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# Antisickling Properties of Two *Calliandra* Species: *C. portoricensis and C. haematocephala* (Fabaceae)

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author JMA designed the study, supervised the research and wrote the final draft of the manuscript, author OOA managed the literature searches, the analyses and wrote the first draft while author MAA performed the statistical analyses. All authors read and approved the final manuscript.*

***Original Research Article***

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## ABSTRACT

**Aim:** To investigate the antisickling potentials of two *Calliandra* species namely *C. portoricensis* (Jacq) Benth *and C. haematocephala* Hassk *in vitro.*
**Study Design:** Evaluation of antisickling activities of medicinal plants on human sickled red blood cells *in vitro.*
**Place and Duration of Study:** Research Laboratory of Drug Research and Production Unit, Faculty of Pharmacy Obafemi Awolowo University, Ile Ife, Nigeria. September 2010 to November, 2011.
**Methodology:** After obtaining ethical clearance, fresh blood samples (5ml) each were collected from confirmed sickle cell anaemia patients who were in a steady state and were attending the routine clinic. Water and 70% ethanol were used separately for the extraction of the leaves and roots of the two plants. The extracts were assessed using the inhibitory and reversal methods *in vitro*.
**Results:** It was observed that there was linear increase in inhibitory and reversal activities of the ethanolic and aqueous extracts of the parts used as the concentration increased. The ethanolic root extract of *C. portoricensis* exhibited the highest activity for inhibitory

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(90.19%) and reversal activities (92.63%) both at 4mg/ml.
**Conclusion:** *Calliandra* species possessed antisickling properties *in vitro* with *C. portoricensis* being the more active plant.

*Keywords: Antisickling; Calliandra haematocephala; C. portoricensis; inhibitory; reversal; sickle cell disorder.*

## 1. INTRODUCTION

Sickle cell disorder is a common genetic condition due to a haemoglobin disorder resulting from inheritance of mutant haemoglobin genes from both parents. This disorder is caused by a point mutation in the β-globin chain of haemoglobin, causing the hydrophilic amino acid glutamic acid to be replaced with the hydrophobic amino acid valine at the sixth position [1]. Sickle cell disorder results in anaemia and crisis that could be of many types including the vaso-occlusive, aplastic sequestration, hyperhaemolytic and other crises [2]. Over the years, several research works have been done and chemical agents for inhibiting or reversing sickle shaped red cell *in vitro* have been proposed. The use of phytomedicines such as *Piper guineensis, Pterocarpa osun, Eugenia caryophyllala* and *Sorghum bicolor* extracts for the management of sickle cell disease have been reported [3]. An investigation into the usage of aqueous extracts of the reddish brown freshly fallen leaves of *Terminalia catappa* reported that the plant can exhibit antisickling activity on Sodium metabisulphite induced sickling [4]. The ability of a compound to increase the gelling time of human HbSS could be taken as a measure of the antisickling potential of the compound and this determined its effectiveness in sickling inhibition and retarding of the aggregation of HbSS erythrocytes in the potential's blood vessels [5]. The extracts of *Xylopia aethiopica* (guinea pepper) and seeds of the African nutmeg were evaluated for *in vitro* antisickling activity [6]. The researchers found that irrespective of whether they used the water, chloroform, methanol or butanol extract of these spices, the extracts were able to prevent red blood cells becoming sickle in shape to varying extents – from 70 per cent to 90 per cent in 15 minutes. Since sickle cell disease is a genetic disorder, no cure had been found but the disease is managed through the use of vitamins and good nutrition. However, of late research has been intensified in order to discover new drugs that will be able to ameliorate the crises conditions associated with this disorder.

The genus *Calliandra* belongs to the family Fabaceae, subfamily Mimosoideae. It is a large geotropical genus from South Africa to the Southern United States. Some species are also found in India, Madagascar and West Africa. The genus comprises of about 200 species of flowering plants, which include herbaceous perennial plants, shrub and rarely small trees growing to a maximum height of 12m and a maximum basal stem diameter of 20 cm [7]. *Calliandra portoricensis* is a shrub or small tree of about 6 m tall with evergreen small bipinnate leaves, whitish- pink scented and globose flowers, which look like small snowballs and Fruits are pods, which are about 4 in (10 cm) long [8]. *Calliandra portoricensis* is also known as 'tude' in Yoruba and is used in ethnomedicine as anticonvulsant [9,10], anti-diarrheal, antispasmodic, antipyretic, antirheumatic and analgesic activities in human beings [11]. The preliminary phytochemical analysis of the extract of *Calliandra portoricensis* reveals that the plant possesses saponins, steroids, tannins, glycosides, alkaloids, anthraquinones, cardiac glycosides, fatty acids, gallic acid, methyl gallate, myricitrin, quercitrin, afzelin, isoquercitrin, caffeic acid, betulinic acid and other related compounds [9]. *Calliandra haematochephala* is similar to *C .portoricensis* the major difference is in the colour of the flower. The flower is reddish- pink and fruit color is brown [12]. Flowers are

