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Phytochemical Screening, Evaluation of Antioxidant and Anti-sickling Activities of Two Polar Extracts of *Combretum glutinosum* Leaves. Perr. ex DC

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Authors' contributions

This work was carried out in collaboration between all authors. Author MS designed the study, performed the statistical analysis. Authors MS and CS wrote the protocol and wrote the first draft of the manuscript. Authors SFN, MDD, IS and RSG managed the analyses of the study. Authors BF, COT, PMG, DF, MF and TND managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Sickle cell disease is a major public health problem in Africa and many other areas across the world. In Senegal, a lot of plants are proposed by traditional healers to manage the sickle cell disease, among them *Combretum glutinosum*. Antisickling activity of *Combretum glutinosum* leaves extracts was studied in this work. Methanolic and ethyl acetate extracts of *Combretum glutinosum* leaves were evaluated on SS sickles type to determine their anti-sickling potential. Antiradical properties of methanolic extract were evaluated using the DPPH radical as oxidant. Total phenolic content of the methanol extract was determined. Phytochemical screening of the crude extract of methanol revealed the presence of tannins, saponins, phenols and flavonoids. Results show a good antisickling effect of extracts with a maximum antisickling revers of 81% and 69% respectively, of the methanol and ethyl acetate extracts at 10 mg/mL versus 67% for arginine at 120 min of incubation. The measured IC_{50} were 0.65 and 0.163 for respectively the methanol extract and ascorbic acid. Antiradical powers 0.155 and 0.62 respectively for methanol extract and ascorbic acid were calculated from the effective concentrations. The results of this study confirm the traditional use of *Combretum glutinosum* leaves in the management of sickle cell disease.

Keywords: Sickle cell disease; oxidative stress; medicinal plant; Combretum glutinosum; antisickling activity; antioxidant activity.

1. INTRODUCTION

Medicinal plants are used in the whole world and have a growing economic importance. The use of plant parts to treat human disease is as old as disease itself [1]. According to the World Health Organisation (WHO), as many as 80% of world population, particularly in developing countries, have used at least one time in their lives, traditional medicines for their primary health care need today and 25% of drugs are based on plants and their derivatives [2]. *Combretum glutinosum* is a very large genus comprising of about 250 species found in both temperate and tropical regions in the world [3]. It is largely distributed in Africa throughout the Sahel belt from Senegal to Cameroon and eastwards to the Sudan. It is a bushy shrub or small tree growing up to 12 m and deciduous species sprouting in the middle of the dry season. The plant parts are used in traditional medicine in Africa to treat many human diseases. In Nigeria, the roots, the stem, the bark and the leaves of *Combretum glutinosum* are used in the treatment of scrotal elephantiasis, dysentery, ring worms, bacterial diseases, syphilis, typhoid fever etc [4]. Antidiarrheal activity of aqueous young leaves extract of *Combretum glutinosum* was demonstrated [5]. Anti-malarial and anti-schistosomal activities of *Combretum glutinosum* were respectively described by Yahaya and Ouattara [2,6]. Therapeutic potential of different parts of *Combretum glutinosum* remains to be explored on several other diseases such as sickle cell disease (SCD).

Indeed Sickle cell disease is a form of haemoglobinopathy that is characterized by chronic hemolytic anemia. SCD is a genetic disease in which the SS individual possesses an abnormal ß-globin gene. A single base substitution in the gene encoding the human ß-globin subunit results in the replacement of ß6 glutamic acid by valine, which leads to the devastating clinical manifestations of SCD. This substitution is followed by a remarkable inflammatory response and oxidative stress within the blood circulation [7]. It also causes a drastic reduction in the solubility of sickle cell hemoglobin when deoxygenated, frequent chest pain and vaso-occlusive complication [8]. SCD occurs world-wide and the African continent bears the highest burden. Hispanic, Arabic, Mediterranean and some Asian populations are also affected. According to Tim [9], approximately 250,000 children are born each year with sick cell disease and over 70,000 individuals in the U.S. suffer from the disease. In Africa, SCD is a major public health problem with a prevalence of SCD trait between 10 and 40% [10]. In Senegal it is between 8 and 10% in the general population [11]. Due to the high mortality rate of sickle cell patients, especially in children, and since chemotherapy has its adverse effects, there is need for rational drug development that must embrace not only synthetic drugs but also natural products. It is acknowledged world-wide that traditional medicine can be explored and exploited for enhanced health management. The ever increasing demand for safer and cheaper herbal recipes in developing country, has led to the extraction and development of several drugs

and chemotherapeutic agent from plants, as well as from traditional herbal remedies [5]

