



Microscopic Features, Mineral Contents, Anti-sickling, Antioxidant and Antibacterial Activities of Stem Bark of *Harungana madagascariensis* Lam. Ex Poiret (*Hypericaceae*)

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Authors' contributions

This work was carried out in collaboration among all authors. Author FBM designed the study, performed the statistical analysis and wrote the protocol. Authors FBM, NKN, KNN, PMN and PKM wrote the first draft of the manuscript. Authors FBM, MMN, PKM, MMM, JVB, NKN, FK and JBM managed the analyses of the study. Authors TFM, PTM, MMN, FBM, JBM, NKN, KNN, GNB and KNN managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2020/v31i2030355

Editor(s):

(1) Dr. Elena Maria Varoni, University of Milan, Italy.

(2) Prof. Marcello Iriti, Milan State University, Italy.

Reviewers:

(1) Daniela Tofani, Roma Tre University, Italy.

(2) Marwa Yassien Badr, Tanta University, Egypt.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/64851>

Original Research Article

Received 25 October 2020
Accepted 30 December 2020
Published 31 December 2020

ABSTRACT

Aims: This study addressed the identification of bioactive compounds, the determination of mineral content and the evaluation of the antibacterial, antioxidant and anti-sickling activities of aqueous extract from stem bark of *Harungana madagascariensis*.

Place and Duration of Study: Centre d'Etudes des Substances Naturelles d'Origine Végétale (CESNOV), Faculty of Pharmaceutical Sciences, University of Kinshasa and Faculty of Sciences, National Pedagogic University in DRC, between October 2018 and January 2021.

Methodology: Phytochemical screening was evaluated by chromatographic methods (TLC and HPLC), the mineral composition by ICP-AES and the antioxidant activities using ABTS and DPPH assays, the antibacterial activity against four bacteria strains using the micro-dilution method; and the *in vitro* anti-sickling activity by the Emmel test.

Results: Phytochemical analysis revealed the abundance in this plant of phytochemicals such as alkaloids, flavonoids, tannins, anthraquinones and triterpenoids. The extract contains sodium, iron, selenium and zinc in proportions of 955.40 ppm, 369.65 ppm, 375.70 ppm and 44.79 ppm respectively as well as copper, chromium and other elements. The antioxidant potential of the stem bark infusion extract from *H. madagascariensis*, evaluated by the ABTS and DPPH tests, showed very high antioxidant activity due to this free radical scavenging capacity linked to IC₅₀ values of 3.08 ± 0.19 µg/mL (ABTS) and 3.53 ± 0.22 µg/mL (DPPH). The antibacterial sensitivity testing with MIC determination performed on a strain isolated from a sickle cell patient and four reference strains showed the MIC values of the extract ranged from 31.25 µg/mL to 250 µg/mL. The lowest MIC value was observed on *S. aureus* ATCC 25923 while the highest MIC value was obtained on *Enterococcus spp* Clinical.

Conclusion: The stem bark extract of *H. madagascariensis* showed high anti-sickling activity at 10.42 µg/mL, which may be due to its phytochemical constituents such as saponins, alkaloids and flavonoids. Obtained results could justify the efficacy of recipes from stem bark of *Harungana madagascariensis* in the management of various infections in traditional medicine and anti-sickle cell disease.

Keywords: *Harungana madagascariensis*; micronutrients; mineral; biological activities.

1. INTRODUCTION

Sickle cell anaemia is a genetic disease, which provokes an aggregation of hemoglobin in the HbSS erythrocytes due to lower oxygen tension, resulting in the characteristic distorted sickled shapes of those erythrocytes. Some studies have tried to reverse the sickle shape of erythrocytes *in vitro* by the incubation of HbSS blood cells with different chemical agents [1,2]. These chemical compounds present a variety of drawbacks as potential therapeutic drugs in the management of sickle cell disease. Sickle cell disease such as sickle cell anemia is most prevalent in sub-Saharan Africa and is now a public health problem for most countries in sub-Saharan Africa where 20 to 40% of subjects carry the sickle cell trait in Central and West Africa. The incidence of sickle cell anemia in the Democratic Republic of the Congo (DRC) is 2% in newborns, or about 40,000 cases per year.

In a context where treatment is only symptomatic, the use of phytotherapy is an effective alternative for these low-income African populations. The use of medicinal plants for the

treatment of various diseases is an important economic advantage. Therefore, the search for active ingredients derived from plants is more relevant than ever. This is mainly due to the fact that the plant kingdom represents an important source of huge varieties of bioactive compounds [3]. These molecules have multiple interests and are used in the food, cosmetic and pharmaceutical industries. Among these compounds we have coumarins, alkaloids, phenolic acids, tannins, terpenes, flavonoids, etc. which have interesting pharmacological properties.

Harungana madagascariensis Lam. ex Poir. (*Hypericaceae*) is a plant, which is used in the treatment of various infections in traditional medicine [4].

