The Pharmaceutical and Chemical Journal, 2016, 3(2):351-358

Available online www.tpcj.org

Research Article

ISSN: 2349-7092
CODEN(USA): PCJHBA

Antisickling and Analgesic Activities of the Aqueous and Ethanolic Extracts of *Entandrophragma Angolense* C.DC. (Meliaceae)

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Abstract Sickle cell disease (SCD) is a public health problem for the sub-Saharan African countries. This study was conducted to evaluate the antisickling and analgesic activities of aqueous and ethanolic extracts from the bark of *Entandrophragma angolense*, a plant used in traditional medicine as an analgesic and anti-inflammatory in the case of stomach or kidneys ache. The treatment of the erythrocytes with sodium metabisulfite at 2% has resulted in a significant increase in sickling cells. After treatment, the aqueous and ethanolic extracts of *E. angolense* decreased significantly the number of sickling cells. The analgesic activity was evaluated according to the test with acetic acid and the tail immersion test in rat. Treatment with the aqueous and ethanolic extracts of *E.angolense* at the dose of 200 mg/kg ($P < 0.05$) significantly reduced the number of writhing induced by acetic acid (1%). The peripheral analgesic action of *E. angolense* is similar to that of paracetamol at the dose of 100 mg/kg. Concerning the tail immersion, the treatment with the aqueous extract (200 mg/kg) has significantly protected the animals against the thermal stimulus comparatively to that of the ethanolic extract at the same dose. Therefore, the aqueous and ethanolic extracts of stem bark of *E. angolense* possess antisickling and analgesic properties. Phytochemical screening of the plant extracts has revealed the presence of tannins, polyphenols, flavonoids, saponins, triterpenes and steroids, which could be responsible for pharmacological properties.

Keywords Entandrophragma angolense, Sickle cell disease, Pain, Antisickling activity, Analgesic activity, Ivory Coast.

Introduction

Sickle cell disease is hemoglobinopathy, the most widespread in Black Africa where its prevalence in some areas of equatorial Africa such as Zaire, Cameroun and Uganda, reaches 30 to 40 % [1-2]. In Ivory Coast, it reaches 14 % of the population [3] in which 2 % are the serious forms. The historical or recent migrations gradually modify its distribution throughout the world. In Asia, apart from the South of India belonging to the sicklemic belt, the areas are not very touched by this disease. In the Northern, central and southern America, the distribution of HbS depends mainly on the African origin of the blacks and their degree of interbreeding. In Europe, only the countries of the Mediterranean circumference present some localities of sickle cell disease [4]. Sickle cell disease, also called sickle cell anaemia, is a hereditary genetic affection with recessive autosomic transmission which is characterized by the deterioration of normal heamoglobin, the protein ensuring the transportation of oxygen in blood. It is due to a specific and single mutation of the gene beta-globin located in chromosome 11 leading to the replacement in position 6 of the beta-globin of the glutamic acid, hydrophilic, present in the heamoglobin A (normal) by hydrophobic valine, resulting then in heamoglobin S [5]. This mutation decreases the affinity of haemoglobin for oxygen and reduces considerably the solubility of haemoglobin S in its non oxygenated form. Thus, when the partial

