European Journal of Medicinal Plants

24(1): 1-10, 2018; Article no.EJMP.41202
ISSN: 2231-0894, NLM ID: 101583475

Evaluation of the Effects of *Clerodendrum polycephalum* Baker Leaf Extracts on Sickle Red Blood Cells

Mojisola C. Cyril-Olutayo[1*], Francis A. Adewoyin[1] and Ayodele O. Ogunyemi[1]

[1]Drug Research and Production Unit, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author MCC designed the study, wrote the protocol, supervised the work and managed the analyses of the study. Author FAA designed the study, provided the materials and supervised part of the work and Author AOO performed the experiments, managed the literature searches and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2018/41202
Editor(s):
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Complete Peer review History: http://www.sciencedomain.org/review-history/25037

Original Research Article

Received 12th March 2018
Accepted 29th May 2018
Published 7th June 2018

ABSTRACT

The mortality rate for people living with sickle cell disease is high and relatively a few patients among them reach adult life, even with a high standard of medical care. Clinical manifestations of the sickle cell disease are diverse and vary, falling into three major categories: anaemia, pain related issues and organ failure. Sickle cell crises or painful episodes may be caused by blood vessel occlusion, damaged organ, triggered by membrane deformation. Patients in West Africa, where sickle cell anaemia (SCA) is prevalent, have for ages been treated with natural products especially herbs as it is still the case in rural communities. The medicinal plant *Clerodendrum polycephalum* used for this study is used ethno medically in treating malaria and pains associated

**Corresponding author: E-mail: mojiolutayo@gmail.com;*

with sickle cell disease. The leaves were collected, oven dried and macerated in methanol for 72 h. The extract was dried and reconstituted in distilled water to give concentrations 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 4 mg/mL. The methanol extract was further fractionated into solvents of varying polarity viz: n-hexane, dichloromethane, ethyl acetate and water. All extracts and fractions were tested for their antisickling properties using the inhibitory and reversal models. Vanillic acid and p-hydroxybenzoic acid were used as the positive controls for the inhibitory and reversal assays respectively. An attempt was made to identify the different classes of compounds present in the extract by using thin layer chromatography technique.
The methanol extract of *C. polycephalum* gave 55.9% inhibitory and 65.63% reversal activities at 4 mg/mL. The antisickling activities were dose-dependent and purification significantly ($p<0.05$) enhanced the reversal activity which was indicated in the polar fractions. This study authenticated the use of *C. polycephalum* in the management of pains associated with sickle cell disorder.

Keywords: Antisickling; Clerodendrum polycephalum; polymerization.

1. INTRODUCTION

Sickle cell anaemia is a hereditary disease which stems from the inheritance of mutant haemoglobin genes from both parents [1]. It is a blood-related disorder that affects the shape of the haemoglobin molecule and causes the entire blood cell to change shape under deoxygenated condition [2]. The use of natural products in attempts at inhibiting sickling could be as old as when the sickle cell disease (SCD) was discovered. Folkloric history has indicated attempts made by inhabitants using plant-derived recipes in parts of Nigeria to treat what they described as “fever of crises”, shifting joint pains, exacerbations especially during rainy seasons and “constant abnormality of the blood,” though relatively few have been validated scientifically [3]. *Clerodendrum polycephalum* is a shrub (family Lamiaceae) used traditionally as a painkiller in most western part of Africa. They usually grow up to 1–12 m tall, with opposite or whorled leaves and its common names include glory bower, bag flower and bleeding-heart [4, 5]. Studies also showed that the plant was used as an antidote for venomous stings, bites, as painkiller and medicine for the treatment of paralysis, epilepsy, convulsion, spams. In spite of the ethnomedicinal importance of this plant, very little information is available on the plant in literature. The leaf sap was known to cure paralysis, epilepsy and also in the treatment of convulsion and spasm. The leaf aqueous extract is traditionally used by people of North East India to alleviate symptoms of diabetes, obesity and hypertension [6]. In Southern Nigeria as well as Eastern Cameroun, the leaf sap is used to wash the face of persons subject to fainting, giddiness and attacks of epilepsy [7]. The powder or paste form and the various extracts of the root, stem and leaves are reported to be used as medicine for the treatment of asthma, pyreticosis, cataract, malaria and diseases of blood, skin and lung. Anti-inflammatory, anti-oxidant, antimalarial, antimicrobial, antitumor, anti-diabetic and anti-diarrheal activities have been investigated and reported in the literature in order to prove their ethnomedical uses [8,9]. The *in vivo* antiplasmodial activity and haematological uses were also investigated by Adewoyin *et al.* [10]. The leaf extract of *C. polycephalum* is given to sickle cell patients to treat malaria and to ameliorate pains during the crisis. This study therefore aims at determining the antisickling property of *Clerodendrum polycephalum* leaf extracts and fractions with a view to authenticate its use in the management of pains in sickle cell disease.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