Chemical investigations on *H. madagascariensis* Lam. ex Poir. (*Hypericaceae*) have shown that more than 200 compounds distributed in different structural classes have been isolated, with flavonoids, terpenoids and alkaloids as the main classes of secondary metabolites. Flavonoids are the most representative and diverse [4]. Given

that free radicals constitute one of the pharmacological targets to be prioritized in the development of anti-sickle cell disease drugs and that flavonoids are recognized essentially for their antioxidant action, modulating the activity of certain enzymes, which allows to validate the *in vitro* anti-sickling activity of the stem bark of *Harungana madagascariensis* Lam. ex Poir. (*Hypericaceae*), a plant used in traditional medicine in Nord Ubangi (DR of the Congo), to determine its various characteristic elements from the cytological and histological point of view of powders (Micrography) and to describe its phytochemical (TLC and HPLC) as well as the mineral composition (ICP-EAS) and to evaluate the antibacterial, antioxidant and anti-sickling activities

2. MATERIALS AND METHODS

2.1 Inclusion Criteria

To be included in the study, blood should come from patients whose sickle cell status had been proven by hemoglobin electrophoresis technique and who had not been transfused within four months prior to blood sampling.

2.2 Plant Material

The plant material consisted of stem bark of *Harungana madagascariensis* Lam. ex Poir. (*Hypericaceae*) harvested in November 2018, in the Luki Biosphere Reserve in Bas-fleuve territory, Kongo Central Province, DRC (Fig. 1 and 2). The plant was identified at the Herbarium

of the “Institut National d’Etudes et de Recherches Agronomiques” (Voucher n°: INERA/LUKI 1785) and confirmed by the “Laboratoire de Botanique systématique et Ecologie des plantes”, Department of Biology, Faculty of Sciences, University of Kinshasa.

The samples were dried at room temperature in the Molecular Bio-Prospecting Laboratory (Department of Biology) for two weeks and ground to a fine powder. Prior to extraction Stem bark were ground and stored in brown covered glass bottles.

2.3 Microscopic Analysis

Micrography is one of the most fundamental methods to control the quality of medicinal plants. It would be very thoughtful to prepare the plate to be observed under the microscope in order to distinguish the distinct elements constituting the powder. Two drops of Steimetz reagent have been placed on a slide and a fine amount of powder was added [5].

This slide is covered with a cover slide in order to homogenize the preparation and then the microscopic examination was carried out. It is important to make a good preparation by carefully wiping the outer surface of the slide and light preparations should be made in order to distribute the tissue well and to avoid superimposition of the cytological structures.

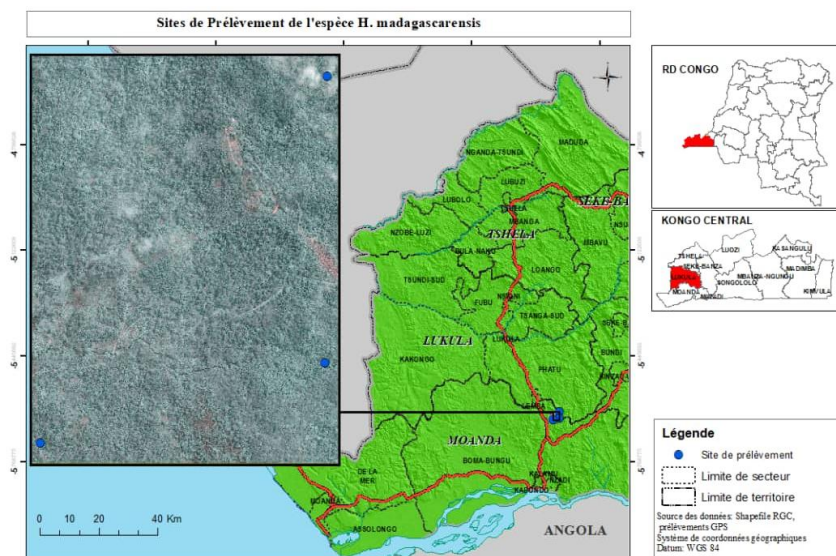


Fig. 1. Harvest site of *Harungana madagascariensis* Lam. ex Poir. (*Hypericaceae*)

Observations on the powders were made using the European Pharmacopoeia reagent, Lactic Acid Reagent (Steimetz Reagent) [5]. Observations were made with a VisiScope BL124 (VWR) microscope, and photographs were taken with the Huawei P30 Pro Smart Phone.

2.4 Preparation of Extracts

Weigh 100 g of powder to which was added 1 L of boiling water (100° C) in an Erlenmeyer flask. The mixture was stirred for 48 hours at room temperature. At the end of stirring, the extract was filtered with a filter paper (Whatman N°1) under vacuum pump (taking into account the viscosity of the mixture). The dry extract, concentrated by lyophilization, was weighed and stored in dark, sterile, airtight vials at 4 °C prior to analysis.

2.5 Chemical Analysis

2.5.1 Phytochemical screening

General phytochemical screening of *H. madagascariensis* Lam. ex Poiret was performed according to Bruneton [6]. Thin Layer Chromatography (TLC) Analysis of 10 µL of solution for 10 mg/mL of methanol and dichloromethane extracts solutions was carried out on normal phase Silica gel 60 F₂₅₄ plates (Merck), using different eluents for the identification of secondary metabolites [7].

2.5.2 High-performance liquid chromatography (HPLC) analysis

Analytical separation on HPLC-DAD was carried out on a Hypersil ODS[®] RP18 column as described by Kapepula et al. [8].