In recent years, the interest in natural antioxidants, in relation to their therapeutic properties, has increased dramatically. Scientific research in various specialties has been developed for extraction, identification and quantification of these compounds from several natural substances namely, medicinal plants and food products [12,13]. The antioxidant activity of a compound corresponds to its ability to resist oxidation. The best known antioxidants are the β-carotene (provitamin A), ascorbic acid (vitamin C), tocopherol (vitamin E) as well as phenolic compounds. Indeed, most synthetic or naturally occurring antioxidants have phenolic hydroxyl groups in their structures; and antioxidant properties are attributed in part to the ability of these natural compounds to scavenge free radicals such as hydroxyl radicals (OH •) and superoxide ($O^{2\bullet}$) [14,15]. Several methods are used to evaluate, *in vitro* and *in vivo* antioxidant activity by scavenging different radicals such as peroxyl radical ($ROO^{\bullet}$) by ORAC and TRAP methods [16]; ferric ions by FRAP [17]; or the ABTS • radical [18] and the method using free radical DPPH• [19]. Because it takes place at room temperature and can eliminate the risk of thermal degradation of thermolabile molecules, the DPPH• radical test is the most recommended for very hydrophilic extracts which are rich in phenolic compounds [20].

To our knowledge, the activities of *Combretum glutinosum* parts have not been investigated against the Sickle cell disease. The objective of this study is on the one hand to assess the potential anti-sickling activity of two polar leaves extracts of *Combretum glutinosum* and on the other hand to measure *in vitro* the antioxidant activity of the most active extract by using the DPPH radical method.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Blood sample, chemistry and biochemistry products

The sickle cells used in this work were obtained from unused SS sickle blood of patients who undergo a diagnostic test for sickle cell disease. These patients had been neither transfused nor treated with hydroxyurea in the last six months. The age of patients ranged from 18 to 35 years in both sexes. Sampling is made at the elbow crease of the patients and the blood was collected in EDTA tubes. Chemical and biochemical products used in this work were obtained from different suppliers (Prolabo, Scharlau, Aldrich or Carlo Erba). All solvents were freshly distilled before use. The human ethics approve was obtained from Cheikh Anta Diop University of Dakar under the number 0228/2017/CER/UCAD on February 03 2017.

2.1.2 Plant material

The leaves of *Combretum glutinosum* used in this work were purchased from traditional healers in Tilene market in Dakar. The identification was carried out at the Laboratory of Pharmacognosy of the Medicine, Pharmacy and Odontology Faculty at Cheikh Anta Diop University of Dakar. The dried leaves were crushed and powdered using a Brabender mill.

2.2 Methods

2.2.1 Extraction of the *Combretum glutinosum* leaves powder

Leaves powder 60 g of *Combretum glutinosum* were extracted with 300 mL of ethyl acetate. The mixture was stirred at room temperature for 48 hours. The macerated matter was filtered and the filtrate evaporated to dryness using vacuum rotary evaporator. The crude methanol extract was obtained from 60 g of *Combretum glutinosum* leaves powder in 300 mL of methanol using the same operation as the one that has been achieved using the ethyl acetate extract. The extracts were labeled as follows: EAE (ethyl acetate extract); ME (methanol extract) and stored in a refrigerator at 4℃ until use. The percentage yield of the extracts was determined by using the following formula:

$$Y=X/WI \times 100,$$

with X = mass of extract and WI = initial mass of the powder.

2.2.2 Phytochemicals screening

Methanol extract was subjected to preliminary phytochemical tests using standard technique to detect the presence of alkaloids, saponins, polyphenols, tannins and flavonoids as described in our previous work [21].

