



Sickling Cells Inhibition and Radical Scavenging Activities of *Zanthoxylum leprieurii*'s (GUILL) Bark Extracts: Comparative Study

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Authors' contributions

This work was carried out in collaboration among all authors. Author KMKT designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors JAA and FY managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: In traditional medicine, several plant species from Rutaceae's family have been used to treat sickle cell anemia. However, more studies are needed to corroborate the antisickling activity of *Zanthoxylum leprieurii* species. The objective of this paper was to evaluate the sickling cell inhibition and radical scavenging activities of hydroethanolic and aqueous extracts of *Zanthoxylum leprieurii*'s bark, a plant species used in the management of sickle cell anemia in eastern Côte d'Ivoire.

Methods: Hydroethanolic and aqueous extracts of the stem bark of *Zanthoxylum leprieurii* were prepared. Qualitative and quantitative phytochemical tests were carried out. In addition, diphenyl 1,

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2 picrylhydrazyl (DPPH) was used to determine the antioxidant potential of these two extracts. The antisickling activity of two extracts was determined by the Emmel method.

Results and Discussion: *Zanthoxylum leprieurii's* bark extracts have demonstrated antioxidant property. The IC₅₀ value of the hydroethanolic extract (0.308±0.06) was lower than that of the decocted (0.434 ±0.06). At 10 mg/mL, the sickling cell inhibition of DZL was 81% ±2.66 while EZL was 89% ±0.44. The presence of alkaloids, sterols, Polyterpenes and phenolic compounds in both extracts could explain the sickling inhibition activity of these extracts. All the extracts revealed an antioxidant and antisickling activities higher than the standard.

Conclusion: The hydroethanolic extract (EZL) demonstrated a higher antisickling activity and exhibited a better free radical scavenging activity. The use of *Zanthoxylum leprieurii's* bark in the traditional management of sickle cell anemia is justified.

Keywords: Sickling cells anemia; antisickling activity; antioxidant; *Zanthoxylum leprieurii*.

1. INTRODUCTION

In Côte d'Ivoire, as in other developing countries 80% of the population use medicinal plants to treat various diseases [1]. Some studies have shown that the Ivorian flora has real therapeutic and nutritional potential that could be used to treat or prevent many diseases [2]. Traditional medicine also uses medicinal plants to treat the symptoms of sickle cell disease, which is a genetic disease. Sickle cell anemia or sickle cell disease is well known as an autosomal recessive genetic blood disorder. It is characterized by a rigid sickle shape erythrocyte. This abnormal shape is due to a point mutation in the β -globin gene. That leads to the substitution of a hydrophilic glutamic acid by a hydrophobic valine residue, at the sixth position of the β -chain of haemoglobin molecule [3]. In a deoxygenated environment, the homozygous individual develops some hemolytic anemia and suffer from pain due to vaso-occlusive crises [4] and have an increasing risk of infections [5]. In Côte d'Ivoire, sickle cell disease is a real public health problem because of the prevalence rate of 14% [6]. This life-long blood disorder has been the subject of an ethnobotanical investigation to identify the plants used by the traditional healers to treat patients suffering from sickle cell anemia, in the eastern Côte d'Ivoire. During this study, attention was focused on plants of the genus *Zanthoxylum*. Indeed, some studies demonstrated anti-sickling properties of plants from Rutaceae family [7]. In traditional medicine, different parts of the Rutaceae family's plants such as the roots, leaves and bark are used to treat people with sickle cell disease [8]. Some compounds have been isolated from Rutaceae family's plants and have been found to have anti-sickling property. This include molecule such as vanillic acid and p-hydroxy benzoic acid [9]. *Zanthoxylum leprieurii* Guill (Rutaceae) also