Clerodendrum polycephalum leaves were collected at the medicinal garden of Drug Research and Production Unit (DRPU) in the Faculty of Agriculture Teaching and Research Farm on the Obafemi Awolowo University Campus in January 2017. It was authenticated at Botany Department, Obafemi Awolowo University, Ile-Ife and a voucher specimen was deposited with reference number IFE 16962.

2.2 Collection of Blood

Blood samples (which would have been otherwise thrown away) collected from confirmed sickle cell anaemia patients in steady state [11], who attend weekly routine check-up at Obafemi Awolowo University Teaching Hospital Complex (OAUTHC) in the Haematology out-patient clinic were used. Ethical approval was obtained for the

use of the leftover blood samples. Samples were always used within 48 h of collection.

2.3 Preparation of Extract

C. polycephalum leaves were oven dried at 40°C for 72 h, grounded into fine powder, weighed and macerated in methanol using an electric shaker for 48 h. The menstruum was filtered and the resulting filtrate concentrated to dryness *in vacuo* using a rotary evaporator at 40°C. The extract was finally freeze-dried to bring it to complete dryness. An aliquot 40 mg of the dried extract was reconstituted in 10 mL of distilled water to obtain the 4 mg/mL concentration. Serial dilutions were made to obtain 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL concentrations used for the antisickling assay.

2.4 Antisickling Assay

2.4.1 Inhibitory assay

In triplicates, 0.2 mL of the blood sample, 0.2 mL of phosphate buffered saline and 0.2 mL of different concentrations of the test extract (4, 2, 1, 0.5 and 0.25 mg/mL) were mixed together in a test tube. The mixture was overlaid with 1mL of liquid paraffin to prevent oxygenation. The mixture was then incubated in a thermostated water bath at 37℃ for 4 h. Freshly prepared 2% w/v sodium metabisulphite solution (0.6 mL) was carefully added to the mixture under paraffin and mixed gently by rolling the test tube between the two palms. The mixture was then incubated for another 1 h 30 mins at 37℃ in a water bath. At the end of the incubation period, the liquid paraffin was carefully removed using Pasteur pipette and the reaction mixture was fixed with 3 mL of 5% v/v buffered formalin solution [12]. Vanillic acid 4 mg/mL was used as positive control.

2.4.2 Reversal assay

In triplicates, 0.2 mL blood sample and 0.2 mL phosphate buffered saline were mixed together in a test tube, 1 mL liquid paraffin was placed on top of the mixture and 0.6 mL of 2% sodium metabisulphite solution was carefully introduced under the paraffin. The mixture was then incubated at 37℃ for 1 h 30 mins. At the end of the incubation period, 0.2 mL of the extract at different concentrations (4, 2, 1, 0.5 and 0.25 mg/mL) was carefully added under the liquid paraffin and incubated at 37℃ for another 6 h. The liquid paraffin layer was removed with a Pasteur Pipette and the remaining liquid fixed with 3 mL of 5% v/v buffered formalin solution [12]. p-hydroxybenzoic acid (PHBA) 4 mg/mL was used as positive control.

