

Original Article

ANTIOXIDANT AND ANTI-SICKLING ACTIVITY OF EXTRACTS FROM THE STEM AND ROOTS BARKS OF *STRYCHNOS INNOCUA* (DEL.)

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ABSTRACT

Objective: In Togo *Strychnos innocua* represents a plant traditionally used to cure sickle cell disease. This study aims to carry out a phytochemical study and evaluate the antioxidant and anti-sickling activity of *Strychnos innocua* root and stem barks to support its use.

Methods: Hydroethanolic and ethanolic extracts of each plant organ were obtained by maceration. Polyphenol content was determined using the Folin-Ciocalteu's reagent and the flavonoid content using Aluminium trichloride (AlCl₃) method. The anti-sickling activity of hydroethanolic extracts of root and stem barks was tested on SS blood samples from sickle cell patients using the Emmel test. Antioxidant activity was assessed by DPPH and FRAP methods on both the hydroethanolic and ethanolic extracts.

Results: Results indicate that the highest levels of polyphenols were measured in the hydroethanolic extract of the root barks (59.32±1.77 mg GAE/g) and the ethanol extract of the stem barks (98.27±2.44 mg GAE/g). The hydroethanolic extract of the root barks and the ethanolic extract of the stem barks showed high levels of flavonoids. Extracts from both organs significantly reduced falciformation (p<0.001) at a rate of 22% (root) and 35% (stem) compared with the control (94%). In addition, root and stem extracts showed slight antioxidant activity.

Conclusion: Results justify the traditional use of *Strychnos innocua* roots in treating sickle cell anaemia.

Keywords: *Strychnos innocua*, Sickle cell disease, Red blood cells, Anti-sickling, Antioxidant

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INTRODUCTION

Sickle cell anaemia is a serious inherited genetic disorder in which the red blood cells are sickle-shaped instead of disc-shaped. It is a genetic haemoglobinopathy of autosomal recessive inheritance affecting the β chain of the protein. It is due to an amino acid change that induces a conformational change of the chain (qualitative abnormalities) which allows it to polymerise when deoxygenated [1]. The potential seriousness of this disease is linked to its anaemic, ischaemic and infectious complications, which are sources of morbidity and mortality in sickle cell disease patients [2]. Sickle cell disease (SCD) is the most common haemoglobinopathy in the world, affecting between 3% and 3.6% of the population. This disease is most common in intertropical Africa (20%) and can reach 40% in some regions [3]. There are more than 312,000 births of sickle cell children worldwide each year, with the vast majority of these births occurring in three countries: Nigeria, the Democratic Republic of Congo and India [4]. In Togo, a study was carried out by Vovor *et al.* [5], on 570 children in the northern of the country and indicated a prevalence of 1.58% and 0%, respectively for Hb SC and Hb SS. Deaths caused by the complications of sickle cell disease are most often recorded in children under five, adolescents and pregnant women [6]. Sickle cell anaemia constitutes a public health issue which needs to be seriously addressed.

Available treatments for SCD are very limited and expensive. Currently, bone marrow transplantation remains the only curative treatment, but it is difficult to be implemented in developing countries, where treatment consists essentially in preventing complications and managing painful episodes using hydroxyurea and blood transfusions [7]. Few existing medical treatments often show serious side effects, which question their effectiveness. In this way, this reveals that African populations are turning to medicinal plants [8]. These medicinal plants are an ideal alternative to chemical drugs, which are often too expensive for people in

developing countries. Researchers have been able to carry out studies proving the effectiveness of a few plants in managing the symptoms of the disease, thereby improving the quality of life of people with sickle cell disease. In Togo, several plants including *Strychnos innocua* are used by populations in the treatment of various pathologies including sickle cell disease. Traditionally, the roots of *Strychnos innocua* are used in association with the leaves of *Raphia sudanica* (Arecaceae) and the fruit shells of *Elaeis guineensis* for the preparation of a decoction that patients take [9]. *Strychnos innocua* is a plant belonging to the Loganiaceae family, a small tree with a straight stem 3 to 14 m high. To our knowledge, this plant has not yet been the subject of studies on sickle cell anaemia. To develop the use of this plant and find a plant-based alternative, this work aims carries out a phytochemical study and evaluate the antioxidant and anti-sickling activity of *Strychnos innocua* root and stem barks.