known as *Fagara angolensis* Engl is a plant species belonging to the Rutaceae family, which contains approximately 150 genus and 900 species. *Z. leprieurii* could grows up to 24 m tall. It is distributed in rain forests from western to Southern Africa [10]. The plant has demonstrated numerous biological properties like antioxidant and antimicrobial properties [11]. Also, some chemical studies showed the presence of acridone and acridone derivatives [12], kaurane diterpenes and coumarins [13], acridone alkaloids, benzophenanthridine [14]. However, unlike other plant species of Rutaceae family such as *Zanthoxylum macrophylla* and *Fagara zanthoxyloides* very few studies have been carried out, in Côte d'Ivoire, to investigate the antisickling potential of *Zanthoxylum leprieurii*. The present study was performed with the aim of investigating the antioxidant property and the antisickling effects by comparing two extracts of *Zanthoxylum leprieurii*.

2. MATERIALS AND METHODS

The blood samples were taken at the Yopougon University Hospital in the Clinical Hematology department and the experimental study took place at in the Biology of Immunity Pole of Pasteur Institute of Côte d'Ivoire (IPCI). As for the extractions and the Phytochemical Screening, they were carried out respectively in Laboratory of Biology and Health at biosciences UFR and the Pharmacognosy Laboratory at UFR of Pharmaceutical and Biological Sciences at the University of Félix Houphouët-Boigny.

2.1 Plant Material

Zanthoxylum leprieurii was harvested from Indenié-Djouablin, a region of eastern Côte d'Ivoire. Plant identification was done at the National Floristic Center (CNF). The stem bark

was washed, cut and then dried at room temperature (25°C). After three weeks of drying, the bark was grounded to powder using a Severin® brand grinder.

2.1.1 Preparation of 70% hydroethanolic *Zanthoxylum leprieurii*'s stem bark extract

One hundred grams (100 g) of the grounded plant was soaked in one liter of 70% hydroethanolic solution. The mixture was then homogenized 10 times for 2 minutes using a Severin® brand blender. The obtained homogenate was drained using a square of cotton fabric and then filtered successively three times on hydrophilic cotton and once on Whatman paper (3 mm). The filtrate was evaporated at 50°C using a Venticell® oven. The resulting extract was named EZL [15].

2.1.2 *Zanthoxylum leprieurii*'s stem bark decoction

One hundred grams (100 g) of grounded plant was brought to a boil for 20 minutes in 2L of distilled water [16], the mixture was cooled at room temperature (25°C) and filtered three times on cotton and once on Whatman filter paper 3. The resulting filtrate was then dried at 50°C in the oven. The dried powder was named DZL.

2.2 Human Material

2.2.1 Inclusion criteria

To be included in the study, the blood should come from homozygous sickle cell patients. The hemoglobin status was proven by the electrophoresis method at Yopougon University Hospital in the Clinical Hematology Department. The voluntary patient shouldn't have undergone blood transfusion for at least two months prior to the blood test, regardless of age and gender.

2.2.2 Collection and conditioning of blood samples

Venous blood sampling of each patient was collected in tube (EDTA). These samples were placed in a cooler containing cold accumulators and then conveyed at 4°C.

2.3 Phytochemical Screening

The screening was carried out using precipitation or/and coloring techniques according to classical methods [17], to characterize the chemical

groups such as sterols, polyterpenes, alkaloids, tannins, polyphenols, flavonoids, quinones and saponins.

2.4 Determination of Total Phenol Content

The total phenolics content was determined using Folin–Ciocalteu assay method [18]. The absorption was read at 745 nm against a blank (the spectrophotometer Jenway 7315, by bibby scientific United Kingdom). A standard range based on of a gallic acid stock solution (0.1 mg/mL) under the same conditions as the assay permits to determine the amount of phenols in the sample (mg GAE/g).

2.5 Determination of Flavonoids Content

The total flavonoid content was determined from the calibration curve made of a 0.01 mg/mL stock solution of quercetin, using direct quantification by aluminium chloride method [19]. A standard range established from a quercetin stock solution (0.1 mg/mL) under the same conditions as the assay is used to determine the amount of flavonoids in the sample.