pressure in oxygen decreases, haemoglobin S becomes very little soluble. It polymerizes with other hemoglobin S molecules and crystallizes in the red blood cell that distort then in sickle. The sickling predisposes the erythrocytes to an early hemolysis [6]. The main acute complications of homozygous sickle cell disease are: painful crisis, severe anaemia, infections, occlusive accidents on the level of the pulmonary and cerebral microcirculation [7]. Painful crisis often dominate the symptomatology in infancy to space itself in adolescence. They associate pains with fever and can be spontaneous or caused by various factors such as the infection, tiredness, the cold, dehydration and all situations causing a hypoxemy. These crises interest mainly the hands and the feet but can also reach the costal and vertebral areas. Crisis of abdominal pains correspond sometimes to spleno-mesenteric infarctions [7]. However, like much of other genotypic diseases, the sickle cell disease at present does not know sufficiently a curative treatment [8]. The ambition of practitioners is to improve the quality of life of patients. So most of them prescribe a purely symptomatic or physiopathological treatment. Only the bone marrow transplant currently reports satisfactory corrections to sickle cell disease patients [9]. However, this therapeutic, very specialized and very expensive approach, is not available for most patients of low income like those of Africa. The currently proposed therapeutic, especially the blood transfusion, the use of desferrioxamine and hydroxyurea, only bring temporary solutions with an increasing risk of contamination by viral or bacterial infectious agents and cytotoxicity [10]. Today the best solution consists in integrating medications containing plants in the health systems of Africa as therapeutic alternative. Indeed, phytotherapy could be currently presented as an alternative being able to offer an adequate treatment to anaemic patients [11]. Natural substances could constitute a possible source of new types of drugs being able to fight against several diseases in general and sickle cell anaemia in particular. Several experimental studies showed the antisickling activity *in vitro* and in vivo of plants [12] and a database of public utility was even made up [13]. In this work, we report the results of the evaluation of the *in vitro* antisickling and in vivo analgesic effects of the bark of *E. angolense*, an ivorian plant whose bark is used in traditional medicine to treat fever and such as analgesic against stomach pain and peptic ulcers, the earache as well as the renal pains [14]. The choice related to this plant is due to the fact that another species of the Entandrophragma kind was studied for its antisickling property [15]. Thus, in this study it is important to check the hypothesis according to which the species collected in Ivoiry Coast would also contain the same active substance which would confer an antisickling activity to it.

Materials and Methods

Plant material

The stem bark of *E. angolense* was collected in the Abidjan area (southern Ivory Coast) in March 2014. It was identified at National Floristic Centre of University Felix Houphouet Boigny where a herbarium specimen of the plant was deposited. The bark was cut out then dried in the shade, at the room temperature during two weeks. Then, it was pulverized using an electric crusher in order to obtain a powder, then it is stored for a later use.

Extraction of the plant material

One hundred grams (100g) of plant powder were boiled during 20 minutes in 2 liters of distilled water. The cooled decoction was filtered three times on cotton wool and once on filter paper Whatman N.3. The filtrate obtained was then dried in a hot air oven at 50°C to give the aqueous extract of *E. angolense*.

The ethanolic extract of *E. angolense* was prepared by maceration according to the method described by Bidié [16]. In fact, one hundred grams (100 g) of ground bark of plant was mixed with 1 L of ethanol 70%. The mixture obtained was stirred during 24 hours at room temperature (25°C) using a magnetic stirrer. The mixture was then filtered three times on cotton and on Whatman N.3 filter paper and dried in a hot air oven at 50°C.

Phytochemical screening

The different groups of compounds (sterols, polyterpenes, alkaloids, tannins, polyphenols, flavonoids, quinones, saponins and cardiac glycosides) have been researched in the extracts of *E. angolense* according to the described methods [17-18].

Biological material

It is composed of samples of fresh blood taken at 20 SCD major patients SSFA2 on the level of the fold of the elbow in tubes containing sodium EDTA. These patients are known and followed in the clinical service of hematology of the CHU of Yopougon. The enlightened and written agreement by each patient was obtained before the blood test.

Experimental animals

Female and male Rats of Wistar strain of body weight ranging between 90-130g were used for this study. The animals were housed in plastical cages and acclimatized for two weeks in the animal house of the Higher Teacher Training School (ENS). They had been maintained under standard conditions (room temperature 25°C ± 3°C, humidity 35 to 60%, light and dark period 12/12 hours). All the animals have free access to water and food.

Biological test of the extracts of plant for the antisickling activity It was carried out according to the principle of the test of Emmel or test of sickling [19]. This test allows the tracking of the carriers of haemoglobin S.

-*In vitro* induction of sickling

A drop of blood is deposited on a strip of glass then three drops of 2% sodium metabisulphite solution. A cover glass is posed on this mixture. An observation under the microscope with the objective x 40 after 15 minutes of contact is made. Then one determines the percentage of sickling cells which was calculated using the formula: (%) Sickling = Number of sickling cells × 100/total cells.