MATERIALS AND METHODS

Plant material

The plant was harvested in April 2021 in Kante in the Kara region. It has been identified in the herbarium of the Botany Department of the Faculty of Science of the University of Lome under the number TOG015914. The stem and root barks of the plant are harvested, washed, and dried in the laboratory at room temperature. They are then ground in a mill (Thomas Muler).

Blood sample

The *Centre National de Recherche et de Soins aux Drepanocytaires* (CNRSD) supplied the SS blood samples for the anti-sickling test. Checks on haemoglobin status are carried out using the haemoglobin electrophoresis method. Samples were taken from patients attending haematology clinics who had not had a blood transfusion or sickle cell treatment for 120 d. The blood was collected in EDTA tubes and used in a fresh state.

Animal material

Wistar rats weighing between 100 g and 300 g were used to assess borderline toxicity. The rats were bred under standard conditions in the animal house of the Department of Animal Physiology at the University of Lome. They were raised in ambient-temperature rooms with a photoperiod of 12 h. The animals had regular access to water and food.

This study has the approval of institute's ethical committee on animal experimentation. The University of Lome has a branch of National Ethic Committee that approved the experimental protocols by using WHO Guidelines for the care and use of human blood and laboratory animals (Ref: CER/UL/AD/MSN/032/2023).

Chemical product

Sodium metabisulphite ($\text{Na}_2\text{S}_2\text{O}_5$), which is a sickling inducer product, has been provided in the form of white powder. Plant extract solutions were prepared with 0.9% of NaCl. Antioxidant tests required 2,2-diphenyl-1-picrylhydrazyl (DPPH) with quercetin as a reference; tripyridyltriazine (TPTZ) as white and iron ferric as the reference.

Preparation of extracts

The various extractions were carried out at a rate of 100 g of plant material for one litre of solvent (ethanol/water: 80/20: v/v and ethanol (96%)) under manual stirred intermittently for 72 h. After 72 h, the extracts were filtered and then evaporated to dryness using a rotary evaporator (BUCHI, Switzerland) under vacuum at 40 °C.

Phytochemical screening

Phytochemical screening is carried out on powders and extracts of *Strychnos innocua* stem and root barks using qualitative phytochemical analysis based on staining and precipitation tests [10, 11].

Determination of polyphenols

Polyphenols are determined spectrophotometrically, using colorimetric method with Folin-Ciocalteu reagent. This assay quantifies the total concentration of hydroxyl groups present in the extract.

The used protocol takes in account the description offered by Ali-Rachedi *et al.* [12] with some modifications. In glass test tubes, a volume of 200 μl of each extract is added, along with a 1 ml mixture of 10-fold diluted Folin-Ciocalteu reagent; after 5 min of shaking 800 μl of a 7.5% sodium carbonate solution is added. The tubes were shaken and kept for 30 min. The absorbance was read at 765 nm and the results expressed as mg gallic acid equivalent per g extract (mg GAE/g).

Determination of flavonoids

Flavonoids are quantified using a method based on forming a highly stable complex between aluminium chloride and the oxygen atoms on carbons 4 and 5 of flavonoids. This protocol used also considers the description provided by Ali-Rachedi *et al.* [12] with a few modifications.

A volume of 100 μl of each extract prepared in ethanol was mixed with 0.4 ml of distilled water and then with 0.03 ml of a 5% sodium nitrite NaNO_2 solution. After 5 min, 0.02 ml of a 10% AlCl_3 solution was added. 0.2 ml of Na_2CO_3 solution (1 M) and 0.25 ml of distilled water were added to the mixture after a 5 min rest. The mixture was vortexed and the absorbance measured at 510 nm on a UV-visible spectrophotometer (Thermo Fisher Scientific) against a blank. The concentration of total flavonoids was deduced from a calibration range established with Quercetin (0-500 $\mu\text{g}/\text{ml}$). Results are expressed as mg quercetin equivalent per g extract (mg QE/g).

