Alternative strategy in the management of SCD is now focusing on the identification of the novel antisickling agents mainly that from medicinal plants. Indeed, traditional medicine continues to play a very significant role in the medical primary health care implementation in developing countries.

It is known that in SCD endemic regions, the use of herbs for the treatment of patients is a common practice. In previous studies of our research team, a number of medicinal plants traditionally used for the management of SCD in DRC were reported. These plant species have been scientifically validated for their beneficial effects in SCD condition including anti-sickling, anti-aggregating/anti-polymerization, radical scavenging (antioxidant) and anti-dehydrating effects. These activities are mainly due to anthocyanins and organic acids (7-11). In the present study, we hypothesized that the naturally occurring anthocyanins and organic acids of Congolese plants *G. tonatis* and *U. heudelotii* could also have antisickling as well as antibacterial properties.

Thus this research work was performed with the aim of evaluating the antisickling and antibacterial activities of anthocyanins and organic acids extracted from *Gardenia ternifolia subsp. jovis-tonantis (*Welw.*)* Verdc. (Rubiaceae) and *Uapaca heudelotii* Baill (Phyllantaceae).

2. MATERIAL AND METHODS

2.1. Plant material collection and identification

The tested plant materials (leaves) used in this study were collected in Democratic Republic of the Congo during a field work in March 2011 and were authenticated by Mr B.L. Nlandu of the INERA (Institut National d'Etudes et Recherches Agronomiques). Vouchers specimens (*Gardenia ternifolia*: ref. N0 Ngb-BC002) and (*Uapaca heudelotii*: ref. N0 H. Breyne 2576) are on deposit at the INERA *Herbarium* of the Faculty of Science (University of Kinshasa).

2.2. Extraction and chemical screening

The dried and powdered plant material (10 g) was repeatedly extracted by cold percolation with 95% ethanol (EtOH) and water (100 mL, twice) for 48 hours. Chemical screening was performed in aqueous and organic extract according to as previously reported protocol (7, 12-14). Fractions were filtered and concentrated to dryness under reduced pressure using a rotary evaporator. Extraction of anthocyanins and organic acids was then done using 100 g of dried powdered plant material following an established protocol [4,15-18]. Anthocyanins extracts were then defatted by n-hexane and all extracts were stored at +4 ℃.

2.3. Biological testing

2.3.1. 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, DRC. 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, as previously reported [1, 3-18]. They were found to be SS blood and were then stored at +4 ℃ in a refrigerator. An informed cons ent was obtained from all the patients participating in the study. 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 anthocyanins or organic acids 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 Chine Group Co LTD) and statistical data analysis were processed using Microcal Origin 7.1 package software as previously reported (1, 7-9).

2.3.2. Determination of antibacterial activity

- ***Microbial strains and media used***

The activity of the plant samples was tested toward *Lactobacillus fermentum* (*L. fermentum* ATCC 9338), *Staphylococcus aureus* (*S. aureus* ATCC 25923), *Enterococcus faecalis* (*E. faecalis* ATCC 19433), *Salmonella typhimurium* (*S. typhimurium* ATCC 13311) and *Escherichia coli* (*E. coli* ATCC 25922). The tested strains were obtained from the

American Type Culture Collection (ATCC, Rockville MD, USA). Mueller Hinton agar and Mueller Hinton Broth purchased from Conda (Madrid, Spain) were used as media

- **Disc diffusion method**

The agar disc diffusion method was used to determine the antibacterial activity of plant extracts as follow: A 1 mL of suspension of an 18 hours culture bacteria containing about $1x10^8$ colony-forming units per milliliter (CFU/mL) were spread on Mueller Hinton agar medium using sterile swabs. Filter paper discs (6 mm in diameter) were soaked in 10 µL of extracts and placed on the inoculated plates and allowed to dry for 30 min, then incubated at 37 ℃ fo r 24 hours. The diameters of the inhibition zones were measured in millimeters (19). Two controls were included in the test: the first was a control involving the presence of microorganisms but without the test extract sample and the last was two standard antibiotics (gentamicin disc, 10 µg/mL for gram-positive bacteria and ofloxacine disc, 20 µg/mL for gram-negative bacteria). Studies were performed in triplicate, and the developing inhibition zones were compared with those of reference discs.