Slides were prepared from the fixed cells after centrifugation. An aliquot of the fixed cells was taken with the use of capillary tube, dropped on a microscope slide and carefully covered with a coverslip. The slide was placed under a bright field microscope and red blood cells (400 sickled and un-sickled erythrocytes) were counted at x400 magnification. The half maximal inhibitory concentration (IC_{50}) was determined and the percentage of sickled red blood cells was calculated as shown below.

$$\% \text{ Sickled cells} = \frac{\text{No of Sickled cells}}{\text{Total no of cells}} \times 100$$

The percentage inhibition/reversal was thereafter calculated from the per cent sickled cells using the formula:

% Inhibition or Reversal = (% Sickled cells of control- % Sickled cells of sample / % Sickled cells of control) x 100

2.5 Preparation of Fractions

The Crude methanol extracts of *C. polycephalum* was fractionated using Vacuum Liquid Chromatography (VLC) with solvents of increasing polarity viz: N-hexane, Dichloromethane (DCM), ethyl acetate and water. Each fraction was dried *in vacuo* and reconstituted in 1% Tween 80 for the antisickling assays.

2.6 Phytochemical Analysis

The crude methanol extracts of *C. polycephalum* was dissolved in methanol for phytochemical analysis. The extract was spotted on a pre-coated silica gel_{254} Thin Layer Chromatography plate (TLC) and developed using DCM: Ethanol 3:2/7:3 solvent system. The plate was viewed under the UV light long wavelength (366 nm) and the distance moved by the spots and colours were noted. The TLC plate was thereafter sprayed with vanillin-sulphuric acid and heated on a hot plate for 1-2 mins [13].

2.7 Statistical Analysis

Each test was performed in triplicates and the results were expressed as mean values and standard deviation (± SD). Results were

subjected to analysis of variance (ANOVA) using SPSS and the level of significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Antisickling Inhibitory Activity of the Methanol Extract

The inhibitory activity of the methanol extract of *C. polycephalum* leaf increases with increased concentrationty.

3.2 Reversal Activity of the Methanol Extract

The methanol extract of *C. polycephalum* leaf, however, showed a significantly higher reversal activity.

3.3 Fractionation of the Leaf of *C. polycephalum*

The yield of each fraction obtained from the fractionation of 14 g of the crude methanol extract of *C. polycephalum* is presented.

The fractions showed low inhibitory activities at 4 mg/mL as shown in Table 4.

This study evaluates the inhibitory and reversal antisickling properties of the crude methanol extract of the leaves of *C. polycephalum* as well as the different fractions at various concentrations. The results showed that the extract could inhibit the sickling of Hb S red blood cells as well as reverse sickled cells to their normal round shape under low oxygen tension. The inhibitory activity of the methanol extract of *C. polycephalum* leaf showed a dose-dependent activity with the lowest activity at 0.5 mg/mL (35.46%) and the highest at 4 mg/mL (55.59%) concentrations. The IC_{50} for the inhibitory activity was evaluated to be 2.02 mg/mL (Fig. 1). Vanillic acid (positive control) showed a significantly $p < 0.05$ higher inhibitory property (92.47%) than the crude extract at the same concentration. Figures 2-5 showed the photomicrograph of blood films before and after treatment with Vanillic acid and *C. polycephalum* at 4 mg/mL. The fractions of the methanol extract, on the other hand, gave low inhibitory activities (Table 3), this implies that purification did not enhance the inhibitory activity of the extracts. The chemical constituents of

Table 1. Inhibitory activities of the methanol extract of *C. polycephalum* leaf at different concentrations

Concentrations (mg/mL)	% Sickled	% Inhibition
0.25	58.39 ± 1.81	35.46
0.5	56.63 ± 2.30	37.41
1.0	41.30 ± 0.80	54.35
2.0	40.24 ± 0.72	55.52
4.0	40.18 ± 0.82	55.59
Positive control (Vanillic acid 4 mg/mL)	9.17 ± 0.12	90.47