2.2.3 Determination of antioxidant activity by the DPPH radical scavenging method

Measurement of the antiradical activity of methanolic extract from *Combretum glutinosum* leaves is performed by the 2,2'-diphenyl-1-picrylhydrazyle (DPPH) test in accordance with the method described by Scherer [19]. DPPH (4 mg) was dissolved in 100 mL of methanol, the solution is then stored for 1 hour in the dark. A concentration range (0.25 to 5 mg/mL) of methanolic extract from the *Combretum glutinosum* leaves and (0.09 to 0.5 mg/mL) of ascorbic acid (reference) was prepared in methanol. A volume of 0.1 mL of *Combretum glutinosum* leaves solution is mixed with 3.9 mL of the DPPH solution. After homogenization, the mixture was incubated in the dark for 30 minutes. The mixture absorbance is measured with a spectrophotometer at 517 nm.

Antioxidant activity is defined as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50% [22]. Results are expressed in the first time as the percentage of the Radical Scavenging Activity (%RSA) by using the following expression.

$$\%RSA = \left[\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right] x100$$

And in the second time, the inhibition concentration, IC_{50} (concentration of extract that traps 50% of DPPH radical) was determined by projection of the absorbance values of methanol extract and ascorbic acid solution on the graphic representing the trapping percentage as function of the concentration. It is possible to deduced from IC_{50} values, the efficient concentration (EC_{50}) and the Antiradical Power (ARP) as described in our previous paper [23].

2.2.4 Determination of polyphenols content

Phenols content of methanol extract was determined by the Folin Ciocalteu method according to a protocol written by Bidie et al. [24]. To a concentration of 0.1 mg/mL of methanolic *Combretum glutinosum* leaves extract, 0.5 mL of Folin Ciocalteu reagent 10% (v/v) is mixed with 2.5 mL of distilled water and 2 mL at 0.5M of sodium carbonate (Na_2CO_3). After 30 minutes incubation at room temperature, the absorbance is read on a spectrophotometer at 765 nm. Gallic acid was used as the reference antioxidant. Concentrations of 0.001, 0.015, 0.025, 0.05, 0.075, 0.1, 0.15, 0.2 mg/mL were prepared from gallic acid in hemolysis tubes. To each tube is added 2.5 mL of Folin Ciocalteu reagent 10% (v/v) and 2 mL of sodium carbonate. The tubes are incubated at room temperature for 30 minutes and then absorbance read at 765 nm. Methanol was used as a blank. Results obtained allowed to draw calibration curve of gallic acid. Projection of the absorbance value of methanolic extract on calibration curve has permitted to determine graphically total phenol content expressed in terms of gallic acid equivalent (mg GAE/g of the sample). All analyses were performed in triplicate.

2.2.5 Antisickling activities

In vitro screening of polar extract of *Combretum glutinosum* leaves for antisickling proprieties with blood samples collected from patients with the confirmed SS sickle cell by Emmel test and electrophoresis, was carried out using a protocol described by Imaga [25] and given in our previous work [20]. Morphological analysis of erythrocytes (100 cells in 4 to 5 fields) is performed using an immersion microscope. When extracts are brought into contact with blood, every 30 minutes up to 120 minutes an erythrocyte count is performed.

2.3 Data Analyses

The results of antisickling activities were evaluated in percentage of residual sickles. For the negative control, since the number of sickle cells increases with time, it was considered that the rate of 100% corresponds to the number of sickle cells obtained in 120 minutes. For samples tested, this number decreases over time; so the rate of 100% is the number of sickle cells at the initial time T0. Evolution of the percentage of residual sickled cells in function of time is given by the following equation:

$$\mathbf{PDR} = \frac{\textbf{Mean of sickles at Tx}}{\textbf{Mean of sickles at T0}} \textbf{ x 100}$$

PDR = Percentage of residual sickles
Tx = 0, 30, 60, 90 et 120 minutes
T0 = initial time

3. RESULTS

3.1 Yield of Extracts

Extraction results in Table 1 show that the percentage yield of methanol extract of *Combretum glutinosum* leaves is higher (7.45%) than the ethyl acetate extract (5.00%). This difference may be justified by the greater polarity of the methanol solvent.

