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The Anti-Sickling and Oxidative Properties of Ethanol Leaf Extract of *Ocimum gratissimum* L. (Scent Leaf)

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Abstract

This study evaluated the anti-sickling potential of the ethanol leaf extract of *Ocimum gratissimum* using microscopy-based assays. The extract markedly reversed 2% sodium metabisulfite–induced sickled red blood cells to normal biconcave forms, and microscopic observations indicated that the percentage reversal of sickled cells was both concentration- and time-dependent. At 250 µg/mL, the percentage reversal of sickled cells at 0, 20, and 30 minutes was 77.00%, 72.31%, and 71.77%, respectively, whereas the control (absence of extract) showed no reversal. At 500 µg/mL, the corresponding reversals were 76.19%, 69.60%, and 74.00%, while at 1000 µg/mL they were 71.49%, 75.92%, and 87.70%, respectively, demonstrating enhanced anti-sickling activity at higher extract concentrations over time. UV–Visible spectrophotometric analysis of oxyhaemoglobin revealed a gradual increase in the 630 nm band, consistent with the oxidation of ferrous (Fe^{2+}) oxyhaemoglobin to ferric (Fe^{3+}) methaemoglobin; the absorbance peak at 650 nm, attributable to chlorophyll, was clearly distinguishable from the haemoglobin oxidation band at 630 nm. In addition, the ethanol leaf extract of *O. gratissimum* exhibited a stabilizing effect on erythrocyte haemolysis.

Phytochemical analysis showed the presence of aromatic and alkylic compounds, which may contribute to intracellular anti-sickling activity by interfering with haemoglobin S polymerization and, through their hydrophobic properties, may also modulate the erythrocyte lipid bilayer membrane.

Keywords: *Ocimum gratissimum*; Anti-Sickling Activity; Erythrocyte Stability; Haemoglobin Oxidation; Phytochemical Constituents.

INTRODUCTION

Sickle cell disease is a debilitating genetic disease that affects over 12 % of Africans and people of African descent (Bhawnani & Yadav, 2023). It is caused by the production of abnormal haemoglobin S due to a mutation in the beta subunit of haemoglobin where valine replaces the normal amino acid glutamic acid at the sixth residue (Ma *et al.,* 2023). This initiates the polymerization of deoxyhaemoglobin S red blood cells, making the blood cells to become rigid and become trapped in small blood vessels, blocking the capillaries and microvasculatures and deprive organs from getting oxygen which could lead to crisis and death (Ballas, 2005). Most conventional drugs used to manage sickle cell disease such as hydroxyurea are costly and have side effects. In developed countries such as the United States of America, an advanced stage of management is the use of bone marrow transplant which involves transplanting the normal beta globin gene into the marrow of the sickle cell patient and generation of normal erythrocytes which can lead to cure. The cost of carrying out bone marrow transplant in Nigeria or Africa in general is enormous, consequently there is need for alternative cheap means of managing sickle cell disease. There has been a growing interest in exploring therapeutic strategies that target oxidation stress pathways to better the clinical complication associated with SCD (Mukherjee et al, 2022). Medicinal plants have been useful and effective in treating many ailments in Africa (Fennel *et al.,* 2004).

Research has shown that sickle cell disease can be managed with crops such as *Cajanus cajan* which is rich in the amino acid phenylalanine (Ekeke and Shode, 1990). Testing for the anti-sickling potentials of many plants will lead to discovering those with anti-sickling potentials. *Ocimum gratissimum* (scent leaf) is a common culinary herb in West Africa (Faluyi, 2020). It is used as a spice in cooking pepper soup, rice, beans, plantain and

other delicacies. The aim of this research work is to investigate the anti-sickling potentials and oxidative properties of the ethanol extract of leaves of *Ocimum gratissimum.*

MATERIALS AND METHODS

The fresh leaves of *Ocimum gratissimum* were purchased from a reputable vendor from Nsukka local market at Enugu state. Extraction of the leaves of *Ocimum gratissimum* was done using the method of Cyril Olutayo *et al.* (2018). The leaves of *Ocimum gratissimum* were air-dried, then chopped into tiny pieces and ground to powder with a lab mortar. Twenty (20) grams of the leaf powder was mixed with 100 mL of 70 % ethanol. The solution was sieved and filtered using filter paper and funnel. The filtrate was concentrated using a water bath at 50^0C to eliminate the ethanol and then stored in a refrigerator.