Key: Mean ± Standard deviation of triplicate analysis

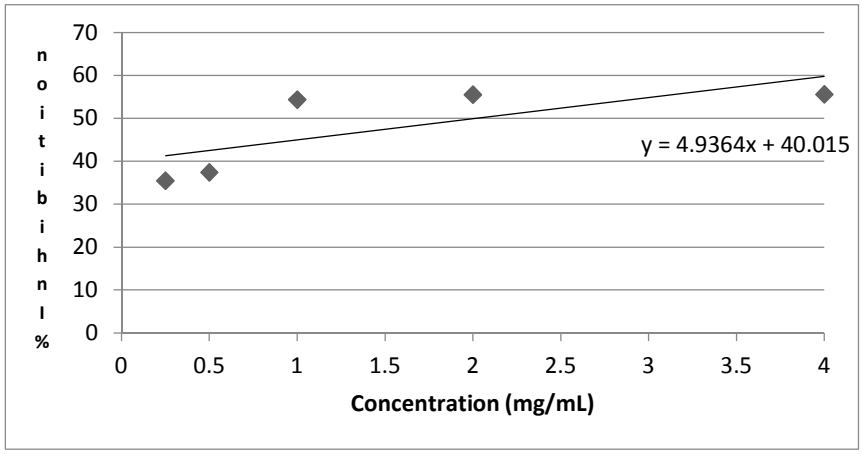

Fig. 1. The IC_{50} of the Inhibitory activity of the methanol extract of *C. polycephalum*

C. polycephalum obviously work in synergy to effect the inhibitory property observed on the Hb S red blood cells. *In vitro* de-oxygenation of RBC by sodium metabisulphite caused progressive aggregation and polymerization of the individual molecules [14] and this process of gelation of haemoglobin molecules increase the formation of sickled erythrocytes (Fig 3). The extract was able to retard the progression of aggregation and polymerization thereby inhibiting the formation of sickle cells.

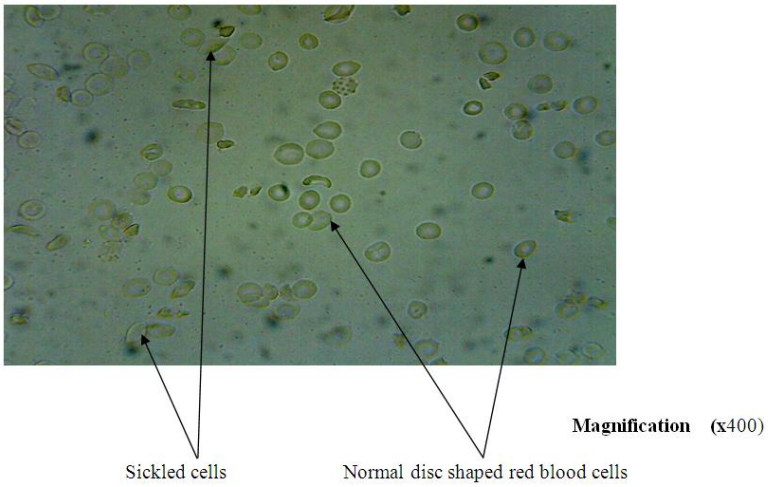

Fig. 2. Photomicrograph of sample blood film of an Hb SS patient in steady state

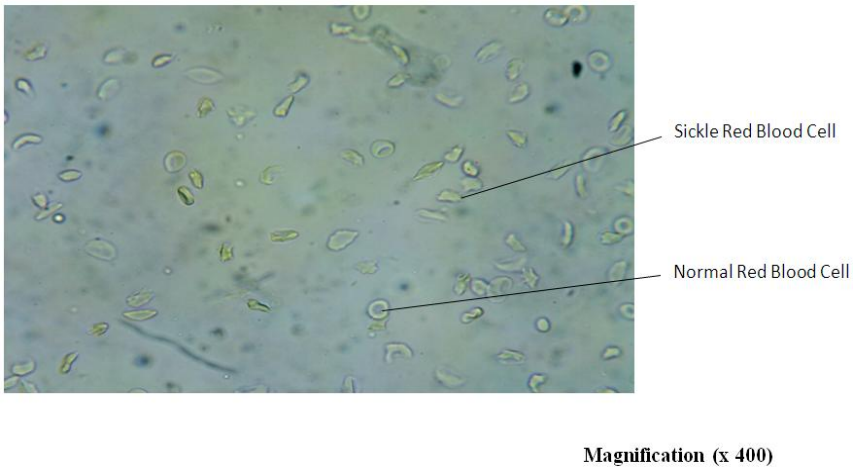

Fig. 3. Photomicrograph of Hb S red blood cells after sodium metabisulphite-induced deoxygenation