Table 1. Yield of extracts

Solvents	Methanol	Ethyl acetate
Extracted Plant powder (g)	4.47	3.00
Percentage yield (%)	7.45	5.00

3.2 Phytochemical Screening Result

The preliminary phytochemical screening of *Combretum glutinosum* leaves shows presence of various bioactive components which are given in Table 2. Results provide evidence of the presence of saponins, phenols, tannins and flavonoids and show the absence of alkaloids. These results are confirmed by Yahaya and Albagouri [2,26] in their phytochemical screening of *Combretum glutinosum* study, they show among other things the presence of saponins, tannins, flavonoids, phenolic compounds and alkaloids. However, the difference is they show the presence of alkaloids. This difference could be explained by a difference of nature of solvent used, the harvest period or the nature of the soil where the plant grows.

3.3 Antiradical Activity

Antiradical activity of methanolic extract of *Combretum glutinosum* leaves is given in Fig. 1. Activity is determined on an average of three measurements per concentration using the statview statistical software by performing a normal analysis of variance (ANOVA) followed by Fischer test. Difference is considered significant if $p<0.05$ versus negative control. The results give IC_{50} = 0.65 and 0.163 respectively for methanol extract and ascorbic acid. Antiradical power calculated from the effective concentration (EC_{50}) informs on the ability of active compounds to inhibit the free radical activities. Indeed the more important the antioxidizing power value of the concerned product is, the higher is its activity. Thus we calculated the anti-radical power of 0.155 and 0.62 for respectively, methanol extract of *Combretum glutinosum* leaves and ascorbic acid Table 3.

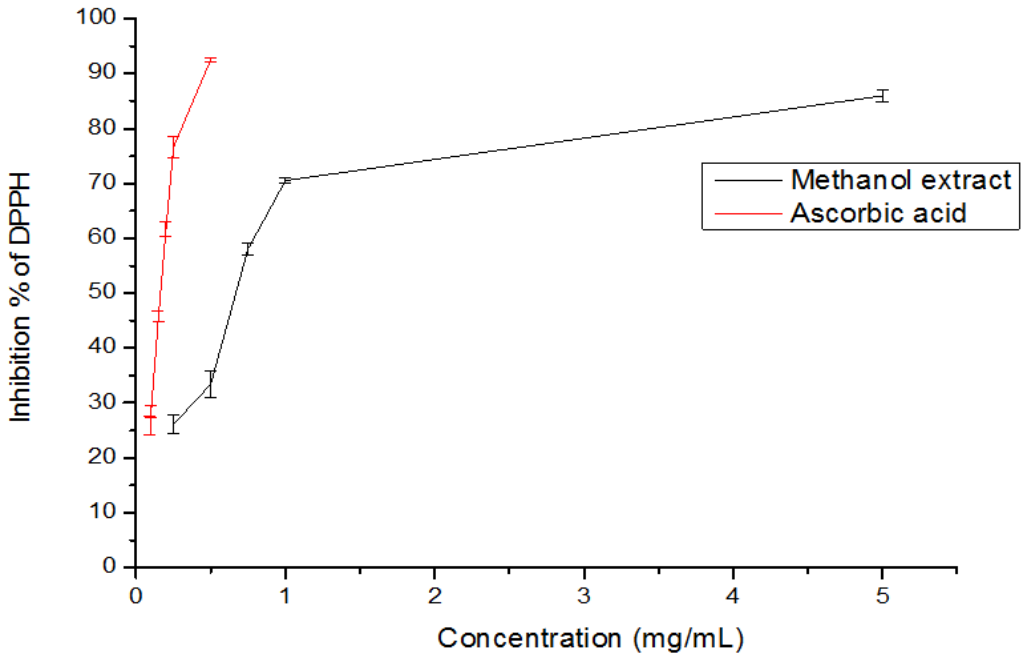

Fig. 1. Percentage inhibition of DPPH radical by *C. glutinosum* and ascorbic acid

Table 2. Results of the phytochemical screening

Alkaloids	Saponins	Polyphenols	Tannins	Flavonoids
-	+	+	++	++

- Absence, + Presence

Table 3. Values of IC_{50}, EC_{50} and ARP from *C. glutinosum* and ascorbic acid

Studied parameters	*C. glutiosum* methanol extract	Ascorbic acid
IC_{50} (mg/ml)	0.65	0.163
EC_{50} (CI_{50} / mol of DPPH).	6.43	1.61
ARP	0.155	0.62

IC = Inhibition Concentration, EC= Effective Concentration, ARP = Antiradical Power

3.4 Polyphenols Content Determination

Determination of phenol content of *Combretum glutinosum* methanolic leaves extract is performed using the regression equation of the calibration curve of gallic acid (Fig. 2), where Y is absorbance of methanolic extract (0.233).