Antioxidant activity

DPPH test

DPPH (2,2 diphenyl-1-picrylhydrazyl) is generally the most widely used substrate for rapid and direct assessment of antioxidant activity due to its stability in the free radical form and the simplicity of the

analysis. The experimental protocol used to study DPPH free radical scavenging activity considered the one described by Rajesh *et al.* [13] with a few modifications. The DPPH solution (Sigma-Aldrich Chemical, Schnellendorf, Germany) was prepared in pure methanol. A range of concentrations (0-60 $\mu\text{g}/\text{ml}$) of quercetin and our extracts (Sigma Chemical, St. Louis, MO, USA) was also prepared in methanol. A volume of 100 μl of our extract solutions or the standard (quercetin) was added to 2 ml of the DPPH solution. After homogenisation, the mixture was incubated for 30 min at the room temperature (25 °C) in the dark. Discolouration compared with the control containing only the DPPH solution was measured at 517 nm against a blank using a UV visible spectrophotometer. The percentage inhibition of the DPPH radical was calculated using the following equation:

$$\text{IP}(\%) = \left(\frac{A_c - A_e}{A_c} \right) \times 100$$

IP: inhibition percentage

Ac: absorbance in the presence of methanol,

Ae: absorbance in the presence of extract or quercetin.

Test FRAP (Ferric reducing ability power)

The iron III reducing power of our extracts was determined using methods described in the literature. It consists in the reduction of the intensely blue tripyridyltriazine-iron complex (Fe^{3+} -TPTZ) to a compound reduced to dark ferrous ions (Fe^{2+}). The Fe^{3+} -TPTZ complex is added to the sample. The antioxidants present in the sample reduce the complex to its reduced form (Fe^{2+} -TPTZ). To test tubes containing 300 μl of freshly prepared FRAP solution (25 ml acetate buffer; 2.5 ml 10 mmol TPTZ in 40 mmol HCl and 2.5 ml 20 mmol $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) were added 30 μl distilled water and 10 μl extract. The reading was taken at 593 nm (Genesys 10S UV-Vis Spectrophotometer, USA), against the blank, after 10 min incubation [14, 15].

Anti-sickling activity: Emmel test

According to the protocol described by Joppa *et al.* [16] a mixture of 50 μl of SS blood and 50 μl of sodium metabisulphite, inducing falciformation and previously prepared at 2%, as well as 50 μl of the hydroethanolic extract solution (2.5 mg/ml) were mixed in a test tube. This mixture was left to stand for 4 h. The control consisted of the mixture without extract. The preparation was mounted between slide and coverslip and read under a light microscope. The test was reproduced on 2 different samples and 2 different slides per sample.

Acute toxicity

The acute oral toxicity of hydroethanolic extracts was studied in female Wistar rats [17]. Three groups of 3 rats were tested. The first group (control group) received distilled water at 10 ml/kg. The second and third groups received extracts at a single dose of 5000 mg/kg. Rats were kept under observation for 15 d with free access to food and water.

Statistical analysis

Results of our various tests are processed using Excel 2019 and GraphPad® 8.0.2 and then expressed as the mean \pm SEM (Standard Error of the Mean). They are said to be significant if their $p < 0.05$

RESULTS

Yields

The extraction yields of the phytoconstituents obtained after exhaustion with ethanol and ethanol (80%) gave the results shown in table 1.

Phytochemical screening

Phytochemical screening revealed the presence of almost all the elements sought (alkaloids, tannins, anthocyanins, flavonoids, heterosides, saponosides, sterols and terpenoids) except anthraquinones in root barks powder and saponosides in stem barks (table 2).

Determination of polyphenols

The quantification of total phenols is determined from the linear regression equation of the calibration curve plotted using gallic acid

as the reference. The highest levels of polyphenols were measured in the stem barks extract (76.96 ± 1.77 mg GAE/g) for the

hydroethanolic solvent (HE) and in the stem barks extract (98.27 ± 2.44 mg GAE/g) for the ethanol solvent (ETOH) (fig. 1).