2.6 Free Radical Scavenging Assay

The DPPH free radical (1, 1-diphenyl-2-picrylhydrazyl) scavenging method was used to determine the antioxidant activity of the two *Zanthoxylum leprieurii*'s extracts (DZL and EZL) [20] with some slight modifications. Briefly, 0.004% (w/v) of DPPH radical in methanol was prepared. Two (2) mL of this solution was added to 1 mL methanolic solution of each extract (DZL and EZL) ranging from 0.05 to 1 mg/mL. The absorbance was read at 517 nm after 30 minutes incubation in the dark and at room temperature using the methanolic DPPH solution as a blank (1 mL methanol in 2 mL DPPH solution). Three tests were performed for each concentration. While the positive control was prepared under the same conditions as the samples. A calibration curve was plotted with ascorbic acid concentrations ranging from 0-0.5 mg/mL.

% of antiradical activity = $[(\text{Abs control} - \text{Abs sample}) / \text{Abs control}] \times 100$

Where Abs control is the absorbance of control and Abs sample is the absorbance of sample. The IC₅₀ value (mg/mL) is the effective concentration of sample at which DPPH radicals were scavenged by 50%. It was graphically determined by linear regression.

2.7 Sickling Inhibitory Activity

The test was performed according to Emmel test which was slightly modified [21]. Confirmed SS Blood was washed for five minutes at 3,000 rpm three times in a row to remove the supernatant. Fifty (50) μ L of washed blood was mixed to 50 μ L of sodium metabisulfite 2% solution and 50 μ L of different plant extracts (5 and 10 mg/ml). After 120 minutes of incubation, morphological analysis was carried out using HumaSCOPE Advanced optical microscope and the residual percentage of sickle cells was determined. The sickling inhibitory activity was expressed in percentage of sickle cells formed in the presence of the plant extracts compared to the number of sickle cells present in the negative control. This activity is determined by the formula noted below:

$$AA = (P0 - P1) / P0 * 100$$

AA: antisickling activity; P0: sickle cells rate in the control; P1: sickle cells rate in plant extract's presence. Triplicate analyses were run for each experiment and each extract. XLSTAT software has been used for the statistical analysis. The typical averages and deviations of the parameters analyzed were categorized using the Tukey and Duncan multiple comparison test.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

The presence of several phytochemicals in some plant extracts of the *Zanthoxylum* family has already been investigated [22]. To confirm the presence of these reported phytochemicals in the bark of *Zanthoxylum leprieurii*, a phytochemical examination was carried out. The results revealed the presence of several classes of bioactive compounds such as catechic tannins, flavonoids, alkaloids, polyphenols, steroids and triterpenes. On the other hand, quinonic substances and gallic tannins were not present in the two plant extracts. Saponins are absent in ethanolic extracts. The qualitative results are in line with some studies. For example, the results of [23] that performed the chemical screening of *Zanthoxylum macrophylla*'s bark obtained similar results with this present study. Also, the oil extracted from the bark of *Zanthoxylum leprieurii* [24] reveal the presence of sterols and polyterpenes. The phytochemical screening of crude extract and derived fractions of *Z. leprieurii* displayed the presence of alkaloids, triterpenes and coumarins [11]. The presence of these

phytochemicals' bioactive groups in the two plant extracts (DZL and EZL) are well known to possess various therapeutic properties [25]. The antioxidant activity of chemical group such as alkaloids, tannins, flavonoids and leucoanthocyanins have been highlighted [26]. The presence of these chemical compounds in both *Zanthoxylum leprieurii* bark's extracts could promote tissue regeneration, which could be helpful in sickled cells case. Moreover, the antioxidant activity of these different chemical groups could fight the oxidative stress associated with sickle cell anemia. Also, among the compounds found in *Z. leprieurii*'s extracts in this study, polyphenols and their derivatives have been cited as having anti-sickling activity [27]. The presence of these chemicals in *Zanthoxylum leprieurii* bark's extracts could explain their varied uses in ethnomedicine.