Phenol content is then equal to X/concentration of methanolic extract. Calculation gives total polyphenol content equal to 150 mg GAE/g sample.

This result is better than those obtained by Yahaya [2]. For the determination of total phenols content from different extracts solvent of *Combretum glutinosum*, the best result, 130 mg GAE/g of extract was given by methanol extract.

3.5 Antisickling Effect of *Combretum glutinosum* Leaves Extracts

Results of the antisickling activity of *Combretum glutinosum* leaves extracts are presented in Figs. 3 and 4. Results show that the two studied polar fractions have interesting activities on sickle cells compared to the negative control for which the number of sickle cell increases over time. We also find that methanol extract which has higher polarity as ethyl acetate extract, presents the best results. In both studied concentrations, methanolic and ethyl acetate extracts have more reversibility of sickling cells than arginine used as positive control.

4. DISCUSSION

Today research is booming in Africa in the development of herbal medicines, drugs and dietary supplements, in the management of tropical diseases and genetic disorder, especially in sickle cell disease where science is still struggling to establish a sustainable treatment. These plant treatments would be for most of the population an alternative to modern medicine because they are not expensive and are accessible to all. Thus, it is after an ethnobotanical study conducted by the traditional healers that *Combretum glutinosum* leaves were selected to study their effects on the SS type sickle.

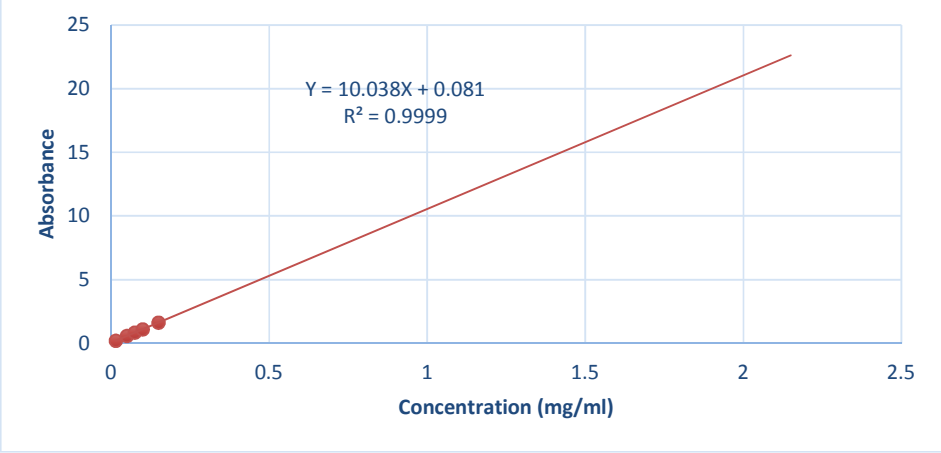

Fig. 2. Calibration curve of the gallic acid (y = 10.038x + 0.081; R^2 = 0.99947)