-*In vitro* antisickling activity of the extracts

A volume of 50µL of total blood was mixed with 50µL of the extract of *E. angolense* diluted to the 1/2 with saline solution then with 50µL of 2% sodium metabisulfite in a test tube, closed with paraffin (to exclude the air).

The concentration of the solutions of the aqueous and ethanolic extracts is 0,1g/mL.

A control was made by mixing 50µL of blood diluted at 1/10 with saline solution with 50µL of saline solution and 50µL of 2% sodium metabisulfite in a closed test tube of paraffin.

At the end of 30mn, 1hour, 1hour 30mn then at 2 hours, a drop of the mixture was deposited on a blade of glass. The whole was then covered with a cover glass.

The observation was made under the optical microscope (with the objective x 40). The abnormal erythrocytes sickling cells take the shape of sickle or banana, with sometimes of the edges fringed in "sheet of holly ". The percentage of sickling cells on each blade of glass was found by counting the total number of sickling cells observed brought back to the total number of red cells.

Evaluation of the antisickling activity

The antisickling activity (A.A) expressed as an inhibition percentage of the sickling was calculated as follows:

$A.A = \frac{Po - P1}{Po} \times 100$

Po = Mean of the percentages of sickling cells on the control blades

Pl = Mean of the percentages of sickling cells on the tests blades.

Analgesic activity study

Writhing test

The analgesic effect of the extracts was evaluated according to the number of abdominal torsions induced by the intraperitoneal injection of 1% aqueous acetic acid following the method described by Siegmund [20].

The rats were deprived of food for 16 hours before experimentation, but had free access to water. They were weighed and divided into 4 groups of 5 animals of the same sex:

Group I: Water distilled at dose of 10 ml/kg

Group II: Paracetamol at dose of 100 mg/kg

Groups III and IV: Aqueous and ethanolic extracts of *E. angolense* respectively at dose of 200 mg/kg.

The extracts to be tested, paracetamol and distilled water were administered orally 1 hour before the intraperitoneal injection of 1% aqueous acetic acid at the dose of 10 ml/kg. After the administration of acetic acid, each rat is observed and the writhing caused by the stimulus (painful syndrome) have been counted for a period of 30 min. The painful syndrome is characterized by movements of stretching of the hind legs and torsion of the abdominal muscles called abdominal cramps. The analgesic effect was evaluated according to the following formula:

$$\% \text{ Inhibition } = \frac{\text{M control } - \text{ M test}}{\text{M control}} X\ 100$$

M= mean of number of writhing

Tail immersion test

The method described by Mohemad *et al.* [21] was used to determine the analgesic effect of the aqueous and ethanolic extracts of *E. angolense* bark .The rats were deprived of food for 16 hours before experimentation, but had free access to water. They were weighed and divided into 4 groups of 5 animals of both sexes. The control group received distilled water while the other groups were treated with the aqueous and ethanolic extracts of the bark (200 mg/kg) and the aspirin at dose of 150 mg/kg. The extracts to be tested, the aspirin and distilled water were administered by oral route. The tail was immersed in a water bath filled with hot water at temperature 55°C. Observations were made before and after administration of drugs at 0 minute, 30 minutes, 60 minutes and 120 minutes respectively. The time (seconds) recorded by the animal to remove its tail of hot water was noted as reaction time.

Statistical analysis

The values were expressed as mean accompanied by the standards errors on the mean (Mean±SEM). The graphic representation of data was carried out starting from the software Graph Pad PRISM 5.0 (Microsoft U.S.A). The statistical analysis of the results was made by using one way ANOVA followed by Dunnett's test to verify the significant difference. $P < 0.05$ was considered significant.

Results

Phytochimical study

Phytochemical analysis of aqueous and ethanolic extracts of *E.angolense* bark revealed the presence of large chemical groups which are: alkaloids, polyphenols, sterols, terpenes, tannins, flavonoids, leucoanthocyanins, quinones, saponins and cardiac glycosides

Biological tests

The treatment of the erythrocytes with sodium metabisulphite (2%) showed a significant increase in sickled cells (52.21% ± 4.89) to T0. After treatment with the extracts of *E.angolense*, a decrease of the percentage of sickled cells was observed in the course of time but it was very accentuated between T0 and T1 (30min) (Figure 1). The results show that the sickling cells having received the aqueous and ethanolic extracts of *E angolense* quickly found their normal form which is round. The difference between the means being significant of T0 to T1, a significant decrease of the sickling cells is observed from approximately 31% for both extracts.