Haemoglobin A and S were obtained from blood drawn from two volunteers whose genotypes were ascertained by cellulose acetate electrophoresis at pH 8.6. The red blood cells were washed three times with 1 % normal saline by centrifugation at 4000 rpm and the serum (supernatant) was discarded with the aid of a Pasteur pipette. The red blood cells at the bottom of the test tubes were used for the microscopic study. The remnant of the red blood cells were lysed with distilled water to get the haemolysate. Sephadex G-25 chromatography was used to extract the haemoglobin from the haemolysate, eliminating membrane aggregate and other substances present.

Microscopic investigation of the anti-sickling properties of ethanol leaf extract of *Ocimum gratissimum* was carried out with a bright field microscope at x40 lens magnification. A drop of 2 % sodium metabisulfite was added to a drop of SS red blood cells. The role of the 2 % sodium metabisulfite was to deoxygenate the red blood cells through conversion of oxyhaemoglobins to deoxyhaemoglobins and convert the cells to sickled shape. The mixture of the 2 % sodium metabisulfite and SS red blood cells were put on the slide of the microscope and a picture was taken as the control. To examine the anti-sickling potential of *Ocimum gratissimum* ethanol leaf extract, a drop of 2 % sodium metabisulfite and SS red blood cells were added to a drop of different doses of the plant extracts (250 µg/mL, 500 µg/mL, and 1000 µg/mL) on the slide and covered with a cover slip. The pictures of the slides containing the different concentrations of the *Ocimum gratissimum* ethanol leaf extract mixed with the deoxygenated SS red blood cells were taken at different time intervals, 0, 20 and 30 minutes. The treated sickle red blood cells were compared to the control. If many

of the sickle red blood cell shapes are converted to their normal spherical shape then the *Ocimum gratissimum* ethanol leaf extract was regarded as an anti-sickling agent.

UV-Visible spectroscopy was used to investigate the oxidative effect of *Ocimum gratissimum* ethanol leaf extract on oxyhaemoglobin. The instrument was set at the range of 400 nm and 700 nm. Normal oxyhaemoglobin spectra shows the presence of 3 peaks at 540 nm, 576 nm and 630 nm. The peaks at 540 nm and 576 nm represent the binding of oxygen to ferrous iron (Fe^{2+}). The 630 nm band reflects oxidation of oxyhaemoglobin to ferric (Fe^{3+}) or methaemoglobin which has lost its capacity to bind oxygen. 900 µL of potassium phosphate buffer was used as the baseline. The oxyhaemoglobin spectra was obtained by dissolving 100 µL of haemoglobin A and S in 900 µL of the same buffer and used as control. 100 µL of the haemoglobin solution was incubated with different concentrations of the *Ocimum gratissimum* ethanol leaf extract (250 µg/mL, 500 µg/mL, and 1000 µg/mL) and scanned at different time intervals. The maximum absorbance of the beta peak (576 nm) and the alpha peak (540 nm) were recorded. The absorbance at the 630 nm band that indicates oxidation was evaluated. The concentration of haemoglobin was determined using Beer/Lamberts law where concentration = A/ (εxb) (A= concentration, ε = extinction coefficient and b = pathlength of cuvette).

The ethanol leaf extract of *Ocimum gratissimum* was also tested for haemolysis of red blood cells. The experiment consisted of eleven test tubes with different volumes of saline and distilled water. 100 µL of SS red blood cells were added to each test tube as the control. The *Ocimum gratissimum* ethanol leaf extract was tested at its highest selected concentration (1000 µg/mL). 100 µL of the *Ocimum gratissimum* ethanol leaf extract was added to each test tube. UV-Visible spectroscopy was used to detect breakage of the red blood cells by the plant extracts and the concentration of the haemoglobin being released in the solution. Haemoglobin has two peaks in the UV-Visible region (540 nm and 577 nm). The 577 nm peak was selected and used to test how the red blood cells were breaking. The haemolysis curve was plotted with the control against the solution with the plant extracts.

The analysis of phytochemicals was performed on a BUCK M910 Gas chromatography equipped with a flame ionization detector.