clustered in globose heads, up to 7 cm across; stamens are long and silky, pink to red. Fruit is a dehiscent pod. *C heamatocephala* is also known as Ule in Yoruba. The chemical constituents of *C heamatocephala* include Betulinic acid, lupeocaffic acid, astilbin, catechin-3-O-rhamnoside and P-hydroxybenzoic acid [13]. The leaf contains pipecolic acid derivatives. Pipecolic acid is a non protein amino acid, and its derivatives have been isolated from the leaves of *C. haematocephala.* Three acylated quercetin rhamnosides were reported from the leaves and stem of *C. haematocephala* and their structures were established as quercetin 2''-*O*-caffeate quercetin 3''-*O*-gallate and quercetin 2'', 3''-di-*O*-gallate [14]. Also, 17 known compounds were reported for the first time from the genus *Calliandra* they are gallic acid derivatives. Leaves of *Calliandra heamatocephala* Hask (Mimosaceae) were used against various microbial infections and active against bacteria thereby justifying their use against skin infections [13]. The betulinic acid acts as an anti-inflamatory agent [15]. Antitumoral and anti-HIV activity of betulinic acid has also been reported [16]. Tyranine is reported present in the leaves at 118 mg / kg fresh weight basis. Six compounds exhibited moderate to strong radical scavenging properties [14]. However, the plant is yet to be authenticated for its antisickling activity hence this study. Therefore, the possible antisickling properties of the *Calliandra* species and the relative antisickling activities of the leaves and roots of the two *Calliandra* species were investigated and assessed respectively.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Reagents

The chemicals used were ethanol (Fluka), methanol (Fluka), distilled water, Sodium metabisulphite (Hopkins and Williams), Phosphate buffered saline pellets pH 7.0, formalin, liquid paraffin, Tween-80 (BDH) and Para-hydroxybenzoic acid (PHBA) (BDH).

### 2.2 Plant Samples

Two *Calliandra* species: *C. portoricensis* and *C. haematocephala* fresh roots and leaves were collected around Mozabique Hall of Obafemi Awolowo University, Ile-Ife in July 2009, and beside antenatal ward of the Obafemi Awolowo University Teaching Hospital Complex, Ile-Ife respectively in July 2009. The plants were identified and authenticated at the Herbarium Units of Faculty of Pharmacy and the Department of Botany of Obafemi Awolowo University simultaneously.

### 2.3 Blood Sample

Ethical clearance was obtained from the Ethical Committee of the Obafemi Awolowo University Teaching Hospital Complex (OAUTHC), Ile-Ife. Fresh blood samples (5ml) each were collected from confirmed sickle cell anaemia patients who were in a steady state and attending the routine clinic. The blood samples were collected into an EDTA anticoagulant containing bottles and mixed gently to prevent lysing of the red blood cells.

### 2.4 Preparation of Plants Materials

Fresh leaves of *C. portoricensis* and *C. haematocephala* were air dried for 24 hours and oven dried at 40ºC for 4 hours to bring about complete dryness. The roots of both plants were washed, cut into small pieces, air dried and finally at 50ºC for 4 hours. All the dried

plants were separately milled into powdered form using grinding machine (Christy) and stored in well sealed amber coloured bottles.

## 2.5 Preparation of Aqueous and Ethanolic Extracts

Powdered plant materials (200 g) each were separately extracted with (2 L) water or 70% (v/v) ethanol (2 L). The suspension were allowed to simmer for 3 hour under reflux and finally allowed to cool. The suspension were filtered under vacuum and concentrated to dryness at 50ºC. Any residual water in the dried extracts was removed using activated silica gel desiccators. Final drying was done by freeze-drying. They were finally stored separately into sample bottles until needed for the experiments. The percentage yields of the extracts of the two *Calliandra* species were determined. A small portion of each of the dried extracts (0.02 g) was reconstituted in distilled water (5ml) to obtain the 4 mg/ml concentration. Serial dilutions were made further to obtain dilutions of 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml concentrations, which were used for the antisickling assay.