Table 2. Reversal activity of the crude methanol extract of *C. polycephalum*

Concentration (mg/mL)	% Sickled	% Reversal
0.25	60.7 ± 0.41	32.91
0.5	59.43 ± 0.21	34.32
1.0	48.13 ± 0.62	46.80
2.0	33.26 ± 0.76	63.23
4.0	31.09 ± 0.15	65.63
PHBA (positive control) 4 mg/mL	18.27 ± 0.45	76.97

Key: Mean±Standard deviation

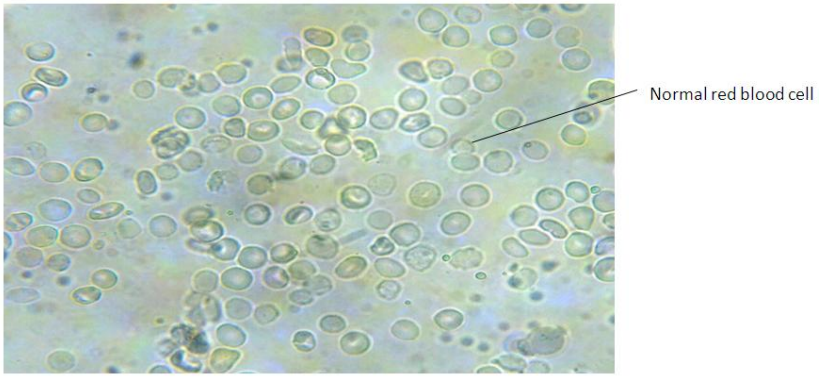

Fig. 4. Photomicrograph showing 90.43% Inhibitory activity of Vanillic acid (positive control) on Hb S red blood cells at 4 mg/mL

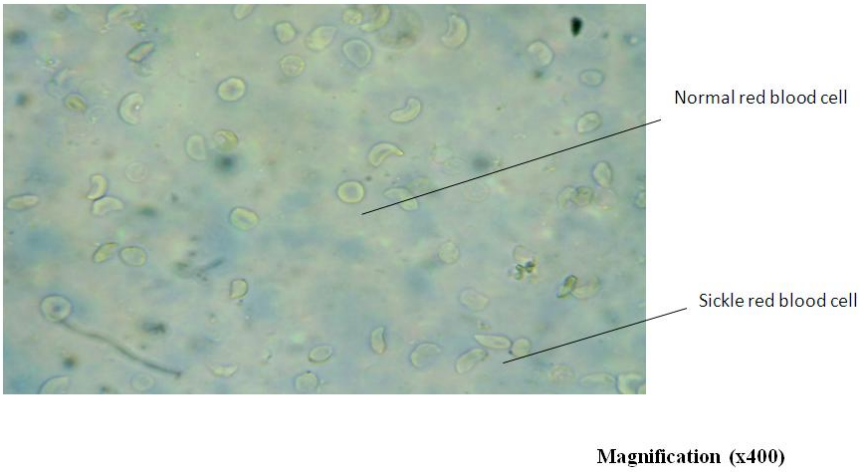

Fig. 5. Photomicrograph of 55.59% Inhibitory activity of the methanol extract of *C. polycephalum* leaf on Hb S red blood cells at 4 mg/mL

Table 3. The yield of the fractions obtained from the crude extract of *C. polycephalum*

Fraction	Yield (g)	Yield (%)
n-hexane	5.18	37.0
DCM	1.75	12.46
Ethyl acetate	0.83	5.96
Aqueous	6.01	43.15