2.5.3 Mineral analysis by ICP-AES

The determination of minerals content was carried out by the method of water and nitric acid and analyzed by Inductively Coupled Argon Plasma Atomic Emission Spectrometry (ICP-AES) [9]. Then 0,3 g of plant powder diluted in 5 mL of distilled water was placed in PM60 Teflon bombs (Analytikjena 40Bar) and heated at 60°C, then 10 mL of nitric acid (65% HNO₃) (Merck) were added. The mixture was allowed to react for 30 minutes at room temperature in the bombs which were covered with caps and then stripped with HNO₃/H₂O (v/v, 1:1). The bombs were then placed in the high frequency microwave mineralizer (Analytikjena AG TOPwave: 2.5 Ghz,

Germany) controlled by microcomputer by choosing the vegetable leave mode as a digestion mode at 180°C, 50bar for 1 hour. At the end of mixing, the digester was stopped by letting the bombs rest for 3 hours until completely cooled. The cooled analyte was thus carefully transferred from bombs by filtration on Whatman filter paper, to previously stripped 50 mL volumetric flasks. The initial volume was diluted to 50 mL with distilled water and 13 mL of analyte were placed in previously stripped 15 mL conical flask for reading by Inductively Coupled Argon Plasma Atomic Emission Spectrometry (ICP-AES) (Optima 8300 Perkin Elmer, USA). The analysis was performed in triplicate. The calibration of the ICP-AES was performed using the working standard prepared from the commercially available standard multi-element solution 3 at two points (1 mg / L and 2.5mg / L, Perkin Elmer, USA). The most appropriate wavelength, gaseous argon flow, plasma stabilization and other ICP-AES instrument parameters for minerals were selected and measurements were made in the linear range of the working standards used for calibration.

Working conditions were: Instrument: ICP-AES (Optima 8300 Perkin Elmer, USA); Power of Rf: 1500 Watt; Plasma gas flow (Ar): 8L /min; Nebulizer: 0.70L / min; Auxiliary gas flow (Ar): 0.2L / min; Viewing size: 5-22 mm; Copy and playback time: 1-5s (maximum 45s); Flow time: 1s (maximum 10s); View: Radial.

2.5.4 Cell free antioxidant assays

The extracts were solubilized in Dimethylsulfoxide (DMSO), thus their effect was compared to a control test performed with DMSO alone. Antioxidant activity was carried out through spectrophotometric ABTS and DPPH that were performed according to the method described by Kapepula et al. [10].

2.5.5 Anti-sickling cell activity

The blood samples used to evaluate the anti-sickling activity of the plant extracts were provided by the "Centre de Médecine Mixte et d'Anémie SS de Kinshasa" in the DRC. None of the patients had been recently transfused with Hb A blood and all experiments were performed with freshly drawn blood. In order to confirm their SS status, the above blood samples were first characterized by hemoglobin electrophoresis on cellulose acetate gel (at alkaline pH) and then stored at ± 4°C in the refrigerator.

The Emmel test was performed to evaluate the capacity of positive control and extracts to correct the sickling of red blood cells as followed: An aliquot of SS blood was diluted with 150 mm physiological phosphate buffered solution and mixed with an equivalent volume of 2% sodium metabisulfite. One drop of the mixture was placed on a microscope slide with or without plant extracts and covered with a slide. Paraffin was applied to completely seal the edges of the microscopic preparation to exclude air (hypoxia). Triplicate analyses were performed for each extract. Red blood cells (RBCs) were analyzed using computer-assisted image analysis software (Motic Images 2000, version 1.3; Motic China Group Co LTD) and statistical data analysis was processed using Microcal Origin 8.5 Pro software, as described above [11]. Betulinic acid was used as positive control.

2.5.6 Antibacterial activity

The antibacterial activity was evaluated using the microdilution method on a 96-well sterile polystyrene microplates and in liquid medium as previously reported [12]. The extract to be tested (10 mg) was dissolved in 250 μ L of DMSO and the final volume was adjusted to 5 mL with Mueller Hinton culture medium. The bacterial suspension was prepared by adding in 2 mL of saline solution (0.9% NaCl) for each strain. Three colonies isolated from strains to be tested namely (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 29922, *Pseudomonas aeruginosa* ATCC 9027 strains and *Enterococcus* spp. (multiresistant strains isolated from sickle cell patients) were generously offered by Institut National de Recherches Biomédicales) and a 24 h incubation allowed to obtain 0.5 McFarland (10^6 cells/mL).

Therefore, the bacterial suspension was diluted in order to have 10^6 cells/mL (1:100 dilution). The microdilution assay was performed in a 96-well sterile polystyrene microplate. Briefly, 100 μ L of culture medium were placed inside wells. After, 200 μ L of each extract to be tested (2.000 μ g/mL) were placed in wells. Thus, take 100 μ L of each extract stock solution for serial dilutions of 2 by 2 up to the ninth column and the last 100 μ L (column 9) are removed. 5 μ L of the inoculum (10^8 CFU/mL) are aseptically removed with a micropipette and added to all wells of the microplate except for wells of the 11th column used as control for the bacterial growth and wells of the 12th column, which were used as control of

sterility of culture medium. Microplates were incubated in an oven at 37°C for 24 hours. After the incubation period, 3 μ L of Resazurin dye (1%) was added to each well and the microplates were kept for a second incubation for 7 hours. The Minimum Inhibitory Concentration (MIC) (first wells with no bacterial growth) was determined 24 hours later. All experiments were performed in triplicate.