Figure 2 shows the effect of the aqueous and ethanolic extracts of *E. angolense* at 50 mg/ml concentration. In the presence of sodium metabisulfite at 2%, the extracts inhibited the formation of sickling cells, this antisickling activity was maximum in 1 hour 30min of contact. Indeed, the maximum of the antisickling activity of the aqueous and ethanolic extracts was 32.94% and 32.75% respectively.

The analgesic activity was evaluated according to two tests: The acetic acid test (chemical stimulus) and the tail immersion test (thermal stimulus). Table 1 shows the response of the rats to the writhing induced by the acetic acid to 1%. The treatment with 200 mg/kg of the aqueous and ethanolic extracts of *E. angolense* significantly ($P<0.05$) reduced the number of writhes. The percentages of inhibition were 32.61% and 30.42% respectively for the aqueous and ethanolic extracts. The peripheral analgesic action of *E. angolense* bark at 200 mg/kg is similar to that of paracetamol at dose of 100 mg/kg (inhibition 38.04%).

Table 2 shows the responses of the animals to the tail immersion. The treatment with the aqueous extract at the dose of 200 mg/kg significantly protected the animals against the thermal stimulus in comparison with that of the ethanolic extract at the same dose. At 200 mg/kg, the aqueous extract effect which inhibited the response of the tail withdrawal is almost with the same degree as that of aspirin at 150 mg/kg.

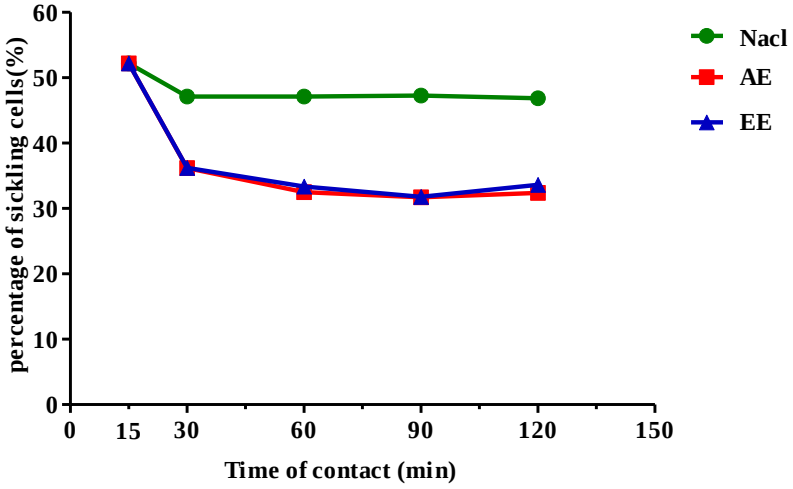

Figure 1: Evolution graph of sickling cells percentage according to the time of contact

Nacl (salin water): Control ; AE : Aqueous extract of *E. angolense* ; EE: Ethanolic extract of *E. angolense*.

At time T0, we have a percentage of sickling cells after a contact of 15 min between blood and sodium metabisulfite.

T1: Percentage of sickling cells after a contact of 30 min between blood, sodium metabisulfite and plant (or NaCl for the control).

T2: Percentage of sickling cells after a contact of 1h between blood, sodium metabisulfite and the plant (or NaCl for the control).
T3: Percentage of sickling cells after a contact of 1h30 min between blood, sodium metabisulfite and the plant (or NaCl for the control).
T4: Percentage of sickling cells after a contact of 2h between blood, sodium metabisulfite and the plant (or NaCl for the control).