- **Determination of Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)**

The Minimum inhibitory concentration was determined by broth micro-dilution method as previously reported (1, 20). 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 to match that of a 0.5 McFarland standard solution. From the prepared microbial solutions, other dilutions with sterile physiological solution were prepared to give a final concentration of 10^6 CFU/mL. Stock solutions of the extracts were prepared in 0.1% (v/v) aqueous tween 80 (Fisher chemicals) at concentrations of 1 mg/mL. The two-fold serial dilutions in concentrations of the extracts were prepared in Mueller Hinton Broth (MHB) (Conda, Madrid, Spain) to give final concentrations ranging from 250 to 1.95 µg/mL.

An aliquot (10 µL) of a $1x10^6$ 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. MTT (30 µL) in aqueous solution (0.01%) was used to evaluate the micro-organism viability. For MBC determination, 10 µL was taken from each well of complete inhibition of bacterial growth after incubation and spot inoculated on freshly prepared MHB and incubated for 72 hours at 37 ℃. The concentration at which no growth was observed on subculture was determined as the MBC.

3. RESULTS AND DISCUSSION

3.1. Chemical screening

The results of chemical screening of *G. ternifolia* and *U. heudelotii* are presented in Table 1.

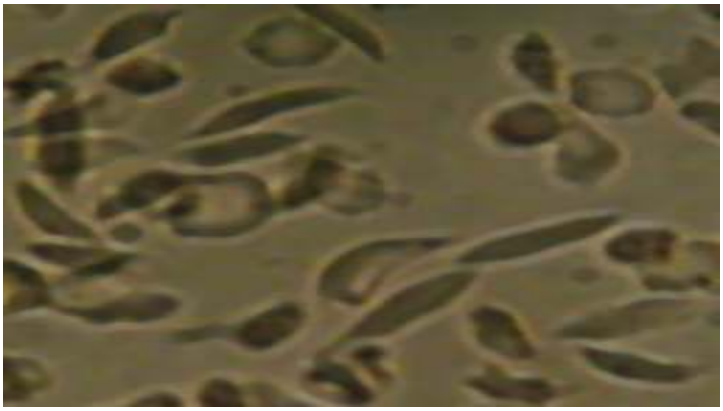
Table 1: Results of chemical screening of *G. ternifolia* and *U. heudelotii*

Chemical groups	Medicinal plant species (used parts)	
	G. ternifolia (leaves)	*U. heudelotii* (leaves)
Total polyphenols	+	+
Anthocyanins	+	+
Leuco-anthocyanins	+	+
Flavonoids	+	+
Gallic tannins	+	+
Catechic tannins	+	+
Coumarines	+	+
Quinones	+	+
Alkaloïds	+	+
Saponins	+	+
Terpenes and steroids	+	+

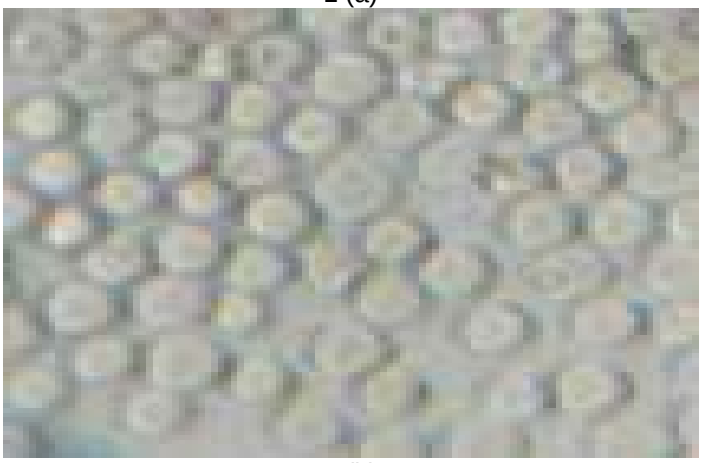
It is deduced from the table 1 that the leaves of both *G. ternifolia* and *U. heudelotii* contain total polyphenols, catechic tannins, alkaloids, saponins, terpenes, steroids, anthocyanins, leuco-anthocyanins, flavonoids, gallic tannins, coumarins and quinines. Compounds which are present in these plant species, are well known for their broad spectrum of pharmacological properties, including antimicrobial, antisickling and antioxidant activities (8-10, 21-24). Hence, the presence of various secondary metabolites in these plants could justify their use as medicine in Africa.

