Evaluation of the anti-sickling activity of Newbouldia laevis P. Beauv extracts

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ABSTRACT

Sickle cell disease is a genetic disease very expanded all over the African block. In Togo like most African countries, it’s one of major causes of morbidity and mortality. The available treatments are very ornamental and are most of the time remedy than cure. However, in our areas, the sicks use medicinal plants for treating themselves. It’s in this scope that this study has been conducted in sight of assessing the anti-sickling activity of Newbouldia laevis extracts (leaves, roots and stem barks) based on Emmel test using 2% sodium metabisulphite. The extracts were also studied for its in vitro antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azobis (2-aminopropane hydrochloride) (AAPH) methods. The results obtained show that the roots and the stem barks extracts have a comparable efficiency. These extracts enabled us to reduce the sickling rate respectively at 17% and 16% against 78% for the control that was incubated in the presence of 0.9% sodium chloride. The leaves extract led to hemolysis and hemolysing concentration was 5mg/ml. Light antioxidant activity in vitro was obtained for the two extracts. Our outcomes proved that in vitro, roots and stem barks of Newbouldia laevis extracts have an anti-sickling activity contrary to the hemolysing leaves extract.

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Keywords: Sickle cell disease, sickling, Newbouldia laevis, Metabisulphite.

INTRODUCTION

Sickle cell disease is a hereditary disease caused by the change of the hemoglobin gene where glutamic acid (Glu) is replaced by valine (Val). The original role of hemoglobin which is the carriage of oxygen in blood is thus affected. This change in the hemoglobin structure is responsible for sickle cell anemia, a hereditary hemoglobinopathy.

This hemoglobin gene change leads to abnormal hemoglobin called hemoglobin S that presents new properties: mechanical instability, decrease in solubility and polymerization (Catonné, 2002). The latter are at the basis of the pathological state of sickle cell disease manifested by the homozygous individual while the heterozygous individual is only a carrier who does not manifest the disease. This pathological state is characterized by a deformation of sickle-shaped red blood cells, which results in hemolytic anemia and vaso-occlusion that the main causes of death in sickle cell patients (Stuart and Nagel, 2004).

Clinical manifestations of sickle cell disease are the following: severe anemia, articulatory pains in the form of crisis appear generally in times of coldness, drought and in altitude. Healthy red blood cells under
microscope have a biconcave disc appearance while the red blood cells of the sickle cell patient at the time of seizures is the shape of sickle. It is a disease that is very disabling on daily basis and that subjects the patient’s body to severe hardships. It is characterized by repercussions on the body parts of the patient such as delay in the growing of parts. Sickle cell disease crises or vaso-occlusive are very common with the patient leading to serious unbearable pains.

According to WHO (2011), 5% of the global population would be the carriers of genes at the basis of hemoglobinopathy notably mainly those who are showing signs of sickle cell disease and thalassemia. Every year, more than 300,000 at birth shows a serious sign of hemoglobinopathy. Among them, sickle cell disease is one mostly presented.

Sickle cell disease is a ubiquitous disease that can be found on all blocks but blacks are known as the original carriers owing to its prevalence that covers 2% in average on the African continent with a lifespan inferior to twenty years old against 0.02% on other blocks (Catonné, 2002; Barthet et al., 2003). Less than 50% of sickle cell disease patients reach five and less than 18% reach adulthood on the African continent (Gbadoe et al., 2001; Onhishi et al., 2001).

As shown by Segbena et al. (2005) in Togo there is an incidence at birth of 1.17% of the homozygous form SS and the national average is 1.3% and there is an increase of HbS/HbC at the northern part of the country (Vvor et al., 2014).

Available treatments are limited. The marrow transplantation remains at present the only curative treatment but it is not possible in developing countries where the care consist to prevent complications and relieve pains (Montalembert and Tshilolo, 2007).

According to Penkert et al. (2018) treatment with hydroxyurea to reduce the risk of stroke, acute thoracic syndrome and also reduce inflammatory molecules remains expensive for most patients. Blood transfusion is also proposed with some measures that are difficult to implement in our developing countries (Rees et al., 2018). For these reasons, sickle cell patients in sub-Saharan Africa resort to medicinal plants that have proved their effectiveness. Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years. This resulted in an intense global search for plant extracts and their constituents for health care (Archana et al., 2015).