Table 1: Extraction yield of plant materials

	Root barks	Stem barks
Ethanol	3.25 %	3.59 %
Ethanol-Eau (80-20)	21.86 %	15.68 %

Table 2: Phytochemical screening of extracts

Phytochemical constituents	<i>Strychnos Innocua</i> root barks	<i>Strychnos Innocua</i> stem barks
Alkaloids	+	+
Tannins	+	+
Gallic tannins	+	+
Anthocyanins	+	+
Flavonoids	+	+
Saponosides	+	-
Heterosides	+	+
Anthraquinones	-	+
Sterols and terpenoids	+	+

(-) negative reaction; (+) positive reaction

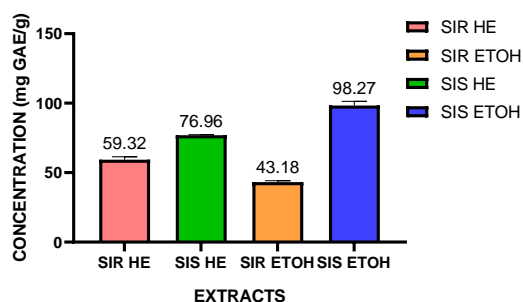


Fig. 1: Polyphenols contents of extracts, SIR HE: *Strychnos Innocua* root barks Hydroethanolic extract, SIR ETOH: *Strychnos Innocua* root barks Ethanolic extract, SIT HE: *Strychnos Innocua* stem barks hydroethanolic extract, SIT ETOH: *Strychnos Innocua* stem barks Ethanolic extract. Each value represents the mean concentration \pm SEM with n=3

Determination of flavonoids

The results obtained after flavonoid determination in the various extracts from our two organs, using the aluminium trichloride method, are shown in fig. 2. For the same hydroethanolic solvent, the root barks extract showed the highest flavonoid content (178.21 ± 7.37 mg QE/g), while for the ethanol solvent, the stem barks extract showed the highest content (213 ± 5.45 mg QE/g).

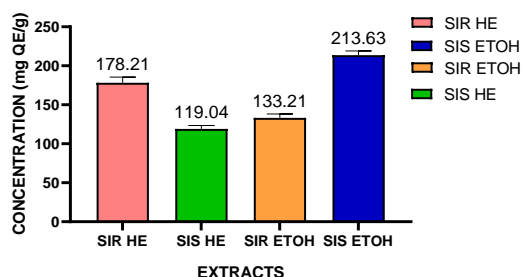


Fig. 2: Determination of flavonoids, SIR HE: *Strychnos Innocua* root barks Hydroethanolic extract, SIR ETOH: *Strychnos Innocua* root barks Ethanolic extract, SIT HE: *Strychnos Innocua* Stem barks Hydroethanolic extract, SIT ETOH: *Strychnos Innocua* stem barks Ethanolic extract. Each value represents the mean concentration \pm SEM with n=3

Antioxidant activity

DPPH test

The results of the DPPH test for the various extracts are shown in fig. 3. The IC_{50} values obtained are (in μ g/ml): 70.27; 1783; 1907.5; 718.80; 1038, respectively for quercetin (reference molecule), hydroethanolic root extract, ethanolic root extract, hydroethanolic stem barks extract and ethanolic stem barks extract.

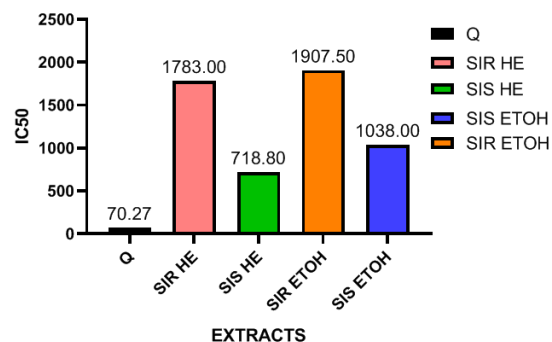


Fig. 3: DPPH test, Q: Quercetin, SIR HE: *Strychnos Innocua* root barks hydroethanolic extract, SIR ETOH: *Strychnos Innocua* Root barks Ethanolic extract, SIT HE: *Strychnos Innocua* stem barks Hydroethanolic extract, SIT ETOH: *Strychnos Innocua* stem barks Ethanolic extract