2.6 Statistical Analysis

All results were expressed as mean values \pm standard deviation (SD). The statistical analysis was performed with GraphPad 7.0 (GraphPad Software, San Diego California, USA). Two-way analysis (ANOVA) and Student's paired t-test were used; multiple comparisons of all data were performed using the "Tukey" Multiple Comparisons Test and the level of statistical significance was set at $p < 0.05$. The IC_{50} values were calculated with GraphPad Prism 7.0 under application of the function "log (inhibitor) vs. normalized response-variable slope" after converting the concentrations into their decimal logarithm.

3. RESULTS AND DISCUSSION

3.1 Microscopic Features

Powders from the stem bark of *H. madagascariensis* treated with Steimetz reagent showed the following botanical microscopic characteristics: Parenchymal cells of the phloem, cork in surface view, sclera, stem parenchyma showing cells with secondary walls; fragment of parenchyma with crystals, parenchymal cells containing a group of brown pigment fibers with calcium oxalate prisms, oil droplets and other elements to be characterized (Figs. 2 and 3). To our knowledge, there is no work that describes the microscopic histological characteristics of de *H. madagascariensis*. Medicinal plant materials are classified according to their microscopic and macroscopic sensory characteristics. Micrography is useful for the identification and authentication of medicinal plants and for detecting adulterated and poor quality medicinal plants [13]. Adulteration of plant samples is a serious problem that involves the intentional or unintentional mixing of a plant species with other plant species of other genera, or even toxic materials. Microscopic analysis is one of the cheapest methods to correctly identify phytomedicines and raw materials of medicinal

plants [13,14]. Thus, the knowledge of the microscopic details of the species being studied is one of the most valuable tools for assessing the quality and identity of these plant species.

However, in-depth microscopic examinations should be carried out particularly to determine the dimensions of the characteristic elements identified. Although micrography alone cannot provide a complete evaluation profile of a medicinal plant, it can nevertheless provide supporting evidence, which in combination with other analytical parameters such as chromatographic fingerprints, can be used to obtain the full range of evidence necessary for

the standardization and evaluation of medicinal plants [14].

3.2 Phytochemicals

Phytochemical screening of the stem bark of *H. madagascariensis* by standard methods using colored reactions (Table 1) and by chromatographic methods (Fig. 4, 5 and 6), revealed the presence of phenolic compounds such as tannins, bound quinones, leucoanthocyanins and flavonoids. Alkaloids, coumarins, saponins and triterpenoids were also identified.

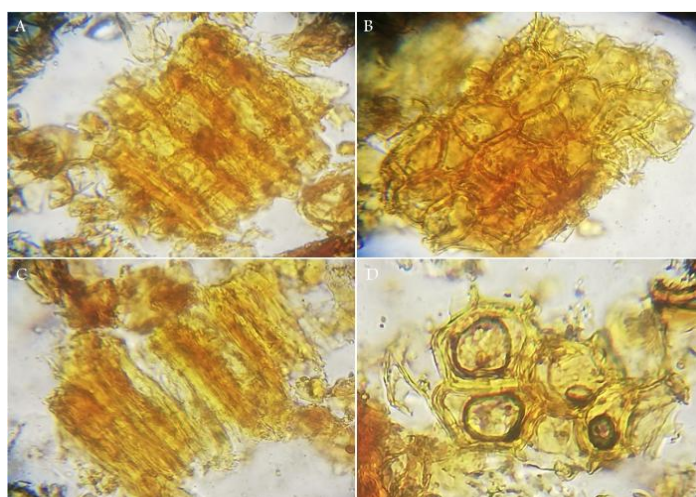


Fig. 2. Phloem parenchymatous cells (A), cork in surface view (B), sclereids (C), stem parenchyma showing cells with secondary walls (D)

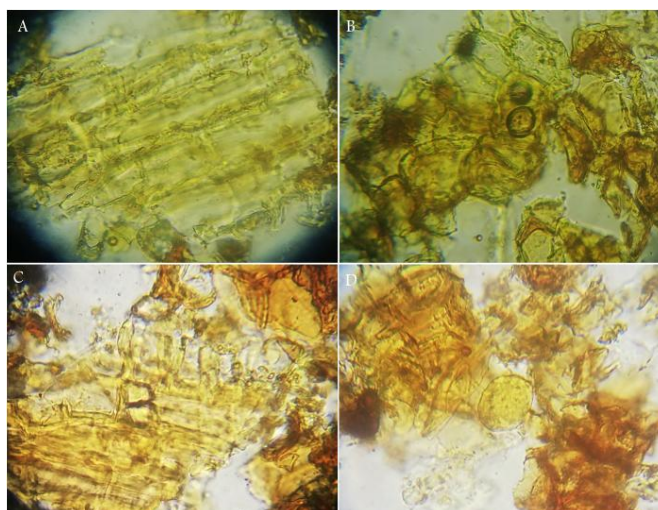


Fig. 3. Fragment of parenchyma with crystals (A), parenchymatous cells containing brown pigment group of fibers with prisms of calcium oxalate (C) and oil droplets (D)