In Togo, for example, leaves of Morinda lucida and Newbouldia laevis roots are used to cure the sickle cell disease crises (Joppa et al., 2008). In effect, Newbouldia laevis roots exert a beneficial effect on sickling with SS patients at the concentration of 30mg/ml (Joppa et al., 2008). Works had been conducted on roots barks of the plant. These works had permitted to show that Newbouldia laevis has a clear anti-sickling effect in vitro and would justify the use of traditional medicine in the treatment of sickling cell disease.

The use of the roots barks of this plant therefore pose a problem of survival of the species. What about other parts of the plant such as the stem and leaves? Would the stem barks and leaves have the same efficiency like the roots barks? The objective of this study was to evaluate anti-sickling effect of whole extracts of stem and leaves of Newbouldia laevis P. Beauv. The study specially was assessment of the anti-sickling effect of whole extracts, phytochemical tests and antioxidant effect of active extracts.

MATERIALS AND METHODS

Materials

Plant material

It is made up of leaves, stem barks and root barks of Newbouldia laevis. This plant material is made at Tsevié, an area that is situated at 35 km at the northern part of Lomé in December 2014. The plant had been identified at the Botany Department of the Faculty of Sciences of the University of Lomé where it is registered in the herbarium and kept under the number 233.
Samples of blood

Blood samples of the S genotype’s patients were obtained at the hematology unit of the CHU Campus laboratory services. The hemoglobinopathy status has been proven upon result electrophoresis of hemoglobin. Only the hematologist after consultation determines the samples to be used. These samples are obtained from people who came for hematological consultation at CHU Campus and having not undergone blood transfusion since four months. The blood is collected in EDTA tubes and used fresh.

Animal material

Healthy Wistar rats weighing 90 g to 100 g were used to collect blood sample for evaluation of active extracts antioxidant effect. These rats (8 male, 8 female) have been raised under standard conditions at the animal house of the Department of Animal Physiology of the University of Lomé. The animals were housed in plastic cages and kept in a conditioned atmosphere at 25±3 °C and humidity 50-55% with 12 h light/dark cycles, they were fed ad libitum with standard pellet diet and had free access to drinking water.

University of Lomé in Togo has a branch of National Ethic Committee that approved the experimental protocols using WHO (World Health Organization) Guidelines for the care and use human blood and laboratory animals. Numbers are not attributed.

Chemicals

Sodium metabisulphite of formula Na$_2$S$_2$O$_5$, 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 DPPH with ascorbic acid as reference; AAPH with PBS as white and ascorbic acid as reference.

Experimental methodology

Preparation of the total plant extracts

Leaves, stem and roots barks are washed, dried and coarsely reduced to powder. The powders obtained are differently put in a mixture ethanol-water at 80% (v/v) for 72 hours with manual stirring: 451 g of leaves in three liters of the mixture; 448 g of stem barks in 3 liters; and 429 g roots barks in 2.5 liters. The supernatants are filtered on cotton and then on filter paper. The filtrates are evaporated under vacuum at 45 °C using Rotavapor R-210. The extraction yields are 13.2% for leaves, 12.81% for stem barks and 11.62% for roots barks respectively.

Phytochemical screening tests

The screening tests of the large phytochemical groups were carried out on the solution of the extract dissolved in distilled water. Concentrations of leaves extracts, stem and root barks are respectively 5mg/ml, 10mg/ml, 10mg/ml. The search for large chemical groups in plant extracts was done by summary qualitative phytochemical analysis from the staining tests (Harbone, 1973; Chabra and Uiso, 1990). Thus, tannins, flavonoids, alkaloids, saponosides, terpenoids, steroids, glycosides and anthracenics are evidenced using these staining tests.

Total phenol determination

Total phenolic content of the extract was determined by the Folin Ciocalteu reaction (Missebukpo et al., 2013). Briefly, a mixture of each extract of *Newbouldia leavis*, FolinCiocalteu phenol reagent 10%, and sodium carbonate (700 Mm) was prepared and allowed to stand at room temperature for 30 min. After that, the mixture was centrifuged and the supernatant was measured at 735 nm. Gallic acid (100, 50, 25, 0 µg/ml) was used as the standard for the calibration curve. The phenolic contents were calibrated using a linear equation based on the calibration curve. The contents of phenolic compounds were expressed as mg gallic acid equivalent (GAE)/g extract.