## 2.6 Inhibitory Assay

The evaluation of both aqueous and ethanolic extracts for antisickling activities were carried out using modified methods of Sofowora et al. [17] and Egunyomi et al. [18]. Vein punctured blood samples from sickle cell anaemia patients not in crises were used. Blood sample (0.2 ml), 0.2 ml of phosphate buffered saline and 0.2 ml of the test extract were mixed together in a test tube. The mixture was overlaid with 1 ml of liquid paraffin to prevent aeration or oxygenation. The mixture was then incubated in a thermostated water bath at 37ºC for 4 hr. Freshly prepared 2% (w/v) sodium metabisulphite solution (0.6 ml) was carefully added to the mixture under the paraffin to create hypoxial state in the medium, which was then mixed gently by rolling the test tube between the two palms. The mixture was incubated for additional ninety minutes at 37ºC in the water bath. The liquid paraffin was carefully removed using a Pasteur pipette and the cell was fixed with 3 ml of 5% w/v buffered formalin solution. The positive control was as described above except Parahydroxybenzoic acid (PHBA) was added instead of extract while the negative control lacked extract but equivalent volume of 5% (w/v) Tween 80 (dissolution solvent of extracts). Each test was performed in triplicate. A drop of each reaction mixture was smeared on a microscope slide and viewed under high powered magnification (x 100) under the oil immersion. Cells were counted on five fields on each slide; the numbers of sickled and unsickled cells were counted to determine the total number of cells. The percentage mean sickling as well as the percentage inhibition activity for each extract were estimated.

## 2.7 Reversal Assay

Mixture of the blood sample (0.2 ml) and 0.2 ml phosphate buffered saline in a test tube was carefully overlaid with 1ml liquid paraffin and 0.6 ml of 2% (w/v) sodium metabisulphite was carefully introduced under the liquid paraffin. The mixture was then incubated at 37ºC for ninety minutes. At the end of the incubation period, 0.2 ml of the extract was carefully added under the liquid paraffin and was further incubated at 37ºC for additional 6 hr. The liquid paraffin layer was removed with a Pasteur pipette and the cells were fixed with 3 ml of 5% (w/v) buffered formalin solution, which was carefully mixed by rolling the test tube between the two palms. The positive control also involved all the procedure described above except that PHBA was added instead of extract while the negative control lacked extract but equivalent volume of 5% Tween 80. A drop of each reaction mixture was smeared on a

microscope slide and viewed under high powered magnification (x 100) under the oil immersion. Cells were counted on five fields on each slide, the number of sickled and unsickled cell were counted to determine the total number of cells. The percentage mean sickling as well as the percentage reversal activity for each extract was estimated using the expression below:

$$\% \text{ Mean Sickling} = \frac{\text{Mean sickled cells}}{\text{Mean total cells}} \times 100$$

$$\% \text{ Inhibition activity} = \frac{\text{Control} - \% \text{ Mean sickled}}{\text{Control}} \times 100$$

$$\% \text{ Reversal activity} = \frac{\text{Control} - \% \text{Mean sickled}}{\text{Control}} \times 100$$

Total number of cells counted= No of sickled + No of unsickled cells

Values are expressed as mean ± SEM of 3 consistent readings. The statistical significance differences were analyzed using Student- Newman- Keuls Multiple Comparison test and analysis of variance. Values of $P < 0.0001$ were considered to be extremely significant.

## 3. RESULTS AND DISCUSSION

The percentage yield of powdered leaves and roots and their extracts shown in Table 1 indicated that 70% ethanol appeared to have better extractive capability than water. The percentage yield of the latter extracts of the roots and leaves of the two *Calliandra* species were higher than the aqueous extracts. The water being inorganic polar solvent present in the 70% Ethanol could have combined with the organic polar nature of the Ethanol thereby demonstrated better extractive capability. As such both the organic and the inorganic salts of the plants which could be putative in a synergistic manner were thus extracted. Water, though an inorganic polar solvent has the capability of extracting organic component of a plant especially the polar component but less of the lipophillic component, but the thermal energy enabled water to extract some of the lipophillic component; but not as much as organic polar solvent under the same thermal condition. The above could explain the relatively higher extractive yields of the 70% ethanol solvent over water. The ethanolic root extract of *C. haematocephala* had the highest yield. This result corroborates the statement that extracts with ethanol extraction were most effective followed by cold-water and hot water extraction respectively [19]. Another experiments also reported that ethanol had higher yield of extracts than water [20]. This could also justify the use of alcohol or local gin as extraction solvent in some ethnomedical preparations in West Africa.

**Table 1. Percentage yield of powdered leaves and roots and their extracts**

| Plant Species | Extracts | Percentage yield (%) |
|---|---|---|
| *C. haematocephala* roots | Aqueous | 7.60 |
| | ethanol | 8.20 |
| *C. haematocephala* leaves | Aqueous | 5.83 |
| | ethanol | 6.72 |
| *C. portoricensis* roots | Aqueous | 4.50 |
| | ethanol | 4.95 |
| *C. portoricensis* leaves | aqueous | 4.53 |
| | ethanol | 6.02 |

The inhibitory activities of the leaf and root extracts of the two *Calliandra* species were compared at various concentration ranges (0.125 mg/ml – 4.0 mg/ml) as shown in Figs 1-4. The inhibitory activities of the extracts were significantly different ($P < 0.001$) from each other and appreciable antisickling activities greater than 70% were recorded at concentration greater than (0.5 mg/ml). The inhibitory activities for the aqueous extracts were relatively lower than that of the ethanolic extracts. This could be due to the better extractive capability of 70% ethanol as discussed earlier. However, both the aqueous (Cpraq) and ethanolic (Cpret) root extracts of *C. portoricensis* exhibited higher inhibitory activities than others. The Cpret extract at high concentration (4.0 mg/ml than the control) was the most active extract with inhibitory activity of 90.19% compared to PHBA which gave 84.04%.