The methanol extract of *C. polycephalum* leaf, however, showed a significantly higher reversal activity. Reversal activity increased with concentration (i.e. activity at 4 mg/mL > 2 mg/mL > 1 mg/mL > 0.5 mg/mL > 0.25 mg/mL (Table 2). At 4 mg/mL, *C. polycephalum* gave a reversal effect of 65.63% with IC_{50} of 1.71 mg/mL (Fig 6). Figures 7 and 8 showed the photomicrographs of the activities of p-hydroxybenzoic acid, positive control, and *C. polycephalum* respectively at 4 mg/mL. Reversal activity of the extract was enhanced by purification. The polar fractions (ethyl acetate and aqueous) were more active than the non-polar. The aqueous fraction gave the highest reversal activity (71.02%) followed by ethyl acetate fraction (62.01%). DCM fraction gave 42.11% activity while the n-hexane gave the least activity of 37.87%. It can be inferred that the components of *C. polycephalum* responsible for its reversal activity were polar compounds extracted in aqueous and ethyl acetate fractions (Table 4). There is no significant difference ($p<0.05$) between the activity of the aqueous fraction and PHBA. Figs. 9 and 10 showed the photomicrographs of the

Hb S red blood cells treated with the aqueous and ethyl acetate fractions respectively. The inhibitory and reversal antisickling activities of the extracts and fractions could be due to the presence of some bioactive compounds present in the extracts. The classes of compound present in the methanol extract of *C. polycephalum* leaf were determined using thin layer chromatography (TLC). Flavonoids appeared as orange spots, terpenoids produced purple reactions and phenolic compounds appear as a yellow colour. The compounds detected in the extract were in accordance with the report of [15,16] who reported the presence of sesquiterpenes and monoterpene hydrocarbons in *C. polycephalum* oil.

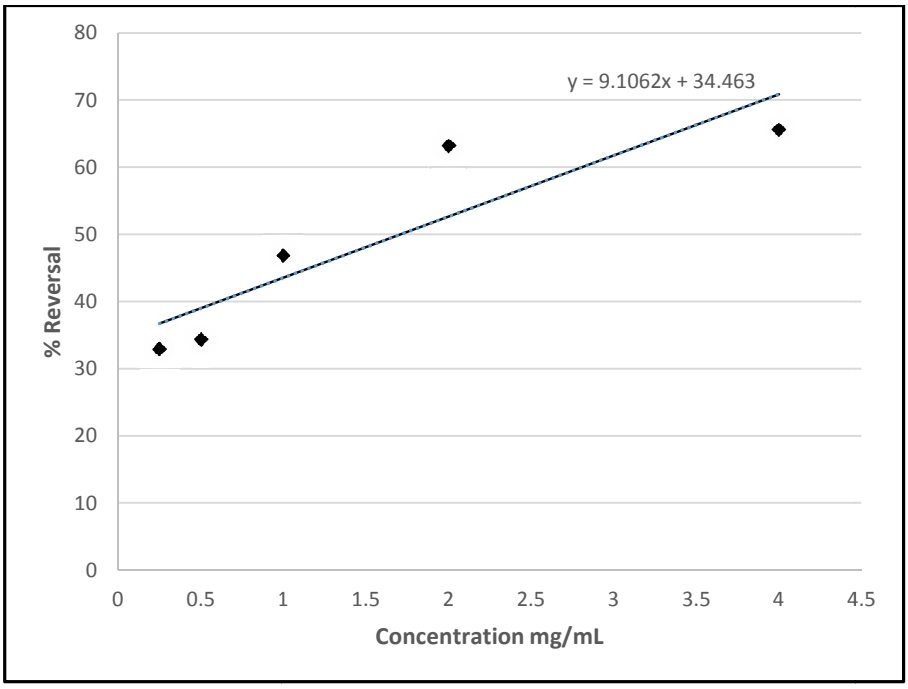

Fig. 6. The IC_{50} of the reversal activity of the methanol extract of *C. polycephalum* leaf