3.2 Content of Total Phenols, Flavonoids and Free Radical Scavenging

Total phenols, flavonoids and radical scavenging activity by the DPPH method of extracts of *Zanthoxylum leprieurii* (DZL; EZL) are recorded in Table 1. Total phenol contents were measured from the equation of the standard curve: $Y = 8.1544X$; $R^2 = 0.9977$. The total phenol content found in DZL was 15.74 ± 0.45 mgGAE/g extract, while that of EZL was 8.87 ± 0.01 . The phenolic content in DZL was twice that of the EZL. Decoction would be better than 70% ethanol extraction to isolate polyphenols from the bark of *Zanthoxylum leprieurii*.

Table 1 also recorded the flavonoid content. These levels were measured from the equation of the standard curve: $Y = 1.3574x - 0.0181$; $R^2 = 0.9976$. Flavonoids are known as free radical scavengers and metal chelators. The hydroethanolic extract of *Zanthoxylum leprieurii* (10.45 ± 0.08 mg CAE/g extract) had a higher flavonoid content than the decoction of *Zanthoxylum leprieurii* (8.30 ± 0.2 mg CAE/g extract).

All the above described extracts (EZL and DZL) were screened for radical scavenging activity against DPPH \cdot . The Fig. 1 below shows the results of the DPPH's inhibition percentage measurement results based on concentration of the tested extracts (EZL and DZL). It showed that the percentage of inhibition of the free radical increases with increased concentration either for EZL and DZL or for ascorbic acid. It's could be said that the radical scavenging activity of both

extracts EZL and DZL has a dose-dependent effect. It is observed that the IC_{50} of the different extracts are higher than that of ascorbic acid for the concentrations from 0.005 to 0.5 g/mL. By expressing the amount of extract required to inhibit 50% of the free radical concentration, the IC_{50} is inversely proportional to the percentage inhibition. The lower the IC_{50} value, the higher the percentage inhibition of an extract. The percentage inhibition of EZL (IC_{50} 0.308 \pm 0.06; 63.65 \pm 0.15 % Inhibition) is higher than that of DZL (IC_{50} 0.434 \pm 0.06; 57.33 \pm 0.12% Inhibition). A low IC_{50} value for an extract indicates its high antioxidant activity [28]. EZL has better free radical scavenging activity than DZL.

3.3 Antisickling Activity

After one hour of incubation (métabisulfite plus red blood cell), all cells in the control group were sickle-shaped (Fig. 2). The percentage of sickle cells was 100%. After treatment of red blood cells with DZL and EZL, it was observed that for both concentrations: 5 and 10 mg/mL, both extracts showed sickle cell inhibition activity. The

decrease in the percentage of sickle cells was observed in Figs. 2, 3, 4, 5 and 6. The percentage of sickle cells ranged from 33% at 5 mg/mL to 19% at 10 mg/mL for the DZL and 29% at 5 mg/mL to 11% at 10 mg/mL for the EZL. The rate of sickle cell normalization increased with the concentration of the extract. Thus, the dose-dependent antisickling activity of both extracts. The sickle cell inhibition activity was determined and recorded in Table 2. At each concentration, the EZL demonstrated higher antisickling activity than the DZL. Thus, at 5 mg/mL, the sickle cell inhibition activity for the EZL was 71% and that of the DZL was 67%. Similarly, at 10 mg/mL, the sickling cell inhibition activity for EZL was 89% and that of DZL was 81%. This anti-sickling activity would be linked to the presence of secondary molecules in the two studied extracts of *Zanthoxylum lepreurii*. Indeed, it is said that free radical scavengers are the most important components of antisickling properties [29].