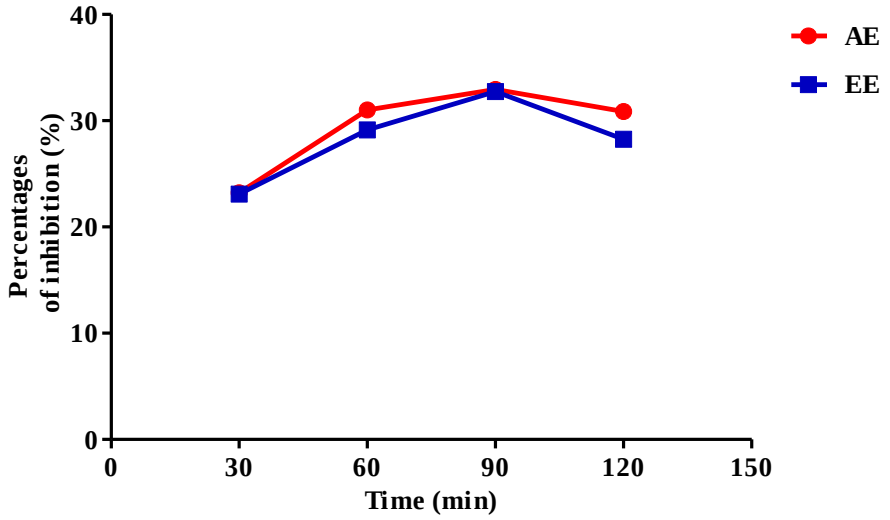

Figure 2: Evolution graph of percentages of inhibition of the sickling according to the time of contact AE: Aqueous extract of E. angolense; EE: Ethanolic extract of E. angolense.

Table 1: Evaluation of analgesic activity of aqueous and ethanolic extracts of *E. angolense* bark by acetic acid induced Writhing method

Treatment	Dose	Number of writhing	% of inhibition
DW	10 mg/Kg	61.33 ± 2,60	--
Paracetamol	100 mg/Kg	38.00 ± 4,04**	38.04
AE	200 mg/Kg	41.33 ± 3,75*	32.61
EE	200 mg/Kg	42.67 ± 4,66*	30.42

All values are expressed in Mean ± SEM, n=5; *$p < 0.05$: statistically significant according to control (DW), **$p < 0.01$: statistically very significant according to the control (DW).
DW: Distilled water; AE: Aqueous extract of *E. angolense*; EE: Ethanolic extract of *E. angolense*.

Table 2: Evaluation of analgesic activity of aqueous and ethanolic extracts of *E. angolense* bark by tail immersion method

Treatment	Time of withdrawal in seconds			
	0min	30min	60min	120min
DW (10ml/kg)	2.53 ± 0.12	2.33 ± 0.19	2.47 ± 0.37	2.77 ± 0.35
Aspirin (150mg/kg)	3.91 ± 0.20	4.25 ± 0.55*	4.27 ± 0.24**	4.35 ± 0.29*
AE (200mg/kg)	2.43 ± 0.18	3.93 ± 0.44	3.96 ± 0.39*	4.65 ± 0.58*
EE (200mg/kg)	2.51 ± 0.25	3.13 ± 0.41	3.41 ± 0.11	3.90 ± 0.17

All values are expressed in Mean ± SEM, n=5; *$p < 0.05$: statistically significant according to control (DW), **$p < 0.01$: statistically very significant according to the control (DW).
DW: Distilled water; AE: Aqueous extract of *E. angolense* ; EE: Ethanolic extract of *E. angolense*.