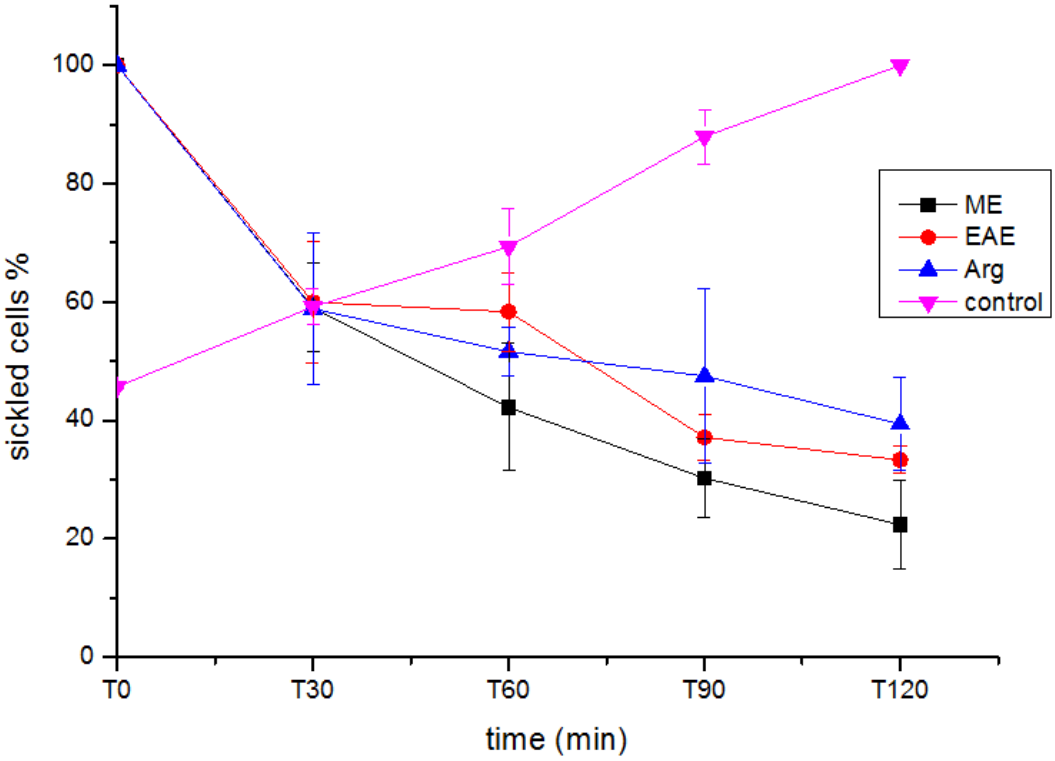

Fig. 3. Antisickling activities of *C. glutinosum* leaves extracts at 5 mg/mL

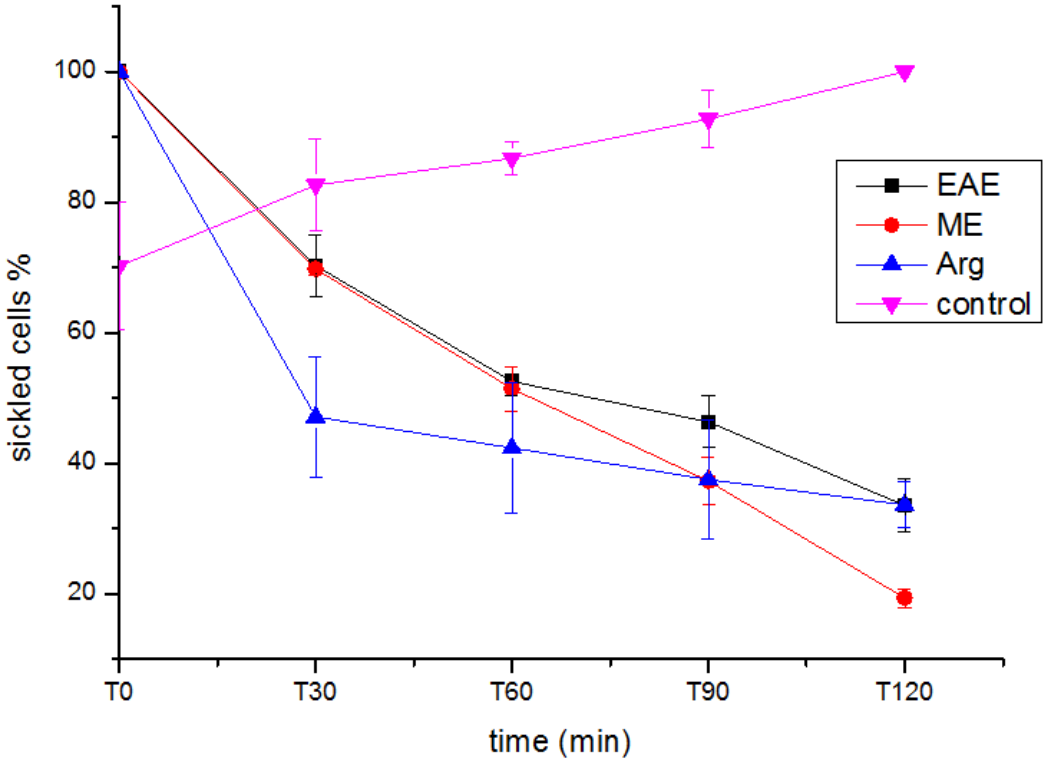

Fig. 4. Antisickling activities of *C. glutinosum* leaves extracts at 10 mg/mL

Methanol and ethyl acetate extracts from *Combretum glutinosum* leaves were active on the reversibility of the sickling of sickle cells. Thus methanol is the most active extract and has 81% sickling reversal in 120 minutes of incubation. Arginine used as positive reference present in the same conditions 67% sickle reversibility. Also arginine is recommended in the management of vaso-occlusive crises. Indeed nitric oxide which plays an important role in the sickle cell disease pathophysiology, has arginine as substrate. It is considered as a vasodilator of blood vessels by acting on the endothelial functions. Therefore, it can reduce the vaso-occlusion crises [27]. Ethyl acetate extract presents a less important activity than methanol, its reversibility rate is 69% at 10 mg/mL in 120 minutes of incubation. The most important polarity of methanol as ethyl acetate explains the most important activity of the former. Methanol extract would contain polar secondary metabolites whose synergetic effects justify this most important activity on the reversibility of the sickling of sickle cells. Phytochemical screening performed on methanol extract, showed the presence of polyphenols, tannins, saponins and flavonoids and the absence of alkaloids. According to Iweala [28], the synergistic effects of a wide variety of biologically active substances and amino acids contained in plants are responsible for the observed antisickling activities. Antisickling results of the methanolic extract of *Combretum glutinosum* leaves (81%) in comparison with those obtained in our previous work on *Leptadenia hastata* [21] and on the *Maytenus senegalensis* [23] which are respectively 77 and 80% sickling reversibility of sickle cells, are more interesting and give bright prospects.

One of the main characteristics of sickle cell disease is the production of a large amount of free radicals leading to severe oxidative stress and consumption of nitrogen monoxide by oxygen free radicals. Oxidative stress also affects the ratio Fe^{3+}/Fe^{2+}, quite high in the sickle cells, is involved in sickle cell hemolysis [29]. Identification of plant antioxidant properties, would