It is also said that the higher the antioxidant activity of an extract of plant, the higher its antisickling activity, because the antioxidant

Table 1. Phytochemical contents and antioxidant activity of *Zanthoxylum lepreurii* extracts

Parameters plants extract	Polyphenols total (mg EAG/g)	Flavonoids (mg EQ/g)	IC_{50} (mg/mL of extract)	%Inhibition At 0.5 mg/mL
EZL	8.9 \pm 0.01 ^a	10.45 \pm 0.08 ^b	0.308 \pm 0.06	63.65 \pm 0.15
DZL	15.65 \pm 0.45 ^b	8.30 \pm 0.2 ^a	0.434 \pm 0.06	57.33 \pm 0.12
L-Ascorbic Acid			0.196 \pm 0.01	66.98 \pm 0.06

Values in the same column with different superscripts are significantly different ($P < 0.05$)

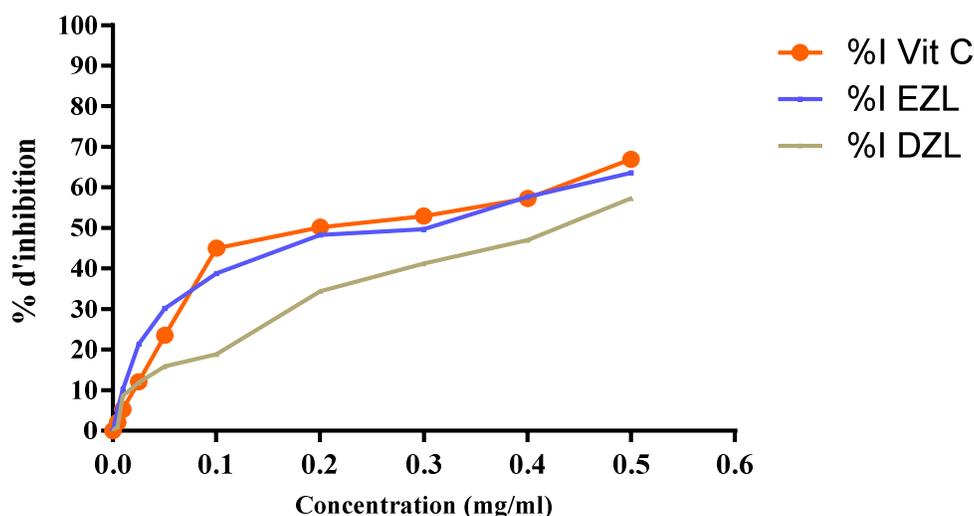


Fig. 1. Inhibition of DPPH by E.ZL, D. ZL and ascorbic acid

Table 2. DZL and EZL antisickling activity

Extracts	Concentration:5 mg/mL	Concentration: 10 mg/mL
EZL	67 % \pm 1.33	81% \pm 2.66
DZL	71 % \pm 0.66	89% \pm 0.44

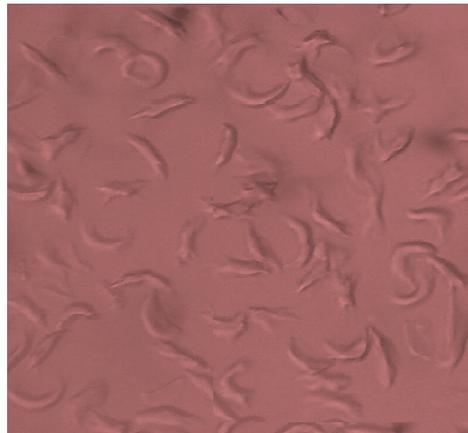


Fig. 2. Morphology of untreated SS blood sickle cell NaCl 0.9% and Na₂S₂O₄ 2% (control) (X40)

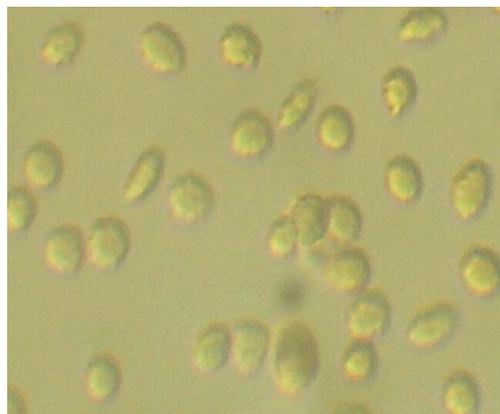


Fig. 3. Morphology of treated SS blood sickle cell with Na₂S₂O₄ 2% and 5 mg / mL of DZL (X40)