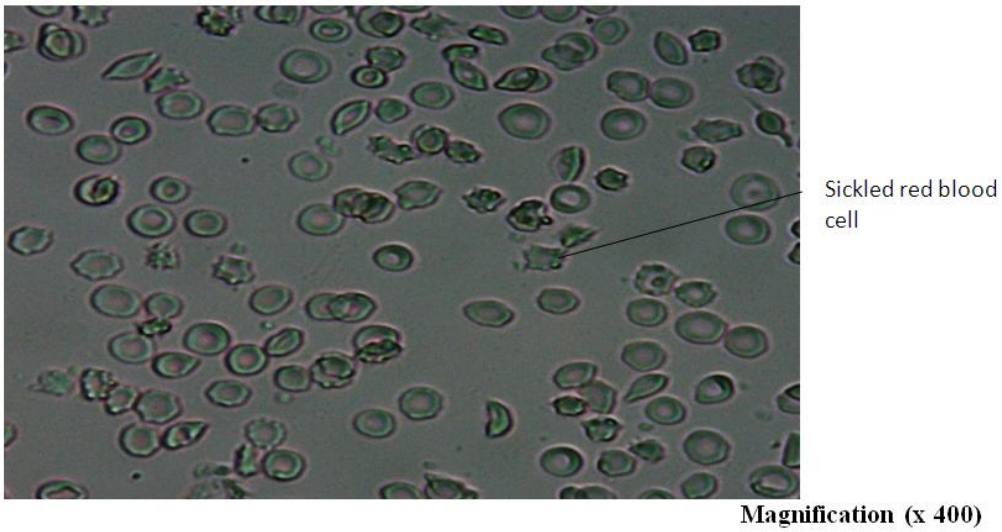

Fig. 7. Photomicrograph of 78.97% reversal activity of PHBA the positive control at 4 mg/mL

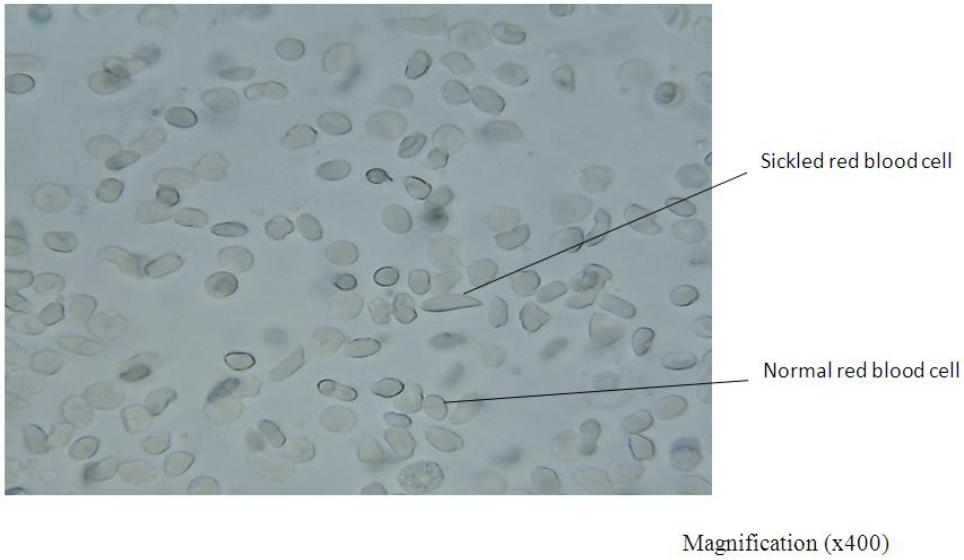

Fig. 8. Photomicrograph of the reversal activity (65.63%) of methanol extract of *C. polycephalum* on Hb S red blood cells at 4 mg/mL

Table 4. Inhibitory and Reversal antisickling activities of the different fractions of the methanol extract of *C. polycephalum*

Fractions (4 mg/mL)	% Inhibition	% Reversal
n-hexane	38.29± 3.06	37.87±0.15
DCM	31.03± 1.66	40.11±0.21
Ethyl acetate	25.82± 2.01	62.01±1.14
Aqueous	38.31± 2.29	71.02±0.19
Vanillic acid (Positive control)	90.43± 0.65	-
p-hydroxybenzoic acid	-	76.97± 0.45