3.2. Antisickling activity

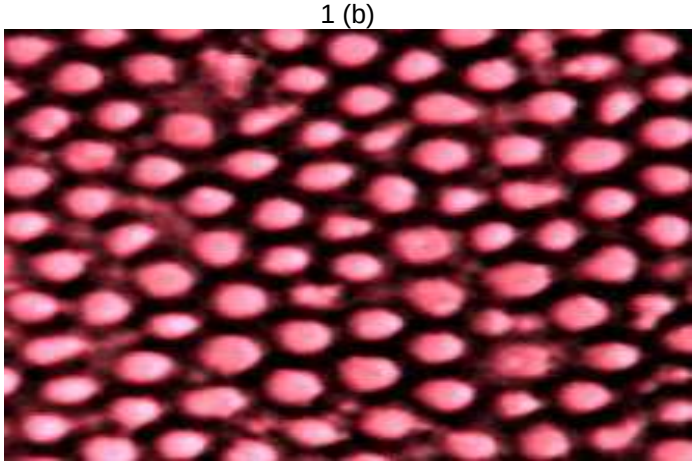
Figures 1a, b, c, and d show the optical micrograph phenotypes of SS blood alone (a) or treated with anthocyanins (b and c) and organic acids crude extracts (d and e) extracted from the two selected plants.



1 (a)



1 (b)



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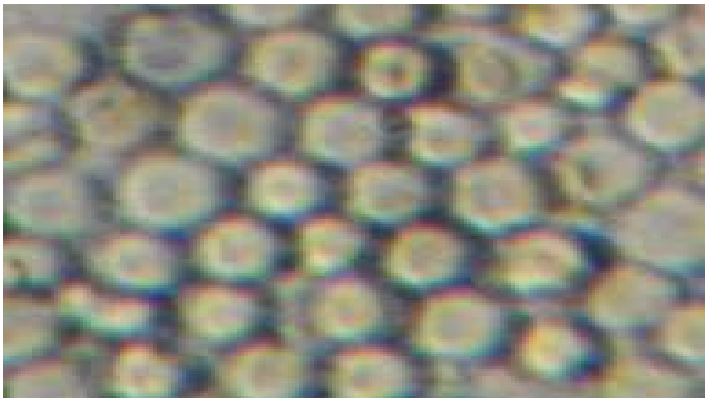

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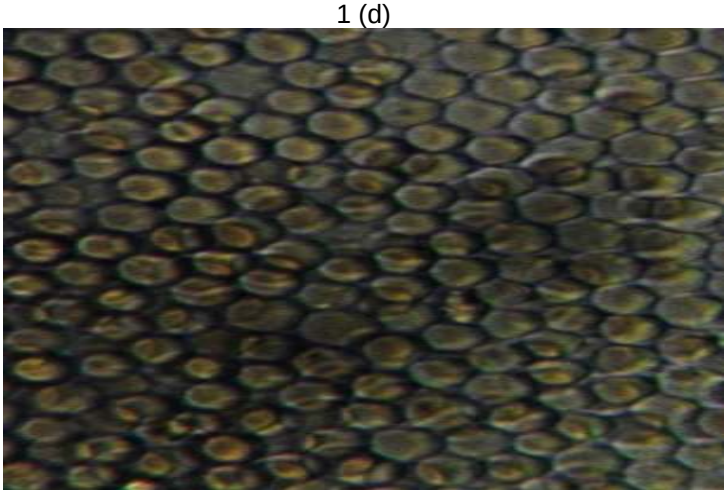

1 (e)

Fig.1: Phenotype of SS red blood cells (SS RBCs) [NaCl 0,9% ; $Na_2S_2O_5$ 2%; X500]*:* (a): untreated SS RBCs (control); (b) SS RBCs treated with anthocyanins extract of *G. ternifolia* (20 µg/mL); (c): SS RBCs treated with anthocyanins extract of *U. heudelotii* (20 µg/mL); (d): SS RBCs treated with organic acids extract of *G. ternifolia* (20 µg/mL); (e): SS RBCs treated with organic acids extract of *U. heudelotii* (20 µg/mL).