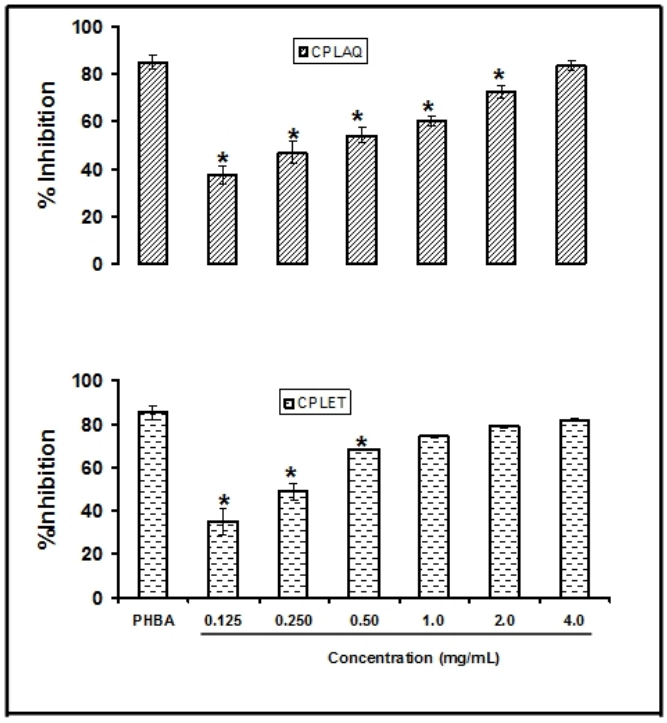


**Fig. 1. The inhibitory activities of ethanol (CPLET) and aqueous (CPLAQ) leaf extracts of *C. portoricensis* when compared with Para-hydroxybenzoic acid (PHBA) [Control]. *p<0.05 when compared with the control (PHBA)**

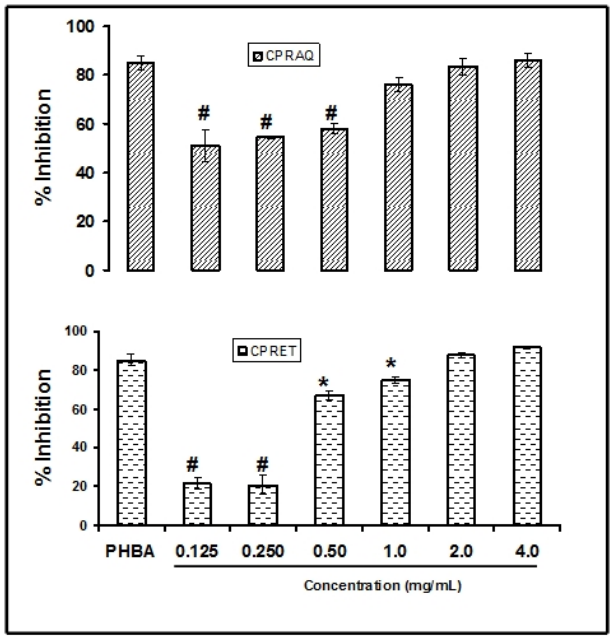


**Fig. 2. The inhibitory activities of ethanolic (CPRET) and aqueous (CPRAQ) root extracts of *C. portoricensis* when compared with Para-hydroxybenzoic acid (PHBA) [Control]. *p<0.001 or #p<0.01 when compared with the control**

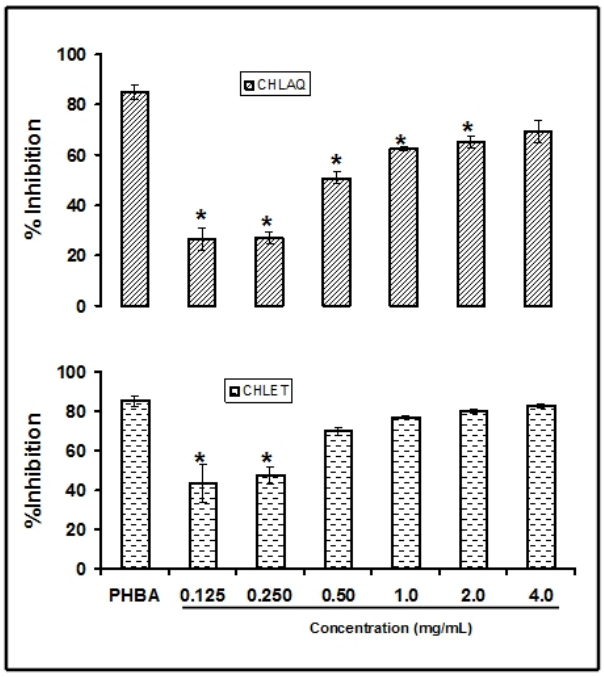


**Fig. 3. The inhibitory activities of ethanolic (CHLET) and aqueous (CHLAQ) leaf extracts of *C. haematocephala* when compared with Para-hydroxybenzoic acid (PHBA) [Control]. *p<0.001 when compared with the Control (PHBA)**