Table 1. Phytochemical screening of *Harungana madagascariensis*

A. Results of chemical screening on the aqueous phase			
Chemical Groups Sought	Reagents used	Observations	Results Stem bark
1. Phenolic compounds			
Anthocyanins	HCl et NH ₄ OH	Red (Acid) and blue or greenish (Alca) colouring	+
Catechetical	Stiasny	Pink precipitate	+
Flavonoids	Cyanidine of Shinona	Colouring orange, red, violet	+
Gallic	Stiasny+FeCl ₃ + CH ₃ COONa	Blue or black tint	-
Leucoanthocyanins	HCl	Cherry red or purplish colouring	+
Linked Quinones	Bornträger	Pink to purplish-red colouring	+
Tannins	FeCl ₃ 1%	Dark blue to black green colouring	+
Total polyphenols	Burton	Blue coloration with blue precipitate	+
2. Alkaloids	Mayer	Precipitation	+
3. Saponines	Foam test	Persistent foam of more than 1 cm after 15 min.	+
4. Mucilages	Ethanol 94%	Precipitation	-
B. Results of chemical screening on the organic phase (Methanol)			
Chemical Groups Sought	Reagents used	Observations	Results Stem bark
1. Triterpenoids	Liebermann	Purple colouring	+
2. Steroids	Liebermann	Purple colouring	-
3. Free quinones	Bornträger and NaOH 10%	Blackish red coloration with precipitate	+
4. Coumarines	Bornträger and NaOH 10%	No fluorescence under UV light	+

Legend: +: Presence of the desired substance; -: Absence of the desired substance

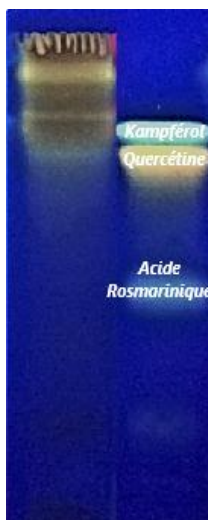


Fig. 4. TLC chromatogram of methanolic extracts of *H. madagascariensis* with rosmarinic acid, quercetin and kaempferol as standards; developed with Dichloromethane/Acetone/Formic acid (85:20:10; v/v/v) and visualized at 365 nm with natural products-PEG reagent. Flavonoids are detected as yellow, orange or green fluorescent spots and phenolic acids as blue fluorescent spots



Fig. 5. TLC chromatogram of methanolic extracts of *Harungana madagascariensis* with D-catechin as standard; developed with Ethyl acetate/Formic acid/Water (100:10:40; v/v/v) and visualized with phosphoric vanillin. Anthocyanins give pink colorings.

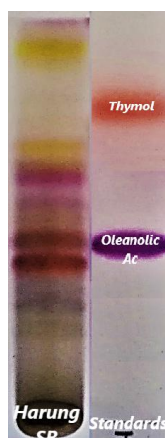


Fig. 6. TLC chromatogram of dichloromethane extracts of *H. madagascariensis* with oleanolic acid and thymol as standards; developed with Toluene/Ethyl acetate (93:7; v/v) and visualized at visible with sulfuric anisaldehyde reagent. Terpenes are detected as violet spots

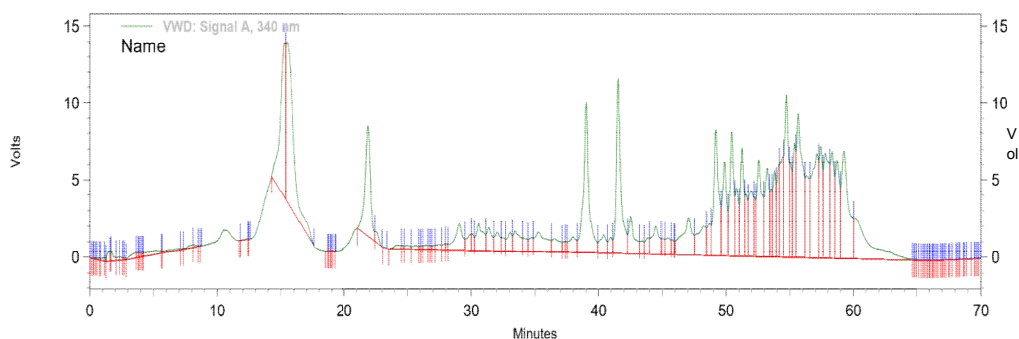


Fig. 7. HPLC profile at 340 nm of the methanolic extract of the stem bark of *H. madagascariensis*. HPLC in the gradient elution system: 0.05% trifluoroacetic acid in water milliQ (A) and acetonitrile (B): 0 min, 100:0 (A:B), 1 min, 93:7 (A:B); 45 min, 60:40 (A:B); 56 min, 40:60 (A:B); 67 min, 100:0 (A:B) and 70 min, stop at 1 mL/min on the hypersil ODS column (4.6 mm × 150 mm)

HPLC profiling of the stem bark extract in chromatographic system used at 340 nm indicated the presence of thirty-six major compounds and four minorities compound having retention times corresponding to rutin (23.722 min), chlorogenic acid (18.867 min), caffeic acid (7.617 min) and quercetin (25.337 min) and thirty-six unknown major compounds (Fig. 7).