RESULTS

Table 1: Phytochemicals present in ethanol leaf extract of *Ocimum gratissimum* (scent leaf)

Ret Time [min]	Area [pA *s]	Amt/Area	Amount [ppm]	Grp	Name
2.617	210.53412	1.40613e-1	29.60385	1	Kaempferol
3.055	129.44804	1.40510e-1	18.18869	1	Catechin
4.567	5.96399	1.39145e-1	8.29861e-1	1	Quercetin
4.768	6.57157	1.36998e-1	9.00292e-1	1	Genistein
6.160	7.40762	1.39420e-1	1.03277	1	Luteolin
6.379	37.41651	1.40004e-1	5.23845	1	Ferulic acid
7.069	4.36262	1.38633e-1	6.04803e-1	1	Artemetin
7.282	1.61948	1.34097e-1	2.17169e-1	1	Gallocatechin
7.459	6.36053	1.39149e-1	8.85062e-1	1	Flavone
7.630	14.59382	1.39772e-1	2.03981	1	Reveratrol
7.884	5.53773	1.38683e-1	7.67988e-1	1	Lunamarin
8.010	2.62720	1.37121e-1	3.60245e-1	1	Retusin
8.517	1.12811	1.31173e-1	1.47978e-1	1	Nobeletin
8.880	9.34233	1.39614e-1	1.30432	1	Ellagic acid
9.326	10.89357	1.39667e-1	1.52147	1	Tangeretein
9.742	3.83840	1.37955e-1	5.29527e-1	1	Epicatechin
9.905	130.06775	1.40536e-1	18.27924	1	Vanillic
10.130	5.21132	1.38379e-1	7.21139e-1	1	Hesperidin
10.295	9.28244	1.39424e-1	1.29419	1	Butein
10.425	1.65181	1.27541e-1	2.10675e-1	1	Apigenin
11.168	4.50330	1.38543e-1	6.23899e-1	1	Naringenin
11.477	5.26400	1.38728e-1	7.30262e-1	1	Myricetin
11.769	2.89167	1.37086e-1	3.96406e-1	1	Hesperidin
12.310	3.36374	1.37984e-1	4.64143e-1	1	Daidzin
12.563	2.44619	1.36842e-1	3.34740e-1	1	Isorhamnetin
13.038	1.87221	1.35265e-1	2.53243e-1	1	Maricetin
13.483	3.23720	1.37384e-1	4.44739e-1	1	Epicatechin
14.101	27.88656	1.40263e-1	3.91145	1	Daidzein
Totals:			**91.83641**		

The result presented in table 1 above indicates the phytochemicals present in ethanol leaf extract of *Ocimum gratissimum* (scent leaf) as detected using the Gas Chromatography- Flame Ionization Detector. 28 compounds were identified. Some of the compounds present are: Gallic acid, Rosmanol, Rosmarinic acid, Nepetrin, Quercetin, Rutin, catechin and Apigenin. Most of these compounds are aromatic and alkylic. These aromatic and alkylic compounds may show their intracellular anti-sickling activity by interfering with haemoglobin S polymerization. The aromatic and alkylic compounds may

also play an anti-sickling role through hydrophobic interactions on the erythrocyte lipid bilayer membrane.

Anti-sickling potential of ethanol extract of *Ocimum gratissimum* leaf

Results from figure 1 to 3 show that *Ocimum gratissimum* has significant anti-sickling property as it converted many of the sickled cells to normal red blood cells. The anti-sickling potential was dose dependent with 1000 µg/mL showing the best outcome with 87.70 % reversed sickling at 30 minute when compared to the control with 100 % sickled cells. Other concentrations (250 µg/mL and 500 µg/mL) also had significant anti-sickling potentials.