Key; Mean ± standard deviation of triplicate analysis

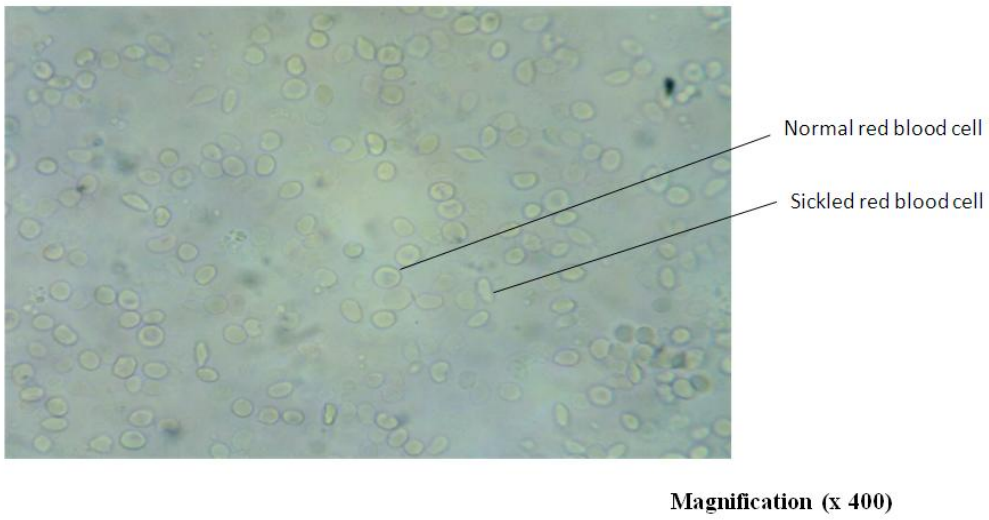

Fig. 9. Photomicrograph of the reversal activity (71.02%) of the aqueous fraction of *C. polycephalum* on Hb S red blood cells at 4 mg/mL

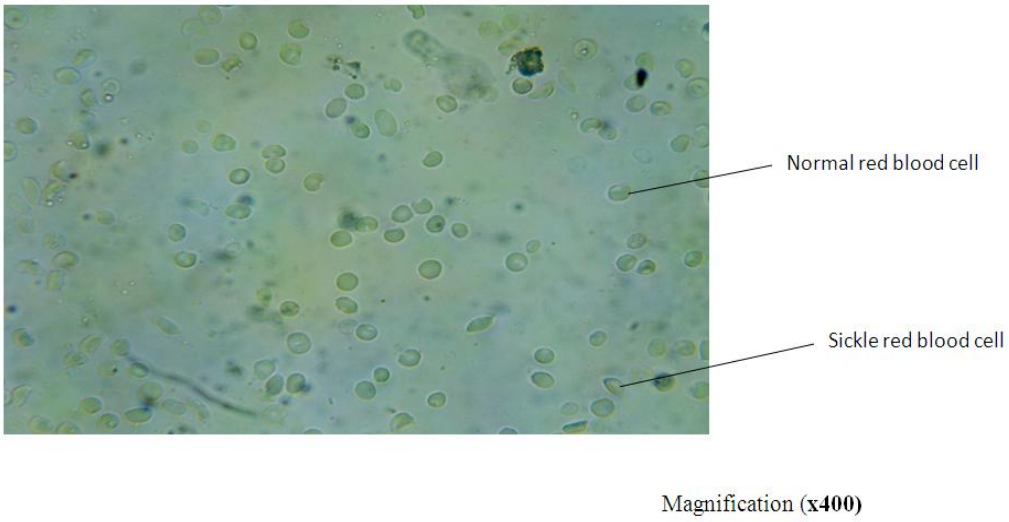

Fig. 10. Photomicrograph of the reversal activity (62.01%) of the Ethyl acetate fraction of *C. polycephalum* at 4 mg/mL

4. CONCLUSION

Pain in sickle cell disease is caused by sickling of red blood cells and the administration of the extract of *C. polycephalum* to Hb SS patients relieves pains. This study showed that the extract works by both preventing and reversing sickling. The constituents implicated are polar compounds although this study did not determine the putative constituents of the extract. It, however, authenticated the use of the *C. polycephalum* in the management of pains associated with sickle cell anaemia. The administration of the extract during crisis probably re-oxygenates red cells, restores the shape and deformability of the cells thereby reducing pain. The exact mechanism of action of the extract needs to be investigated.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/25037