Discussion

Under the conditions of hypoxia (lack of oxygen), the aqueous and ethanolic extracts of the plant showed a capacity to change the shape of red blood cells $SSFA_2$ *in vitro* by transforming them from the sickling form (abnormal) to the biconcave form (normal). The phytochemical screening carried out on the bark of *E. angolense* showed that they contain flavonoïds especially anthocyanins. The presence of anthocyanins in this plant could justify the antisickling activity partly observed. Indeed, Mpiana and its allies recently showed that the antisickling activity of the majority of the congolese medicinal plants was allotted to this chemical group [22-23]. It has been recently shown that the anthocyanins would reduce not only the polymerization of HbS but also they would also act by stabilizing the membrane of the erythrocytes [6-24]. Anthocyanins would inhibit the polymerization of haemoglobin S while engaging in a reaction with this protein which would be competitive with that of polymerization. Moreover, the antioxidant properties of these pigments would enable them to prevent the peroxydation of the membrane lipids and to then prevent the lysis of the erythrocytes. However, other authors claim that phenylalanine, the acid p-hydroxybenzoïc and its derivatives as well as the maslinic, oleanolic and betulinic acids would be at the basis of the antisickling activity extracts of the plants [25]. It may be that these compounds are present in *E. angolense* bark. Ibrahim *et al.* [26] reported that saponins, in addition to the carboxylic acids and of the flavonoïds can be responsible for the antisickling activity leaves *of Hymenocardia* acida. These results indicate that our active ingredients reduce the ellipsoïdity of the sickling cells in hypoxic conditions. Thus, our drugs would prevent all the complications related to the sickling such as the pain, the inflammation, anaemia. The main symptom of sickle cell disease is pain. This pain appears when the red cells affected and deformed are blocked in the vessels and the bones. Among the other symptoms, there are anaemia and the sensitivity to the infections. To reduce or eliminate the painful sensations, analgesics such as aspirin and paracetamol are used.

In addition the abdominal contractions induced by the injection of the acetic acid and the tail immersion test were used to evaluate the analgesic effect of the aqueous and ethanolic extracts of *E.angolense*. The contractions induced by intraperitoneal injection of the acetic acid is a method used to study peripheral analgesic effect of a substance. The pain caused by the injection of the acetic acid with 1% is due to the release of serotonin, histamine, the bradykinin, the substance P and prostaglandins (PGE2, PGF2). These chemical mediators stimulate the peripheral nociceptive neurons and induce the increase in vascular permeability [27]. Both extracts inhibited the abdominal contractions significantly.

The aqueous and ethanolic extracts with 200 mg/kg appreciably have the same analgesic effect with respectively 32.61% and 30.42% of inhibition. Their effect is comparable with that of paracetamol at the dose of 100 mg/kg with a percentage of inhibition of pain 38.04. This analgesic effect could be related to the inhibition of the release of the chemical mediators.

The tail immersion of rats maintained in hot water at 55°C caused a biphasic reaction. The first phase is initiated immediately after the tail immersion and is characterized by the stimulation of the fibers C and the release of the substance P and the bradykinin. The second phase is due to the local inflammatory pain caused by the production of serotonin, histamine and the prostaglandins [28]. Central analgesics such as opioids inhibit both phases while the peripheral analgesics (non-steriodal anti-inflammatory) antagonize the inflammatory phase [29]. *E.angolense* bark extracts inhibited both phases caused by the thermal stimulus. The analgesic effect was more significant in the second phase; this suggests that both extracts act as non-steriodal anti-inflammatory. Thus, the two extracts could reduce the production of the various chemical mediators intervening in both phases. However, the analgesic effect of the aqueous extract was more important than that of ethanolic extract.

Conclusion

Natural substances take more and more a place of choice in therapy. Indeed the plants constitute real chemical factories of which it is necessary to make the maximum of profit for the well-being for the populations especially the poorest.

In vitro, aqueous and ethanolic extracts of *E angolense* at the concentration of 50 mg/ml inhibited the formation of sickling cells caused by the sodium metabisulfite. The maximum antisickling activity was 32.94% for the aqueous extract and 32.75% for the ethanolic extract after 90 minutes of contact of the erythrocytes with the extract; the two extracts have the same effect. Moreover, the significant results achieved during the analgesic study showed that the aqueous and ethanolic extracts of *E angolense* have analgesic properties.

Thus, the therapeutic virtues of the plant could come from the phytochimical compounds and also from its possible vitamin and mineral components. Finally, *E. angolense* could have a positive effect on the treatment of sickle cell disease because while decreasing the sickling cells, it prevents pain.

Acknowledgement
We express gratitude to the Department of Biochemistry of University Felix Houphouet-Boigny of Abidjan (Ivory Coast), the clinical service of hematology of the CHU of Yopougon and to the authorities of Superior Normal School Abidjan for providing the facilities for conducting this research.

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