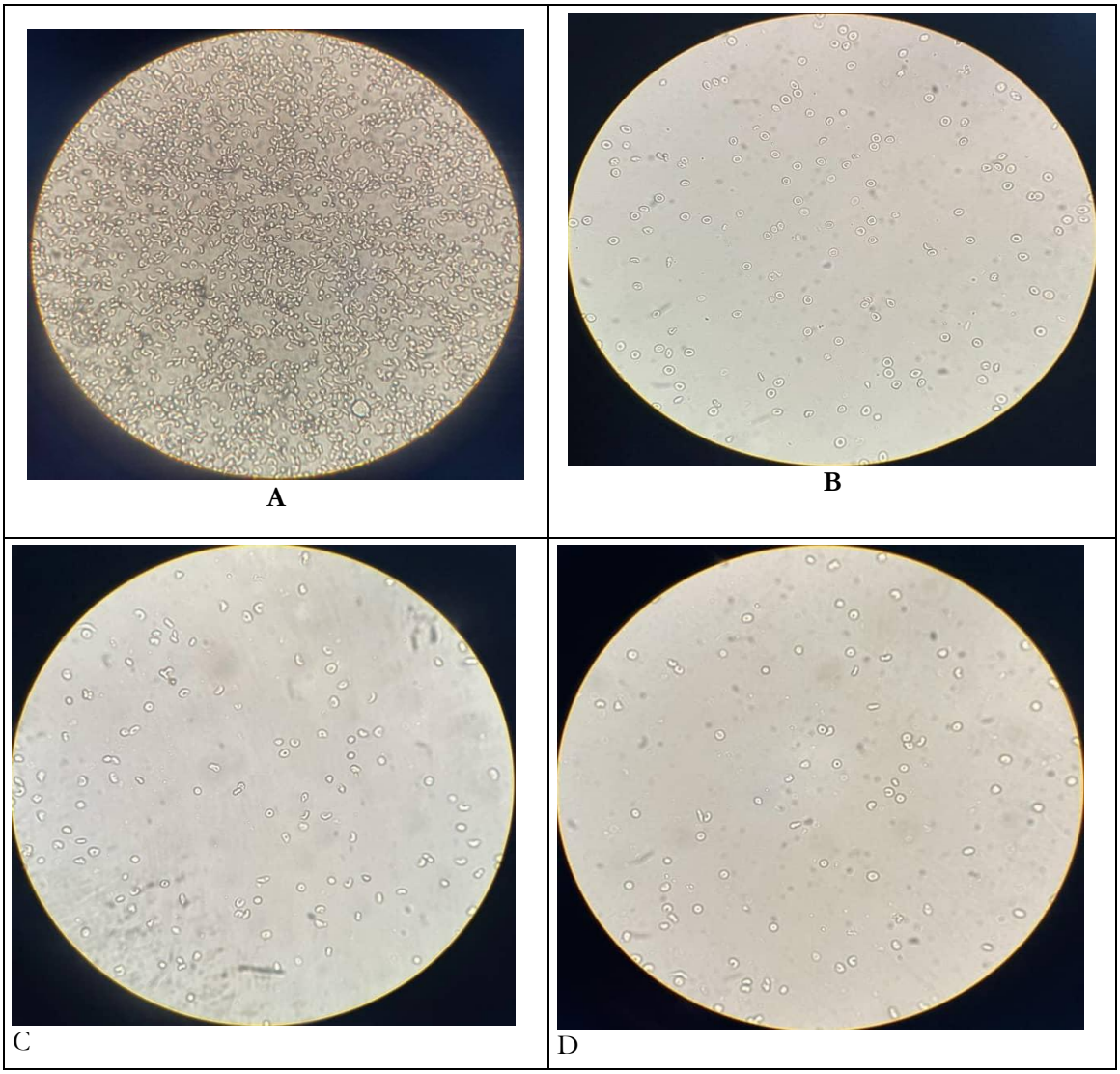


Figure 1: Microscopy of 250 µg/mL of ethanol leaf extract of *Ocimum gratissimum* on SS red blood cells at 0, 20 and 30 minutes.

A is the control (2 % sodium metabisulphite + SS red blood cells), % sickled red blood cells = 100. B showed 250 µg/mL of ethanol leaf extract of *Ocimum gratissimum*+ 2 % Sodium metabisulphite + SS red blood cells at zero minute, % reversed sickled red blood cells = 77. C showed 250 µg/mL of ethanol leaf extract of *Ocimum gratissimum*+ 2 % Sodium metabisulphite + SS red blood cells at 20 minutes, % reversed sickled red blood cells = 72.31. D showed 250 µg/mL of ethanol leaf extract of *Ocimum gratissimum*+ 2 % Sodium metabisulphite + SS red blood cells at 30 minutes, % reversed sickled red blood cells = 71.77