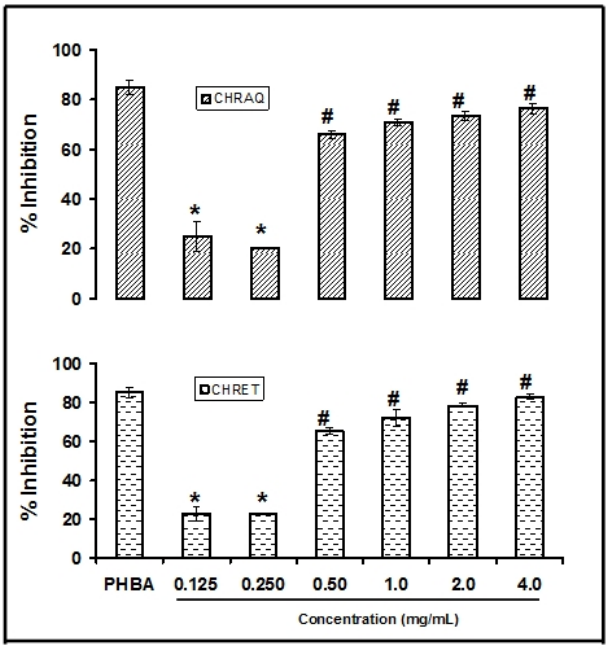


**Fig. 4. The inhibitory activities of ethanolic (CHRET) and aqueous (CHRAQ) root extracts of *C. haematocephala* when compared with Para-hydroxybenzoic acid (PHBA) [Control]. *p<0.001 when compared with the control (PHBA) and #p<0.05 when compared with the doses of 0.123 or 0.250 mg/mL groups**

Plate 1 showed Red blood cells (RBC) of HbSS patient under hypoxial state. The film showed lots of sickle cells with the deformed RBC with sickle shape. However, in the Plate 2 at highest concentration of 4 mg/ml virtually all the RBC maintained the spherical, ovoid shape of normal RBC which was indicative of the capacity of the extract to inhibit the sickling of red blood cells.