Total flavonoids content of the extract

Total flavonoids content was determined according to aluminum chloride colorimetric method (Missebukpo et al., 2013). Each extract (2 ml) of *Newbouldia*
leavis (5, 25, 50, 75, 100 µg/ml) in methanol was mixed with 2 ml of methanol, 2 ml of 10% aluminum chloride, 2 ml of 1 M sodium acetate. The mixture was at room temperature for 30 min, the reaction mixture absorbance was measured at 440 nm with a double beam. Rutin (5 to 100 µg/ml) was used as the standard for the calibration curve. The levels of total flavonoids contents were determined in triplicate and the result was expressed as mg rutin equivalent (RE)/g extract.

**Study of anti-sickling activity: Emmel test**

According to Joppa et al. (2008), in a test tube, 50 µl of blood and 50 µl of sodium metabisulphite (previously prepared at 2% in 0.9% sodium chloride solution and 50 µl of the extract solution are mixed at a concentration of 30 mg/ml. Then the mixture is left on rest for 4 hours. The control consists of mixture without extract. The preparation is mounted between the core and the coverslip and bound to the microscope. The percentage of sickling is calculated by reporting sickle cell count on the total number of erythrocytes multiplied by one hundred.

**Research of the minimum hemolysing concentration of leaves extract**

Based on the leaf extract solution at 30 mg/ml concentration, we are able to prepare solutions to various other low concentrations (0; 2.5; 7.5; 10; 12.5; 15 mg/ml). The mixtures are let to stand within two hours then centrifuged at a speed of 2500 tours for 5 minutes. After the passage in the centrifuge, a series of preliminary observations with the naked eye was made. After its observations, the solutions contained in each test tube are introduced one after the other into the spectrophotometer tank. The optical density (absorbance) of each supernatant was thus measured at 540 nm to determine the hemolysis rate at the different concentrations.

**Evaluation of the antioxidant effect**

**DPPH test**

A DPPH solution was prepared at 100 mmol/l. The preparation was accurate if reading the optical density (DO) at 517 nm gave an absorbance of between 1.030-1.035 (Gurpreet et al., 2006; Metowogo et al., 2014). Six range points (ranging from 3.125 to 200 µg/ml) of ascorbic acid diluted in methanol are prepared. The extract was also prepared at various concentrations in methanol. To 1.5 ml of DPPH was added 0.25 ml of extract of each concentration or ascorbic acid range points. Point 0 as a white-reactant was a mixture of 1.5 ml of DPPH and 0.25 ml of methanol. The mixture rigorously has been shaken for 10 minutes and the absorbance read at 517 nm. For each concentration, 3 replicates were made. The percentage inhibition was calculated according to the formula below: Percent inhibition = (initial absorbance – sample absorbance)/ initial absorbance x 100.

**AAPH test 'ex vivo’**

Blood collected at the retro-orbital sinus level of the Wistar rats in the NFS tubes (5-6 ml/rat) or SS blood are centrifuged at 1500 rpm for 10 minutes and the resulting red blood cell pellet was washed three times with 5 volumes of PBS (pH=7.2) each time (Dai et al., 2005). During the last rinse, the tubes are centrifuged at 3000 rpm for 10 minutes. The pellet obtained after this last rinsing was diluted in 4 volumes of PBS. Six range points ranging from 0 to 500 µg/ml of ascorbic acid are prepared. The dissolve extract in PBS was prepared with concentrations ranging from 100 µg/ml to 1500 µg/ml. Tubes filled with 0.5 ml of red cell suspension are prepared. 0.5 ml of each concentration of extract or ascorbic acid range points was added to this suspension. To the mixture was then added 0.5 ml of AAPH and the whole was incubated well for 3 hours at 37 °C. After the incubation, the mixture was centrifuged at 3000 rpm for 5 minutes. The absorbance of the supernatant was read at
540nm. The white consisted of 1.5ml of PBS and the positive control of 1.5ml of the mixture PBS, AAPH, suspension of red blood cells in the proportion of 1/3 each. The percentage inhibition was calculated according to the formula below: Percent inhibition = \[
\frac{[\text{initial absorbance (AAPH)} - \text{sample absorbance}]}{\text{initial absorbance}} \times 100
\]

Statistics analysis
The results are processed by the Graphpad 6.02 software and averaged ±SEM (Standard Error Mean). They are said to be significant if their p < 0.05.

RESULTS
Phytochemical study
Phytochemical screening carried out on the hydroethanolic extracts of *Newbouldia laevis* revealed the presence of polyphenols (flavonoids, tannins), anthracenic heterosides (except in leaves), saponosides, terpenoids and steroids (Table 1).