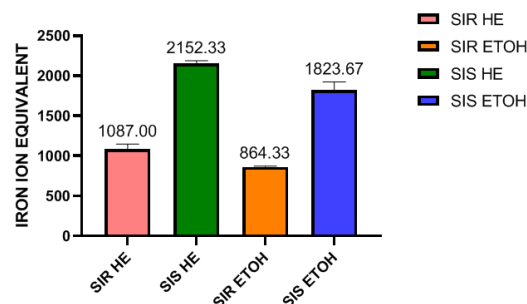


Fig. 4: Test FRAP, SIR HE: *Strychnos Innocua* root barks hydroethanolic extract, SIR ETOH: *Strychnos Innocua* root barks ethanolic extract, SIT HE: *Strychnos Innocua* Stem barks Hydroethanolic extract, SIT ETOH: *Strychnos Innocua* Stem barks Ethanolic extract. Each value represents the mean concentration \pm SEM with n=3

FRAP test

The results of the antioxidant activity, carried out by the FRAP method, of the different extracts reveal that for root and stem barks, it is the hydroethanolic extracts that show a strong reduction in Fe^{3+} $1087 \pm 34.02 \mu\text{mol/ml}$ and $2152.33 \pm 20.342 \mu\text{mol/ml}$ respectively (fig. 4). Furthermore, it can be noted that with both extraction solvents (hydroethanolic and ethanolic) it was the stem barks extract that strongly reduced the Fe^{3+} ion.

Anti-sickling activity of hydroethanolic extracts

Fig. 6 shows the results of the antifalcemic activity of the various extracts of *Strychnos innocua* at a concentration of 2.5 mg/ml. Preliminary studies made it possible to determine our effective concentration, which is 2.5 mg/ml, and to see that ethanolic extracts

did not show very good activity. The results indicate that all our extracts have significant activity on the falcification of red blood cells. For example, after 4 h of incubation, the control, consisting of blood and metabisulphite, showed a 94% rate of sickle cell formation. This rate was reduced in the presence of the different hydroethanolic extracts to 22% for root barks and 35% for stem barks.

Acute toxicity

Rats administered a single dose of 5000 mg/kg body weight of hydroethanolic extracts of the root and stem of *S. innocua* showed no clinical signs of toxicity and no behavioural changes for 48 h after dosing and for 14 d after dosing. This single dose of our extracts did not cause any deaths. Hydroethanolic extracts of root and stem are therefore not toxic by the oral route at a single dose of 5000 mg/kg.

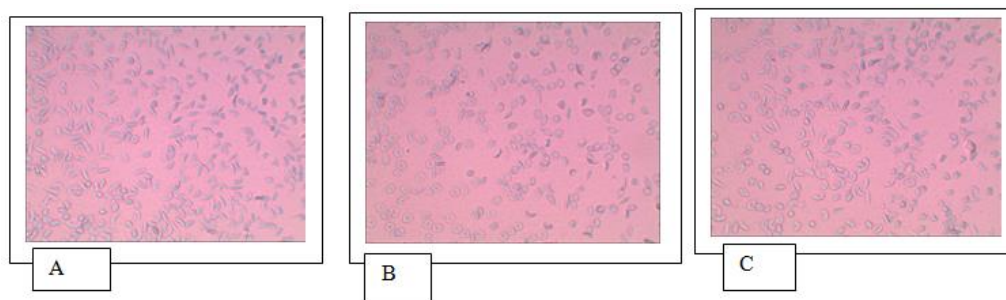


Fig. 5: Photograph of red blood cells from control blood (A) and blood treated with hydroethanol extracts of roots and stem barks (2.5 mg/ml) (B and C respectively)

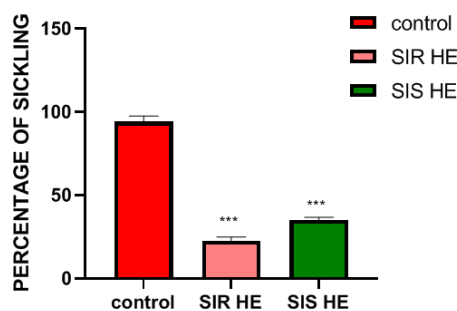


Fig. 6: Percentage of sickling in the presence and absence of hydroethanolic extracts of root barks and stem of *S. innocua* at concentrations of 2.5 mg/ml. The reduction in the falcification of SS red blood cells was significant for both extracts. Each value represents the mean of the percentage of falcification \pm ESM with $n=3$ *** $p<0.001$ (control vs extracts)