indicate its favourable action on the management of sickle cell disease. Indeed, we have demonstrated *in vitro* that the methanol extract of *Combretum glutinosum* leaves inhibits the activity of DPPH radical with an IC_{50} of 0.65 versus 0.163 for ascorbic acid. Polyphenol content determined from the calibration curve of gallic acid is 150 mg GAE/g. These results show that the methanol extract acts also on the reduction of the oxidative stress generated in sickle cell disease.

Oxidative processes, which trigger the production of free radicals, resulting in tissue damage, are a major contributor to diminished health, and manifested in a wide spectrum of disease [30]. The medicinal value of plants is related to their phytochemical component content and secondary metabolites, including: phenolic compounds, flavonoids, alkaloids, tannins, and other stress gene response products. The flavonoids present in the *Combretum glutinosum* leaves represent a large family of polyphenols and the involvement of the latter in the treatment of oxidative stress has been proven [31]. Antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators [32]. The information presented above suggests to us that *Combretum glutinosum* leaves with high phenol content could, not only be a solution in the management of sickle cell disease, but also beneficial to all other pathologies generated by oxidative stress.

5. CONCLUSION

Combretum glutinosum leaves have been reported by the traditional medicine healers in Senegal to have antisickling activities. This necessitated scientific evaluation of the concerned part of the plant against the homozygous sickle. Methanol and ethyl acetate extracts of *Combretum glutinosum* leaves have a significant sickle cell reversal activities. These results confirm the traditional use of the *Combretum glutinosum* leaves in the management of sickle cell disease. This alternative therapeutics using phytotherapy has proven to, not only reverse sickling cells but also acts on the oxidative stress. Future studies should isolate and study the bioactivities compounds responsible for the potential antisickling in this plant.

ETHICAL APPROVAL

Ethical approval from Cheikh Anta Diop University of Dakar (0228/2017/CER/UCAD).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

Fig. 1. Picture of *Combretum glutinosum* Perr.ex DC

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