Various phytochemical studies conducted on *H. madagascariensis* have also revealed the presence of alkaloids, lignans, coumarin amides saponins, tannins, reducing sugar, phenols, anthraquinones, cardiotoxic glycosides, resins as secondary metabolites that are also of chemotaxonomic importance to the species. Other metabolites such as flavonoids, sterols and

terpenes have also been isolated from *H. madagascariensis* [4,15-17].

3.3 Minerals

ICP-AES analysis on the stem bark of *H. madagascariensis* showed the presence of plant mineral among which trace elements in varying proportions (Figs. 8 and 9). The analysis of the minerals revealed the presence of calcium, magnesium, potassium, phosphorus and sodium.

Trace element analysis of the stem bark extract of *H. madagascariensis* revealed the presence of phosphorus, manganese, iron, copper, zinc, selenium and cobalt. The elements occurred in different proportions, most significant were iron, selenium and zinc in proportions of 369,65 ppm, 375,7 ppm and 44,79 ppm respectively (Fig. 9).

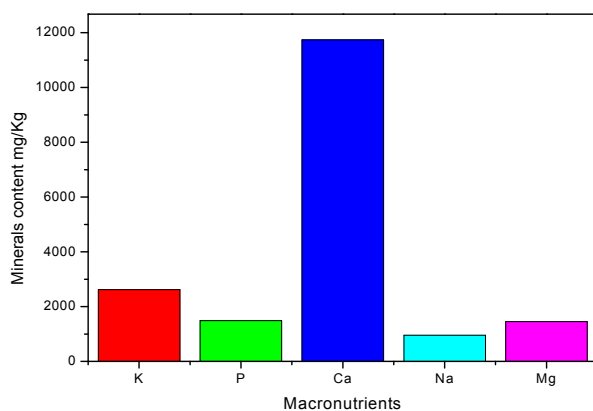


Fig. 8. Macronutrients content of the stem bark extract of *H. madagascariensis*

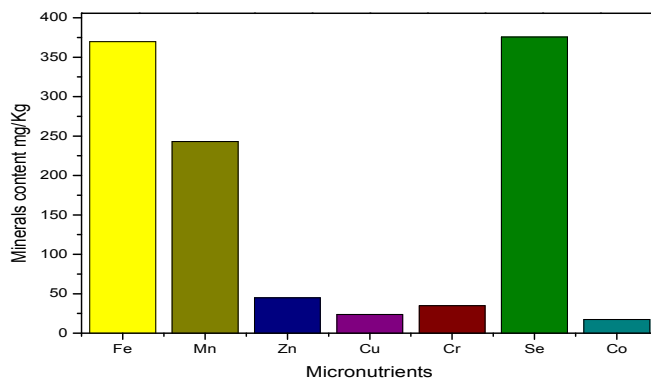


Fig. 9. Micronutrients content of the stem bark extract of *H. madagascariensis*

Plants require macro- and micronutrients, each of which is essential for a plant to complete its life cycle and their availability can fluctuate greatly in both space and time due to environmental factors such as weather, climate and physicochemical properties [18]. Minerals are required to meet a wide variety of essential metabolic and structural functions in the human body and their requirements and metabolism can be altered by chronic diseases [19,20]. These results corroborate those of Duke and Ayensu [21] and Olusayo et al. [22] who reported that the high iron content of 35.69, 15.52 and 35.21 mg/100 g found in the root bark of *Bridelia cathartica*; in the fruit extract of *H. madagascariensis* and *Lannea stuhlmannii*, respectively, are responsible for their use in the treatment of anaemia in East Africa.

3.4 Antioxidant Activities

The evaluation of antioxidant activity using ABTS and DPPH assays showed that infusion extract from the stem bark of *H. madagascariensis* had the ability to scavenge free radicals connected with their IC₅₀ values (Table 2).

Radical-scavenging activities do not vary significantly in each type of test. In ABTS and DPPH assays, such the cases of standards used (gallic acid and quercetin), the IC₅₀ values of ABTS assay were weak than those of DPPH assay. This difference is attributed to the reaction mechanisms. Indeed, ABTS reacts simultaneously with hydrophilic and lipophilic compounds while DPPH reacts only with hydrophilic compounds of the analyzed matrix. Obtained results showed very low IC₅₀ values, which testify to their more powerful scavenging radical effect. Lower IC₅₀ values indicate higher radical scavenging activity [23]. Previous studies reported the antioxidant capacities of some parts of studies plant. Harungana phenolic compounds such as harunganin, ferruginin and diterpenoids compounds were identified to be responsible of

antioxidant activity of *H. madagascariensis* [24-26].

Our result corroborate those of previous studies on extracts of other medicinal plants of the family of *Hypericaceae* [25-26] and on *H. madagascariensis* [27]. *H. madagascariensis* could therefore play a role in the prevention of oxidative stress-related diseases in areas where it is widely consumed as an aromatic plant without any adverse effects on nutrition.

The antiradical activities of the bark of *H. madagascariensis* stems could be correlated with their chemical composition. This activity could be attributed to the presence of polyphenols which are the most widespread secondary metabolites in the plant kingdom. They possess numerous biological properties including antioxidant capacities for which they are indicated in the management of various pathologies including sickle cell disease [28]. Free radical scavenging is one of the most important properties of plant extracts. Since our bodies may not produce enough antioxidants, it is necessary to use them every day to get rid of reactive species and thus oxidative stress. Reactive species (ROS) induced by oxidative stress can ultimately lead to apoptotic or necrotic cell death [29].