DISCUSSION

The objectives of this work were to highlight the anti-sickling and antioxidant activities and to carry out a phytochemical study of root and stem barks extracts from *Strychnos innocua*, a plant that is little known in Togo. Initially, yields of ethanolic and hydroethanolic extracts from the same organ were compared. It was found that the extraction rate with the hydroethanolic solvent was higher than with ethanol. Therefore, this means that the solvent had a strong influence on the extraction rate. Furthermore, for the same solvent used for different organs (root and stem barks), a difference in extraction rate was observed, which means that the extraction rate depends on the organ used. According to the work of Souhila *et al.* [18], water combined with ethanol results in better extraction of the compounds.

Phytochemical screening of extracts revealed the presence of alkaloids, flavonoids, tannins, anthocyanins, heterosides and sterols. These compounds are all known to be the main constituents responsible for the antioxidant activity of plants [19]. However,

anthraquinones are absent from the roots and saponosides from the stem barks. The richness of the *S. innocua* species in these major groups of active chemical compounds could therefore justify the traditional use of this plant to treat numerous illnesses such as constipation, abdominal pain, pulmonary tuberculosis, rheumatism [20].

Results of the polyphenol assay (fig. 1) show that the hydroethanolic extract of the root barks contains a high level of polyphenols ($59.31 \pm 1.77 \text{ mg GAE/g}$) compared with their ethanolic extract ($43.18 \pm 0.973 \text{ mg GAE/g}$). On the other hand, for stem barks, the ethanolic extract had the highest polyphenol content ($98 \pm 1.728 \text{ mg GAE/g}$). Whatever the extraction solvent (hydroethanolic or ethanolic), the highest polyphenol content was found in the stem barks extracts. It should be noted, however, that the variation in polyphenol content depending on the organ is significant. Several factors can influence phenolic compound content. The qualitative and quantitative distribution of phenolic compounds varies between species, organs, tissues and physiological stages [21].

Analysis of fig. 2 shows that the total flavonoid content varies from one hydroethanolic extract to another and from one plant organ to another. For hydroethanolic extracts, it is the root barks extract that has the highest total flavonoid content ($178.208 \pm 7.372 \text{ mg QE/g}$). But for ethanolic extracts, it was the stem barks extract that had the highest total flavonoid content ($213.625 \pm 5.449 \text{ mg QE/g}$). Some authors indicate that the flavonoid content in plant extracts depends on the polarity of the solvents used to prepare the extracts [22]. From this perspective, it is obvious that stem barks have a high total flavonoid content (166.34 mg QE/g on average) compared with root barks (155.71 mg QE/g on average). This unequal distribution of flavonoids could be explained by the fact that stem barks are more exposed to sunlight than roots. In fact, some studies have demonstrated that flavonoids protect plant tissues from the harmful effects of solar radiation [23].

The DPPH test on extracts from the two organs of *S. innocua* (fig. 3) shows their antioxidant activity. The obtained IC_{50} (50% inhibitory concentration) values obtained make it possible to classify the DPPH radical scavenging capacity of the extracts tested with quercetin. The results indicate that for root barks, the hydroethanolic and ethanolic extracts have IC_{50} values equal to 1783 and 1907.50 $\mu\text{g/ml}$,

respectively. Similarly, IC₅₀ values of 718.80 and 1038 µg/ml were obtained for the hydroethanolic and ethanolic extracts of stem barks, respectively. The IC₅₀ for the reference molecule, quercetin, was 70.27 µg/ml. The antioxidant power is inversely proportional to the IC₅₀ value, implying that our extracts have low antioxidant power compared with the reference (Quercetin). In addition, irrespective of the extraction solvents, stem barks extracts showed better antioxidant activity than root barks extracts. This could be the consequence of the high polyphenol content observed in the different stem barks extracts.