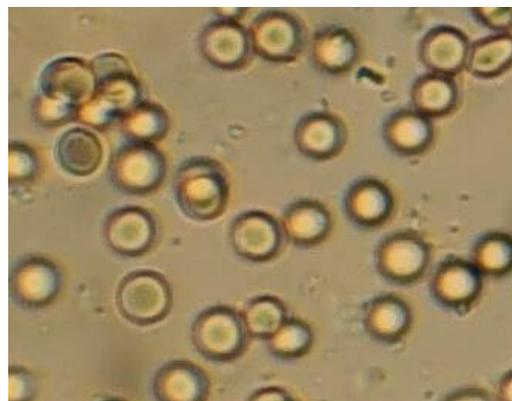


Fig. 4. Morphology of treated SS blood sickle cell with Na₂S₂O₄ 2% and 10 mg / mL of DZL (X40)

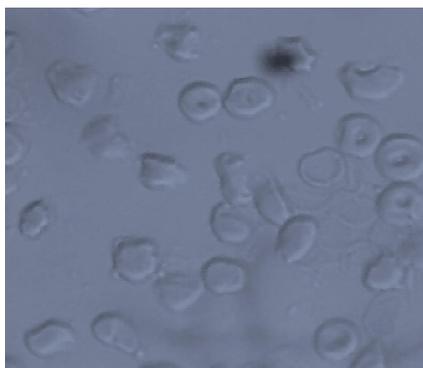


Fig. 5. Morphology of treated SS blood sickle cell with Na₂S₂O₄ 2% and 5 mg / mL EZL (X40)

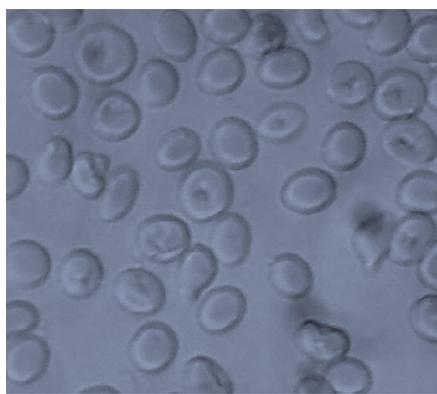


Fig. 6. Morphology of treated SS blood sickle cell with Na₂S₂O₄ 2% and 10 mg / mL of EZL (X40)

agents could reduce oxidative stress that contributes to sickle cell crisis and increase the membrane protection of the cells. Mpiana have shown that the anthocyanins, natural pigments, are partly responsible of the antisickling activity [30]. Also, Mazasa showed that anthocyanins have the ability to interact with proteins. Their possible interaction with the hemoglobin S could enter into competition with the polymerization of this hemoglobin and thus prevent polymerization of sickle cells [31]. The antioxidant activity of anthocyanins is also known. It could act on the Fe^{3+}/Fe^{2+} ratio, which is high in the sickle cells or the stability of the erythrocytes' membrane [32]. A comparison of antisickling activity of EZL and DZL shows that Under the conditions of our study, could be attributed to different concentrations of the antioxidant molecules such as polyphenol, flavonoids identified in EZL and DZL.

4. CONCLUSION

From the present study, it could be concluded, that *Zanthoxylum leprieurii*'s bark contain phenolic and flavonoid that would be responsible

of its antioxidant and antisickling activities. The hydroethanolic extract (EZL) demonstrated a higher antisickling activity and a better free radical scavenging activity than the decocted. This study justifies the use of *Zanthoxylum leprieurii*' by the traditional healers to treat sickle cell anemia. The isolation the identification and the toxicity assessment of the active molecule on sickle cell anemia in *Zanthoxylum leprieurii* will be considered in further study.

CONSENT AND ETHICAL APPROVAL

An agreement was obtained from the ethic committee and an informed consent approved by each patient wishing to participate to the study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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