Today, in pharmacognosy the properties of polyphenols are widely studied where they are recognized as having various biological activities among others (antibacterial and anti-sickle cell activity) [28,30,31]. Polyphenols, as antioxidants, act by different ways which are: direct scavenging of reactive oxygen species (ROS), inhibition of enzymes involved in oxidative stress and chelation of oligo-metals responsible for ROS production, and protection of antioxidant defense systems [31]. The radical scavenging activity is an essential indicator of the antioxidant activity of the different forms of use in the traditional pharmacopoeia of this plant species.

Table 2. IC₅₀ values (µg/mL) of infusion extracts from stem bark of *H. madagascariensis* Lam. ex Poiret on ABTS and DPPH assays (Mean ± SD, n = 6).

Samples	IC ₅₀	
	ABTS (µg/mL)	DPPH (µg/mL)
<i>H. madagascariensis</i> (Stem Bark)	3.08 ± 0,19	3,53 ± 0,22
Quercetin	1.42 ± 0.04	3.21 ± 0.99
Gallic acid	0.71 ± 0.08	1.07 ± 0.10

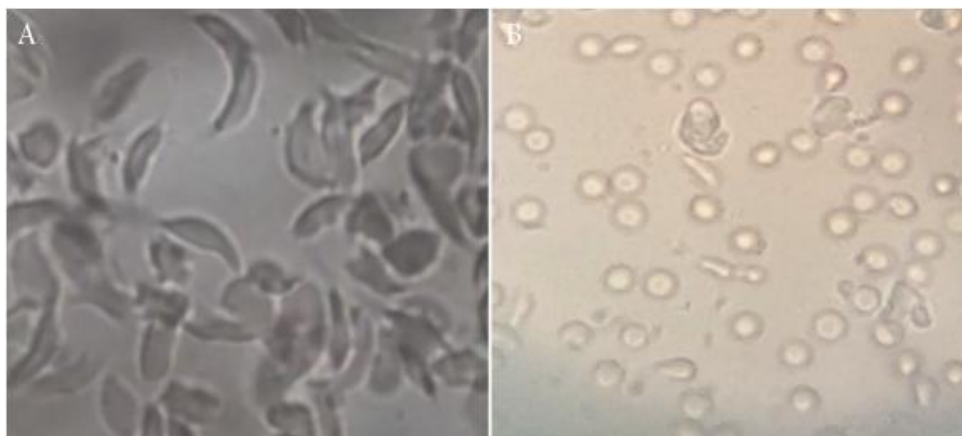


Fig. 10. Morphology of non treated erythrocytes (A); treated erythrocytes with 10.42 µg/mL of stem bark extract (b) from *Harungana madagascariensis* Lam. ex Poiret. [X 500; NaCl 0.9%; Na₂S₂O₅ 2%].

3.5 Anti-sickling Activity

The anti-sickling activity test showed that in the absence of plant extract, the erythrocytes are sickle-shaped, confirming the sickle-cell nature of the blood sampled. However, in the presence of the aqueous extract from *H. madagascariensis*, the sickle cell erythrocytes revert to the normal biconcave form (Fig. 10). These results indicate that stem bark of *H. madagascariensis* could have an anti-sickling effect on the erythrocytes.

The results showed that the infusion extract from stem bark is very active at concentration of 10.42 µg/mL and this activity could be due to the phytochemicals such as phenolic compounds and triterpenic acids identified in the plant. Previous studies have also reported identified phytochemical groups in the stem bark of *H. madagascariensis* [21,27,32]. Alkaloids and saponins from *H. madagascariensis* have been reported to possess anti-a potentials. [32] Saponins inhibit platelet aggregation or thrombosis while vitalizing and promoting blood circulation [21,27]. Also, saponins are membrane active compounds that lyse erythrocytes or other bio-membrane, it is possible that the erythrocytes could be initially lysed by the saponins rich plant extract and during the time, the cells were capable to cleave some of the terminal sugars from the saponin via production of glycosidic enzyme thereby detoxifying it [33].

The anti-sickling activity of phenolic compounds and triterpenic acids from Congolese plants were largely reported previously [23,34-38]. Tshilanda

et al. [38] revealed that ursolic acid was the major biologically active compound responsible of anti-sickling activity of *Ocimum gratissimum*. Phenolic acids such as lunularic acid and rosmarinic acid, anthocyanins showed interesting anti-sickling activities at low concentrations and could be responsible for this activity in these natural products [37,38].

Obtained results corroborate those reported by Maskovic et al. [39] who stated that the stem bark extract of *Hypericum perforatum* raised the level of haemoglobin and erythrocyte and they attributed this activity to its vitamin and mineral constituents. *H. madagascariensis* anti-sickle activity may also be attributed to the presence of minerals elements such several studies have shown that three trace elements are strongly implicated in hemoglobin deficiency or anemia. These are zinc, copper and selenium [39]. These micronutrient work together with vitamin E as an antioxidant. They protect cells from damage caused by radical species. Selenium may help to protect against certain diseases caused by oxidative stress, including cancers and sickle cell anemia. The hydro-ethanolic bark extract of *H. madagascariensis* was reported to protect the sickle red blood cell membrane [40]. Mpiana et al. [40] reported that this plant species is traditionally used to treat sickle cell disease in the Tshopo province in DRC.