Quantitative phenolic compound study
Total phenolic and flavonoids contained are approximately the same in the two different extracts. The following table (Table 2) shows the content of total phenols and total flavonoids.

Effects of hydroethanolic extracts on sickling

Effect of the stem and roots barks
Our extracts reduced sickling after 4 hours of incubation. We got a sickling rate of 78% in the control consisting of metabisulphite and sodium chloride without extract. This sickling rate is reduced to 17% for roots barks extract, 16% for stem barks against 78% for the control (Figure 1).

Effect of the leaves extract
Leaves extract induced hemolysis of SS blood.

Search of minimum hemolysing concentration
Observation of the slides under the microscope showed that a hydroethanolic extract of the leaves at concentrations ranging from 5 to 30 mg/ml caused hemolysis of the SS blood (Figure 2). The study of the absorbance of hemoglobin after hemolysis has shown that the highest absorbance is observed with concentrations ranging from 5 to 30 mg/ml (Figure 3). These results made it possible to deduce that the minimum hemolysing concentration is 5 mg/ml.

Study of antioxidant activity of the active extracts
The leaves extract being hemolysing, studies of the antioxidant activity did not continue. Only extracts of roots and stem barks were used in this study.

DPPH test
The anti-radical activity of the hydroethanolic extracts of *Newbouldia laevis* roots and stem barks was evaluated by the DPPH reduction method. The IC$_{50}$ values of these extracts are very high compared with ascorbic acid used as a reference drug (Table 3).

AAPH test
The anti-hemolytic effect of hydroethanolic extracts of *Newbouldia laevis* roots and stem barks was evaluated *ex vivo* by incubation of SS blood and Wistar rats blood in the presence of a chemical oxidation initiator AAPH. The extracts inhibit the haemolysis in a dose-dependent manner and this activity is compared with ascorbic acid by their IC$_{50}$ (Table 4).
Table 1: Identification results of the major phytochemical groups present in the hydroethanolic extracts of *Newbouldia laevis*

| Phytochemical groups          | Roots barks extract | Stem barks extract | Leaves extract |
|-------------------------------|---------------------|--------------------|---------------|
| Tannins                       | +                   | +                  | +             |
| Flavonoids                    | +                   | +                  | +             |
| Alkaloids                     | -                   | -                  | -             |
| Saponosides, Terpenoids, Steroids | +               | +                  | +             |
| Anthracenic heterosides       | +                   | +                  | -             |

+ : present; - : absent

Table 2: Total phenolic and flavonoids contained in the different extracts

| Extract                  | Total phenolic (mg GAE/g) | Total flavonoid (mg RE/g) |
|--------------------------|---------------------------|---------------------------|
| Stem barks extract       | 29.80                     | 12.20                     |
| Roots barks extract      | 35.20                     | 16.20                     |

Figure 1: Percentage of sickling in the presence and absence of hydroethanolic extracts of *Newbouldia laevis* roots and stem barks at concentrations of 30 mg/ml.

The SS whole blood is incubated between slide and covership in the presence of 2% sodium metabisulphite and with an equal volume of extracts for 4 hours. The reduction of sickling of SS red blood cells is significant for both extracts. Each value represents the mean of the percentage of sickling ±ESM with n=3 ***p<0.001 (control vs extracts).
Figure 2: Percentage of hemolysis of leaves extracts of Newbouldia laevis at different concentrations

Figure 3: Absorbance of SS hemoglobin at 540 nm wavelength by leaves extract concentration of Newbouldia laevis.

Table 3: IC₅₀ values of ascorbic acid and hydroethanolic extracts of Newbouldia laevis roots and stem barks showing inhibition of DPPH oxidation.

|                  | Ascorbic acid | Roots barks | Stem barks |
|------------------|---------------|-------------|------------|
| CI₅₀ (µg/ml)     | 108.20        | 1339.99     | 1346.03    |
Table 4: IC_{50} values of ascorbic acid and hydroethanolic extracts of *Newbouldia laevis* roots and stem barks showing their inhibition of AAPH-induced hemolysis.

|                | Ascorbic acid | Roots barks | Stem barks |
|----------------|---------------|-------------|------------|
| CI_{50} (µg/ml) |               |             |            |
| Rat blood      | 145           | 913         | 769        |
| CI_{50} (µg/ml) |               |             |            |
| SS blood       | 98            | 615         | 642        |