In addition, the FRAP test was carried out to assess the reducing power of extracts from the two organs. The results (fig. 4) show that the hydroethanolic extracts had the best-reducing power, equal to 1087±34 µmol/ml for the roots and 2152.33±20 µmol/ml for the stem. These results confirm those obtained with the DPPH test. The presence of reducing agents in the plant extracts causes the Fe³⁺ ion to be reduced to the Fe²⁺ form. The reducing power of *S. innocua* extracts is probably due to the presence of hydroxyl groups in the phenolic compounds, which can act as electron donors. Antioxidants are considered to be reducers and inactivators of oxidants [24].

Sickle cell disease is increasingly becoming a major public health problem in many countries in sub-Saharan Africa. Several researchers have demonstrated that tropical plants are a good therapeutic alternative in the treatment of sickle cell disease. The effectiveness of these plants is often due to the presence of biologically active substances capable of reversing sickle cell disease [25]. According to our results, hydroethanolic extracts of root barks and stem of *Strychnos innocua* normalize the sickle cells after induction of falciformation by sodium metabisulphite (2%). These results confirm the traditional use of this plant in the management of sickle cell anaemia [9]. At a concentration of 2.5 mg/ml, root barks extract reduced sickle cell disease by 22%, compared with 35% for the stem extract. Both organs were, therefore, active in sickle cell disease. To our knowledge, this is the first time that such a study has been carried out on this plant, and given the satisfactory results obtained, we suggest the use of stem barks instead of roots to preserve the species, which is becoming increasingly rare.

The observed anti-sickling activity is thought to be due to the richness in secondary metabolites of the two organs used. The results of the phytochemical screening revealed the presence of flavonoids, tannins and anthocyanins in the root and stem barks. These compounds belong to the polyphenol family. Polyphenols are recognised as being responsible for anti-sickle cell activity by several authors [26, 27]. According to Ghosh *et al.* [28], flavonoids improve endothelial function by activating the synthesis of vasorelaxant nitric oxide, which could therefore play an important role in the treatment of sickle cell disease. Vaso-relaxation leads to improved blood circulation, helping to prevent vaso-occlusive crises. In addition, several studies have shown that polar extracts more significantly reduce falciformation [29, 30]. As our extracts are obtained with a water-ethanol mixture, they are full of polar secondary metabolites whose synergistic effects would justify this greater activity on the reversibility of sickle cell disease.

The acute (limit) oral toxicity study of hydroethanolic extracts of *Strychnos innocua* stem and root barks, after 14 d of observation, revealed no signs of toxicity. There were no behavioural changes or deaths in the rats. These results show that the median value or LD₅₀ of these two extracts is greater than 5000 mg/kg. Indeed, previous studies have shown that any product with an oral LD₅₀ greater than 5000 mg/kg can be considered non-toxic [31]. In this study, the LD₅₀ of the extracts was greater than 5000 mg/kg. This suggests that they are practically non-toxic and therefore have a significant safety margin. This, by extrapolation, oral intake of hydroethanolic extracts of *Strychnos innocua* stem and root barks at a single high dose can be considered safe in humans.

CONCLUSION

Strychnos innocua is a plant mainly found in Togo. The roots of this plant were identified during ethnobotanical surveys as a plant with virtues for sickle cell anaemia. This study analysed the effectiveness of the roots of this plant in reducing sickle cell disease. In addition to the roots, it was also proved that the stem barks can significantly

reduce sickle cell disease. They should therefore be used instead of the roots to preserve the species. A study of the antioxidant activity of hydroethanolic and ethanolic extracts from the root and stem barks of *S. innocua* using the FRAP and DPPH methods showed that both organs possess antioxidant activities. Consequently, the combined anti-sickling and antioxidant activity of *Strychnos innocua* could synergistically act to reduce oxidative damage and alleviate the symptoms associated with sickle cell anaemia. With the purpose to isolate and identify bioactive molecules for the rational use of plant extracts, we are currently involved in further studies in order to come out with obvious results.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICTS OF INTERESTS

All the authors no conflicts of interests to declare

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