Figure 1 shows that the control (a) contains in majority sickle-shaped RBCs, confirming the SS nature of the blood used. Mixed together with anthocyanins and organic acids extracts (b-d), the majority of SS RBCs are reversed normal-shape. At the extract dose of 6, 25 µg/mL, the calculated normalization rates were found to be 70% (for anthocyanins extract of *U. heudelotii*), 80% (for organic acids extract of *U. heudelotii*), 68% (for anthocyanins extract of *G. ternifolia*) and 72% (for organic acids extract of *G. ternifolia*) as it can be seen on the figure 2.

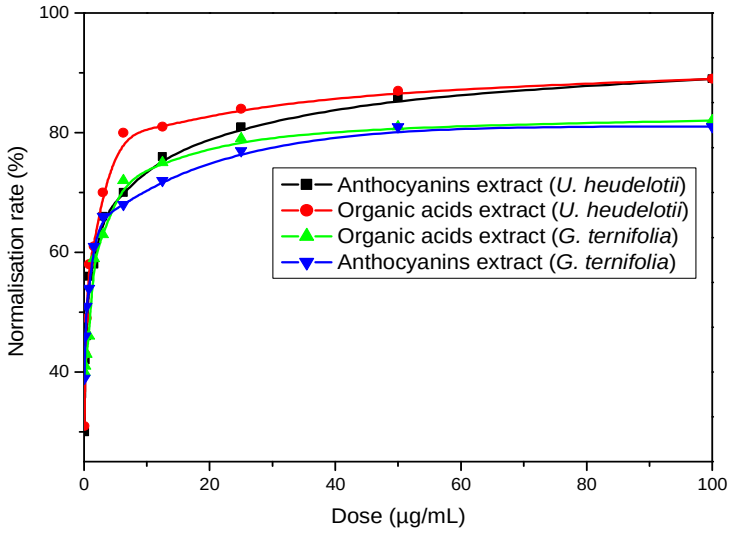

Fig.2: Evolution of the normalization rate of sickle erythrocytes with dose extracts from selected medicinal plant species (NaCl 0,9%; Na2S2O5 2%; X500). %Normalization rate = [number of drepanocytes of untreated SS blood (control) - number of drepanocytes of treated SS blood (sample)/ number of drepanocytes of untreated SS blood] ×100.

Our results indicated that anthocyanins and organic acids are the major antisickling agents of the tested plants. These results confirm those already reported by our research team on anthocyanins and organic acids from other plants used in Congolese folk medicine for the management of SCD (3-12). As previously reported, anthocyanins have the ability to interact with proteins (15). Interaction of these secondary metabolites with hemoglobin S could chemically compete with the polymerization of this abnormal hemoglobin thus preventing the sickling of sickle erythrocytes. In addition, anthocyanins (for which intestinal catabolism gives phenolic acids) are also known for their antioxidant properties and could affect the Fe^{3+}/Fe^{2+} higher ratio in sickle cells and the stability of erythrocytes membrane by preventing the oxidation of membranes phospholipids (16).

3.3. Antibacterial activity

The antibacterial activity of extracts from selected plants against *L. fermentum, S. aureus, E. faecalis,* and *S. typhimurium* was determined. The results are shown in Table 2.