**DISCUSSION**

The yields of extraction show the similar rate for roots and stem barks. The achieved results show that the hydroethanolic extracts of *Newbouldia laevis* P. Beauv roots and stem barks normalize hypoxia-induced bark sickle cell disease created by the addition of 2% sodium metabisulphite. Our results confirm those obtained by Joppa et al. (2008). Indeed, the *Newbouldia laevis* roots barks extract at the concentration of 30 mg/ml (the same as in our study) allowed inhibition of sickling of 90% against 83% in our case. We obtained inhibition of sickling of 84% with stem barks. To our knowledge, it is for the first time that such a study of this part of the plant has been conducted. In view of satisfactory results, compared with roots barks, we can advise the use of stem barks instead of using roots barks. This will preserve the species and save in the search for plant material for the treatment of sickle cell disease.

The anti-sickling activity of *Newbouldia laevis* roots and stem extracts is thought to be due to the polyphenols present in the plant. Indeed, the phytochemical screening carried out made it possible to know our extracts contain flavonoids and tannins. These polyphenolic compounds are known to be responsible for the anti-sickling activity. This hypothesis has been reported in the literature by many authors (Mpiana et al., 2009b; Kambale et al., 2013; Sawadogo et al., 2017). However, other authors claim that phenylalanine, p-hydroxybenzoic acid and its derivatives as well as the maslinic, oleanolic and betulinic acids would be at the basis of the activity of anti-sickling extracts of plants (Tshibangu et al., 2011). It is noticed that *Newbouldia laevis* contains these compounds are. The use of plants to cure sickle-disease is a hope. Besides, access to plants is easier whereas a lot of proposed pills are expensive, toxic and often inefficient.

The normalization of the deflagens is generally attributed to the inhibition of the polymerization of hemoglobin S. It is, indeed, known that the formation of intra-erythrocytes tactoids is at the basis of the phenotypic modification of SS red cells (Kambale et al., 2013). The interaction of certain metabolites extracted from plants with hemoglobin S would inhibit its polymerization thus preventing the sickling of erythrocytes.

*Newbouldia laevis* leaves extract hemolyzed SS blood at low concentrations up to 5 mg/ml. The leaves could contain chemical compounds responsible for hemolysis. It has been reported that under the same conditions of oxygenation, the red blood cells of the sickle cell SS are less deformable than those of healthy subjects (Brandao et al., 2003). The extract of leaves would be toxic and harmful for the erythrocyte of the subject SS. This may aggravate the situation of sickle cell sufferers because it is a prone to anemia and thus the risk of blood transfusion.

Oxidative damage may therefore play a role in the pathogenesis of sickle cell disease. Oxidative bioavailability of nitric oxide (NO) and inhibiting the antioxidant and vasodilator properties of nitric oxide would exacerbate hemolysis with sickle cell disease sufferer. In addition to anemia, vaso-occlusion, resulting from polymerization and adhesion of erythrocytes to the epithelium of the blood...
vessels, results in seizures of pain, fever and chest pains (Sall et al., 2016). For this reason we have evaluated in our study the *ex vivo, in vitro* and antioxidant antiradical properties of the extract on normal rat red blood cells and sickle cell disease S. the results show a low anti-radical activity *in vitro* and a low antioxidant activity *ex vivo* on the rat red blood cells against a more or less marked anti-oxidant activity *ex vivo* on the cell sickle disease sufferer S. the results obtained show that the anti-sickling activity extract is not dependent on its antioxidant activity.

**Conclusion**

This study was to evaluate the antisickling activity of the hydroethanolic extracts (leaves, roots and stem barks) of *Newbouldia laevis* P. Beauv.

Roots and stem barks of *Newbouldia laevis* have anti-sickling activity. The phytochemical screening of hydroethanolic extracts indicated the presence of polyphenols and the absence of alkaloids. The leaves are devoid of anti-sickling activity and are hemolyzing. The phytochemical screening of the hydroethanolic leaves extract indicated the presence of polyphenols and the absence of alkaloids and anthrachne heterosides. The antioxidant power of the hydroethanolic extracts of roots and stem barks is low for normal blood in Wistar rats. It is more important for the SS blood. Other bio-guided fractionation and subacute and chronic toxicity studies are needed to better understand the mechanisms of action and the anti-sickling potential of these extracts.

**COMPETING INTERESTS**

The authors declare that they have no competing interests.

**AUTHORS’ CONTRIBUTIONS**

All the author have contributed equally.

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