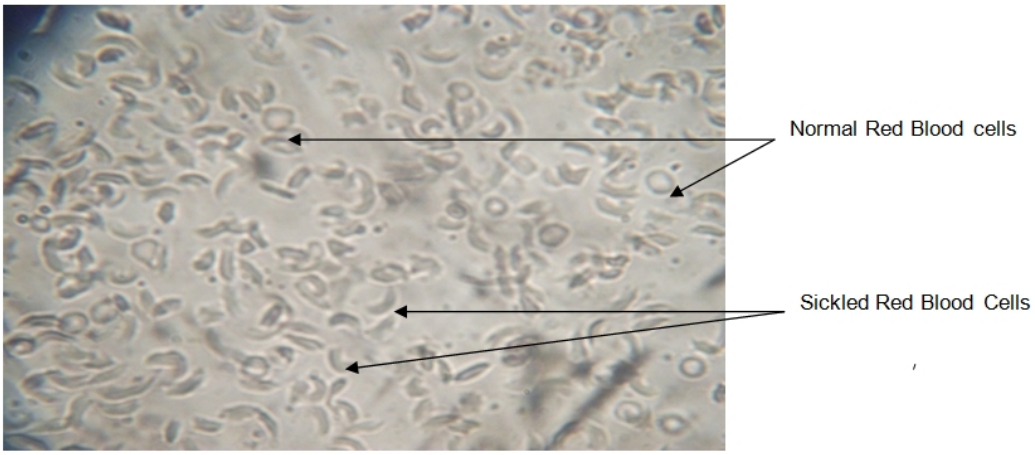


**Plate 1. Untreated control showing sickled red blood cells**

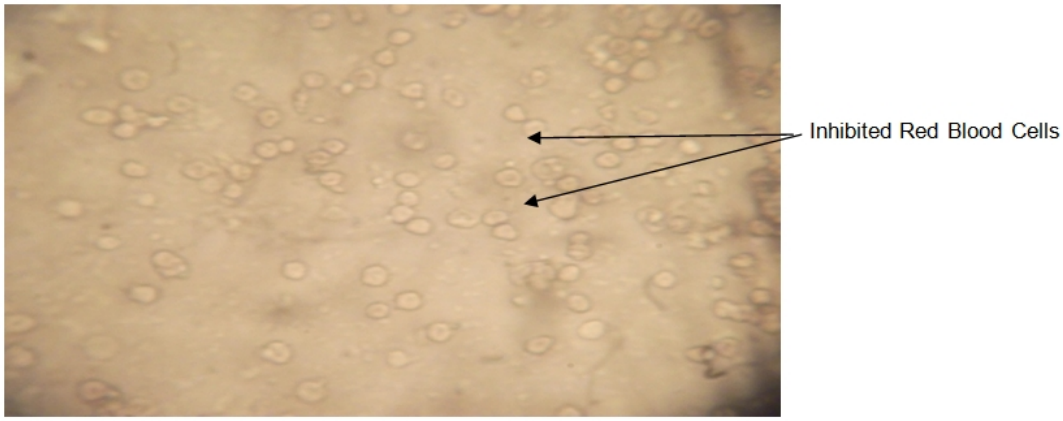


**Plate 2. Inhibitory activity (90.19 %) after treatment with Cpret at 4mg/ ml**

The relative reversal activities of the leaf and root extracts of the two *Calliandra* species were compared and presented in Figs. 5-8. The result indicated that all the extracts showed a significantly (P< 0.001) increase in antisickling activity as the concentration increases from 0.5 mg/ml – 4 mg/ml. The ethanolic leaf extracts (Cplet and Chlet) of the two species exhibited better reversal of sickling activity at varying concentrations compared to the aqueous leaf extracts (Cplaq, Chlaq and Cplet) possessed the highest reversal of the sickling activities of HbS to normal red blood cells shape (87.59%). However, the activity of the root extracts of *C. portoricensis* was found to be more pronounced than *C. haematocephala* with ethanolic root extract (Cpret) given higher antisickling activity. Both extracts from *C. portoricensis* seemedto have higher reversal activity in comparison with that of *C. haematocephala.* Thus Cpret (ethanolic root extract) was the most active with 92.63% reversal ability compared to the PHBA (positive control) which was 90.86%.

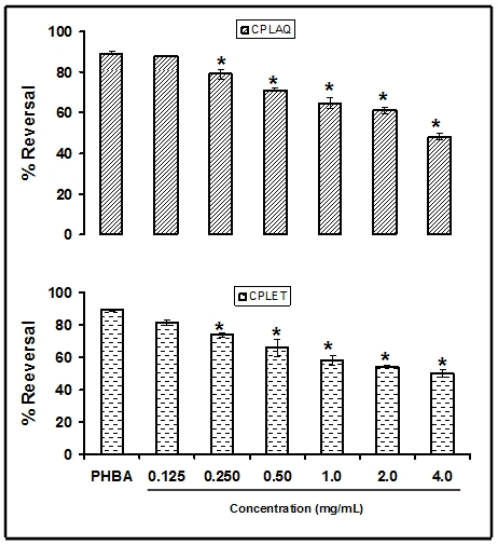


**Fig. 5. The reversal activities of the ethanol (CPLET) and aqueous (CPLAQ) leaf extracts of *Calliandra portoricensis* when compared with Para-hydroxybenzoic acid (PHBA) [Control]. *p<0.001 when compared with the control (PHBA)**

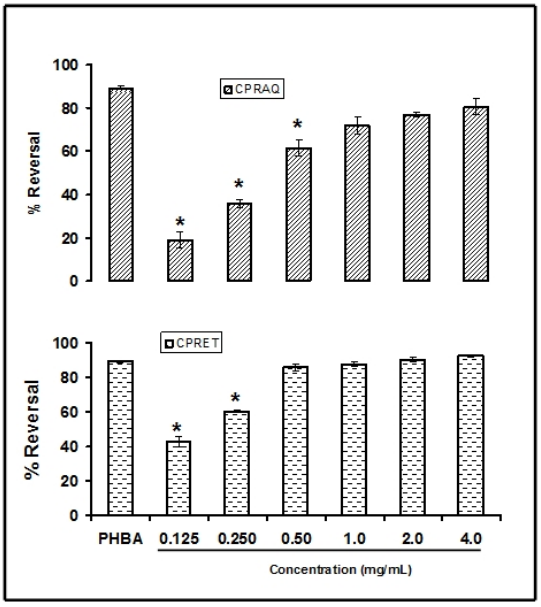


**Fig. 6. The reversal activities of the ethanol (CPRAQ) and aqueous (CPRET) leaf extracts of *Calliandra portoricensis* when compared with Para-hydroxybenzoic acid (PHBA) [Control]. *p<0.001 when compared with the control (PHBA)**