Table 2: Bacterial inhibitory growth effects of extracts from *G. ternifolia* and *U. heudelotii*

Reference bacterial strains	*Gardenia ternifolia*						*Uapaca heudelotii*					
	Anthocyanins extract (µg/mL)			Organic acids extract (µg/mL)			Anthocyanins extract (µg/mL)			Organic acids extract (µg/mL)		
	25	**50**	**100**	**25**	**50**	**100**	**25**	**50**	**100**	**25**	**50**	**100**
L. fermentum	-	+	+	+	+	+	-	-	+	-	+	+
S. aureus	-	+	+	-	-	+	+	+	+	+	+	+
E. faecalis	-	-	+	-	-	-	-	+	+	+	+	+
S. typhimurium	-	+	+	+	+	+	-	-	+	+	+	+
Activity (%)	**58**						**75**					

Legends - : Inactive extract (inhibition zone = 0 mm); +: Active extract (inhibition zone > 15 mm);
Positive control: gentamicin 10 µg/mL (inhibition zone = 19 mm), ofloxacine, 20 µg/mL (inhibition zone = 26 mm); %S: sensitivity percentage.

The antibacterial activity of extracts is closely dependent on the tested plants and the reference bacterial strains. Overall, *Uapaca heudelotii* was more active than *Gardenia ternifolia* (% average activity equal to 75 vs 58) (table 2). It should also be noted that *L. fermentum* and *S. typhimurium* showed inhibition zones to all *G. ternifolia* tested extracts while *S. aureus* and *E. faecalis* showed in contrary more less inhibition zones toward *U. heudelotii* extracts. Considering the strains globally, it should be noted that *E. faecalis* was the bacteria strain which has been showed less number of inhibition zones. *E. faecalis* is a Gram-positive bacterium belonging to the family of *Enterococcaceae* which is reported in the literature to be involved in drug resistance towards antibiotics such as aminoglycosides, cephalosporins, clindamycin, oxacillin and cotrimoxazole (25).
However, it should be noted that at low dose (25 µg/mL), organic acids extracts of *U. heudelotii* inhibited the growth of this bacterium. This result showed that the secondary metabolites from the plants under study can serve as a source of new antibacterial agents. The results revealed also that *S. aureus* was the most sensitive strain.

3.4. Determination of Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The results of MIC and MBC are shown in Table 3.

Table 3: Minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of *Gardenia ternifolia and Uapaca heudelotii*.

Reference bacterial strains	MIC/MBC (µg/mL)			
	Gardenia ternifolia		***Uapaca heudelotii***	
	ACE	**OAE**	**ACE**	**OAE**
E. coli ATCC 25922	125/500	125/>500	62.5/250	125/>500
S. aureus ATCC 25923	62.5/250	125/>500	31.25/62.5	125/>500

Legends: ACE Anthocyanis extract; OAE Organic acids extract

The Gram positive bacterium *S. aureus* was more sensitive to Anthocyanis extract (ACE) than the Gram negative *E. coli*. (table 3). The higher sensitivity of Gram-positive bacteria could be attributed to their outer peptidoglycan layer which is not an effective permeability barrier. Gram-negative bacteria possessing an outer phospholipidic membrane carrying the structural lipopolysaccharide components make the cell wall impermeable to lipophilic solutes while porins constitute a selective barrier to hydrophilic solutes with an exclusion limit of 600 Da (20, 26). *S. aureus* is the bacteria most implicated in septicemia and osteomyelitis in SCD patients (2). Medicinal plant extracts displaying at the same time antibacterial (against *Staphylococcus aureus*) and antisickling effects could be useful in the management of Sickle cell disease. It has been reported that human gut microflora have the ability to metabolize anthocyanins into smaller phenolic acids which are more bioavailable and stable end-products (27). As anthocyanins metabolic stable end-products, they could have less antibacterial activity than their parents. The present study confirmed this hypothesis.
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. Such important nutraceuticals will contribute to solve health problem.

4. CONCLUSION

The present study provided evidence for the antibacterial and antisickling activities of anthocyanins and organic acids extracts from *Gardenia ternifolia* and *Uapaca heudelotii* and bring working data for future investigations that will lead to the characterization of chemical structure of anthocyanin and organic acid extracts from these plants.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

Authors KN Ngbolua and DST Tshibangu wrote the first draft of the manuscript, PT Mpiana designed the study, SO Mihigo wrote the protocol , BK Mavakala and MC Ashande managed the analyses of the study and LC Muanyishay managed the literature searches. All authors read and approved the final manuscript

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee

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