The calculated mean values of the radius, surface area and perimeter of SS erythrocytes before and after treatment with *H. madagascariensis* stem bark extract at 10.42 µg/mL are presented in the Table 3.

From this table, it can be noted that the anti-sickling activity is reflected at the cellular level by the reappearance of the radius value, the increase in cell surface area and the decrease in its perimeter. Indeed, the non-treated erythrocytes do not have circular form so the soft could not give the mean radius of red blood cells. In presence of extract, erythrocytes recovered their circular (biconcave) form that conducts to the reappearance of the radius value.

3.6 Antibacterial Activity

The results of the evaluation of the antibacterial activity of the stem bark extract from *H. madagascariensis* are shown in Table 4.

The antimicrobial potential of infusion from stem bark of *H. madagascariensis* have been evaluated for the control of certain infections. *H. madagascariensis* was active against certain strains of *E. coli*, *S. aureus*, *P. aeruginosa*, and *Enterococcus spp.*

These bacteria showed sensitivity to the tested extract (MIC 31.25; 62.5 and 250 µg/mL, respectively). These MIC seem to be lesser than those reported by other authors on strains of *B. subtilis*, *E. coli* and *S. typhi*, but not on

P. aeruginosa, which showed a sensitivity toward tested extract (MICs of 2.0 and 15.6 mg/mL; and MBCs of 2.0-3.9 mg/mL and 15.6-31.3 mg/mL, respectively, for the cold and hot extracts). *S. aureus* showed sensitivity to the hot extract only [21] and the ethyl acetate extract of *H. madagascariensis* showed antibacterial properties against *S. intermedius*, with a MIC of 50 µg/mL and an MBC of 125 µg/mL. For *Pseudomonas* strains, the extract had a MIC of 250 µg/mL (*P. aeruginosa*) [26]. For the multi-drug resistant strain (*Enterococcus spp.*), it was shown to have minimal inhibitory concentrations ranging from 6.25 mg/mL to 25 mg/mL [25-26]. Phytochemicals like phenolic compounds, terpenoids present in studied extract, can act by interacting with the cell membrane or cell wall of microorganisms, leading to the change of membrane permeability and therefore cell destruction or enter bacterial cells and promote the coagulation of their contents [31,41]. Therefore, phytochemicals can be used as natural antimicrobial in traditional plant extracts. Tannins are effectively known to possess interacting antimicrobial activities [12].

Bioactivities of extracts from *H. madagascariensis* demonstrates the need for the validation of traditional recipes for a scientific evidence.

Table 3. Values of the radius, surface area and perimeter of SS erythrocytes before and after treatment with 10.42 µg/mL of *H. madagascariensis* stem bark extract (Mean ±SD, n=6)

Measured parameters	None treated SS erythrocytes	Treated SS erythrocytes	Observations
Radius (µm)	-	11.0 ± 0.4	Reappearance
Surface (µm ²)	297.1 ± 0.8	325.3 ± 0.11	Increase
Perimeter (µm)	115.7 ± 0.5	59.2 ± 0.12	Decrease

Table 4. Minimum inhibitory concentrations MICs (µg/mL) of extract from stem bark of *H. madagascariensis* (data from three experiments in triplicate, MIC defined as the lowest concentration for which no growth was observed in every tested well).

Aqueous extract of <i>H. madagascariensis</i>	MIC values (µg/mL) of microorganism (Gram)			
	<i>E. coli</i> (-) ATCC	<i>S. aureus</i> (+) ATCC	<i>P. aeruginosa</i> (-) ATCC	<i>Enterococcus spp</i> (+) Clinical
Stem bark	62.5	31.25	62.5	250

(*): MIC values below 500 µg/mL: High antibacterial activity; 500 to 1000 µg/mL weak activity and over 1000 µg/mL inactive [10].

4. CONCLUSION

The phytochemical analysis and the evaluation of the antioxidant, antibacterial anti-sickling activities of infusion extract from stem bark of *H. madagascariensis* has given interesting results. In accordance with previous works concerning the antioxidant as well as the anti-sickling properties of the extract of *H. madagascariensis*, it is clear through the present study that this extract could really protect the red blood cells membrane and could thus consolidate the priority given to traditional medicine, helping people suffering from different type of anemia such as sickle cell anemia. However, there are few reports about the chemical and biological studies of Congolese *H. madagascariensis*. In addition, the scientific validation for the popular use of this deserve needed further chemical investigation. The identification and the characterization of active principles from this species would be of great scientific merit. The antioxidant and antibacterial activities of the studied species may have potential therapeutic interest and could justify their use in traditional medicine and local aromatic resources, but in future, bioactivities studies especially *in vivo* studies are useful for demonstrating the benefits of these extracts in sickle cell disease.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT AND ETHICAL APPROVAL

Consent forms were obtained from all patients participating in the study, and research procedures were approved by the Ethics Committee of the Department of Biology of Faculty of Sciences (University of Kinshasa).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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