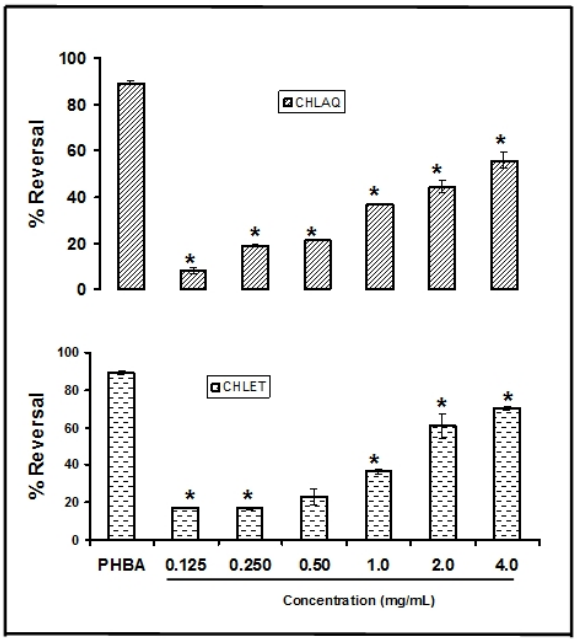


**Fig. 7.** Reversal activities of ethanolic and aqueous leaf extracts of ***C. haematocephala*** **when compared with Para-hydroxybenzoic acid (PHBA) [Control]. *p<0.001 when compared with the control (PHBA)**

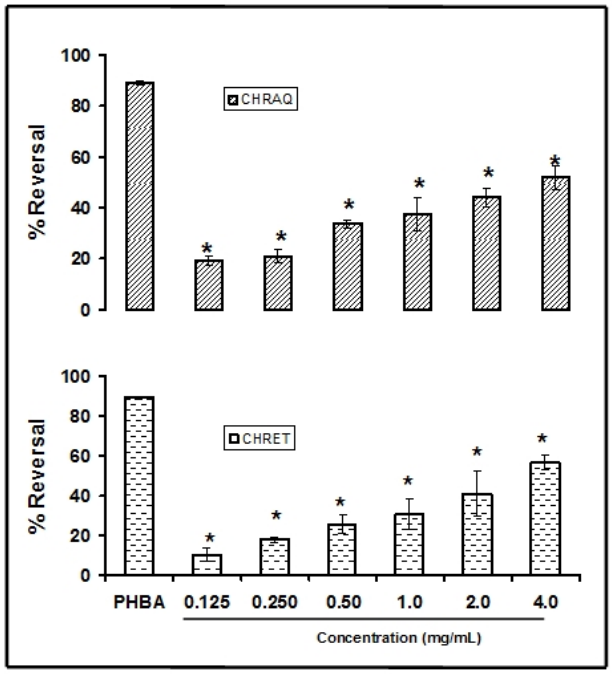


**Fig. 8**. R**eversal activities of ethanolic (CHRET) and aqueous (CHRAQ) root extracts of *C. haematocephala* when compared with Para-hydroxybenzoic acid (PHBA) [Control]. *p<0.001 when compared with the control (PHBA)**

Plates 2a and 2b showed the photomicrograph of red blood cells (HbSS). The untreated control showed high percentage of sickled cells under hypoxial condition while plate 2b showed the reversed normal spherical shaped RBC after treatment with the ethanolic root extract of *Calliandra portoricensis* (Cpret).

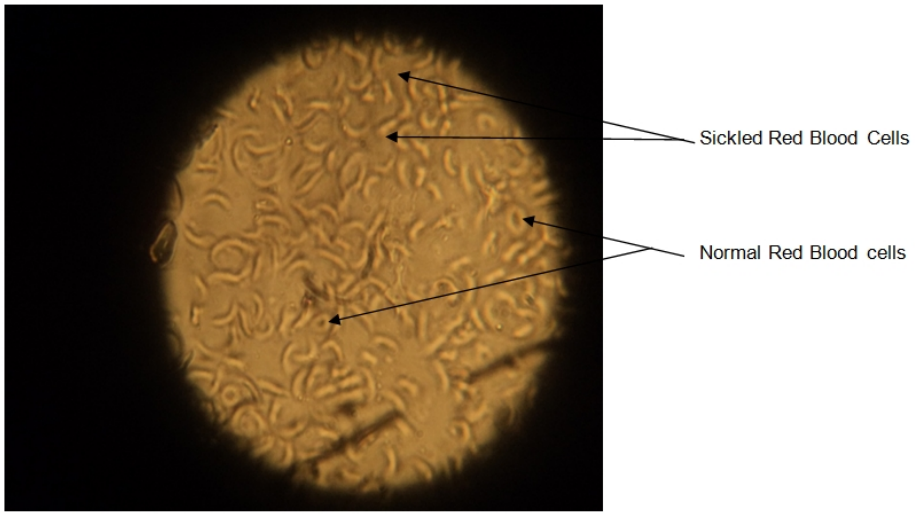


**Plate 2a. Untreated control showing sickled red blood cells**

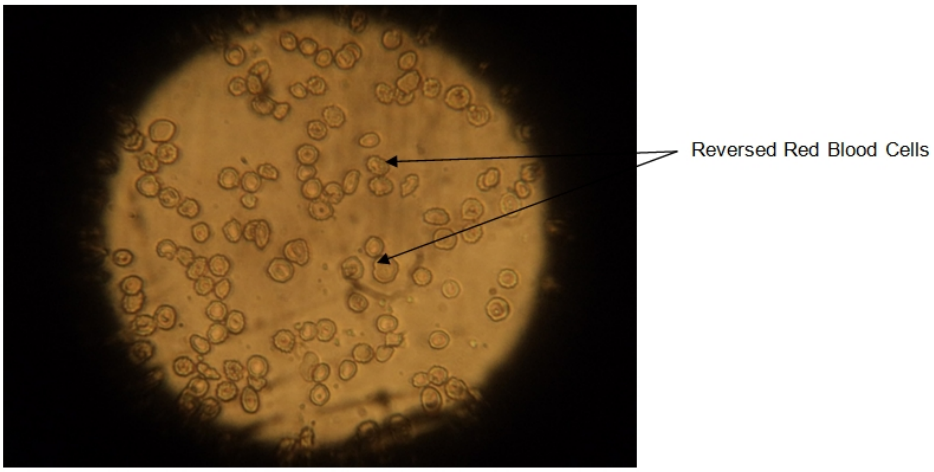


**Plate 2b. The reversed sickled red blood cells on treatment with Cpret with a reversal activity of 92%**

This study showed that the putative antisickling compound(s) were hydrophilic in nature. Both extracts still showed very good activities in the inhibitory and reversal tests. The antisickling assays of the leaves and roots of the two *Calliandra* species indicated that both the inhibitory and reversal activities of ethanolic and aqueous extracts had a linear correlation in activity. Generally, as the concentration increases, the activities also increased significantly ($P< 0.001$). It was observed that both the ethanolic and aqueous the leaf extracts of *C. portoricensis* had a weak reversal of sickling activity as the concentration increases (Fig. 5) unlike the results obtained for reversal activity of both root extracts of *C. portoricensis* and the extracts of either root or leaf of *C. heamatocephala*.

In this experiment sickling condition was potentiated by low oxygen levels, in the medium due to the addition of Sodium metabisulphite. increased acidity and dehydration of the blood are also implicated in the causation of sickling of HbS. The constituents of the aqueous and ethanolic extracts of the plant are highly oxygenated due to the presence of poly hydroxyl constituents of the plant such as Gallic acid, methyl gallate, myricetin, quercetin, myricetin 3-*O*-â-D-4C1-glucopyranoside, afzelin, isoquercetin, myricetin 3-*O*-(6''-*O*-galloyl)-â-D-glucopyranoside, myricetin 2''-*O*-gallate, quercetin 2''-*O*-gallate, afzelin 2''-*O*-gallate, myricetin 3''-*O* gallate,afzelin 3''-*O*-gallate (17), 1,2,3,4,6-penta-*O*-galloyl-â-D- 4C1-glucopyranose, myricetin 2'',3''-di-*O*-gallate, quercetin 3- *O*-methyl ether [16]. Hence they have the potential of possessing antioxidant properties. Caffeic acid which is reported to be present in the plant was known to exhibit antioxidant property *in vitro* and *in vivo* and also possess immunomodulatory and anti-inflammatory activities [21]. This is similar to p-hydroxybenzoic acid earlier reported to be the active antisickling principles of *Zanthoxylum xathoxyloides* Waterm [22].

The presence of these compounds in *Calliandra* could be responsible for the observed antisickling activity. Similarly the presence of Gallic acid and methyl 3, 4, 5-Trihydroxy benzoic acid found in the plant could also contribute to the antisickling property of the plant because gallic acid has been reported to protect human cells against oxidative damage. This indicates that the *Calliandra* may indeed have a great potential in the management of sickle

cell disorder. This study has confirmed the antisickling properties of the two plants species. However, the more active plant has been identified as *C. portoricensis*.

## 4. CONCLUSION

Species of the plant genus, *Calliandra* especially *C. portoricensis* can be regarded as a potential antisickling plant that can play essential role in the management and treatment of sickle cell disorder.

## CONSENT

Not applicable.

## ETHICAL APPROVAL

However no human subject was used in this study, sickle cell blood of patients attending sickle cell clinic of the Obafemi Awolowo University Teaching Hospital Complex Ile – Ife, Nigeria were used after seeking and obtaining the approval and consent of the authority of the Hospital.

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## COMPETING INTERESTS

The authors wish to declare that there is no existence of any competing interest in the design or execution of this research

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