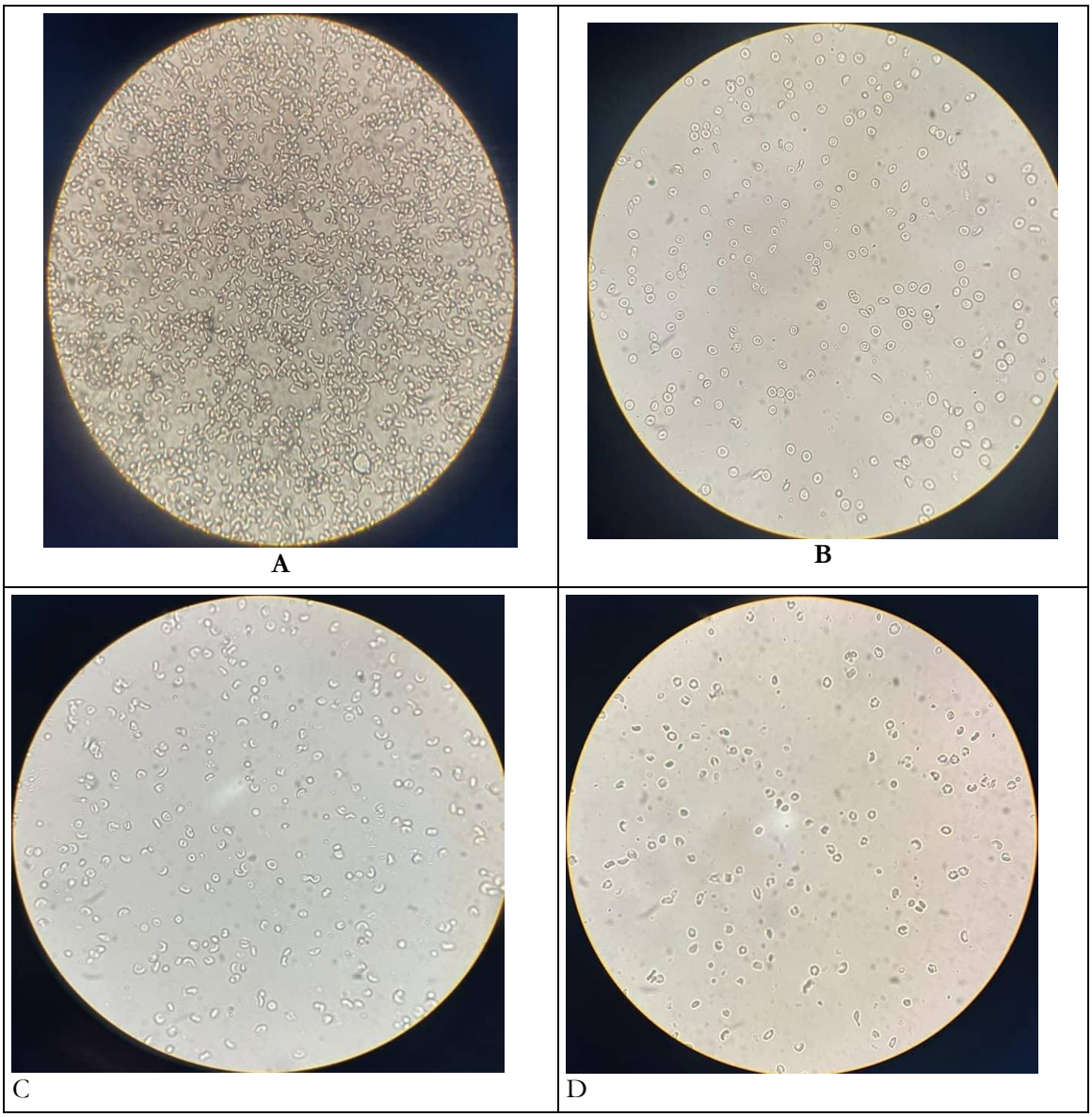


Figure 2: Microscopy of 500 µg/mL of ethanol extract of leaves of *Ocimumgratissimum* on SS red blood cells at 0, 20 and 30 minutes.

A is the control (2% Sodium metabisulphite + SS red blood cells), % Sickled red blood cells = 100. B showed 500 µg/mL of ethanol leaf extract of *Ocimum gratissimum*+ 2 % Sodium metabisulphite + SS red blood cells at 0 minute, % reversed sickled red blood cells = 76.19. C showed 500 µg/mL of ethanol leaf extract of *Ocimum gratissimum*+ 2 % Sodium metabisulphite + SS red blood cells at 20 minutes, % reversed sickled red blood cells = 69.6. D showed 500 µg/mL of ethanol leaf extract of *Ocimum gratissimum*+ 2 % Sodium metabisulphite + SS red blood cells at 30 minutes, % reversed sickled red blood cells = 74.

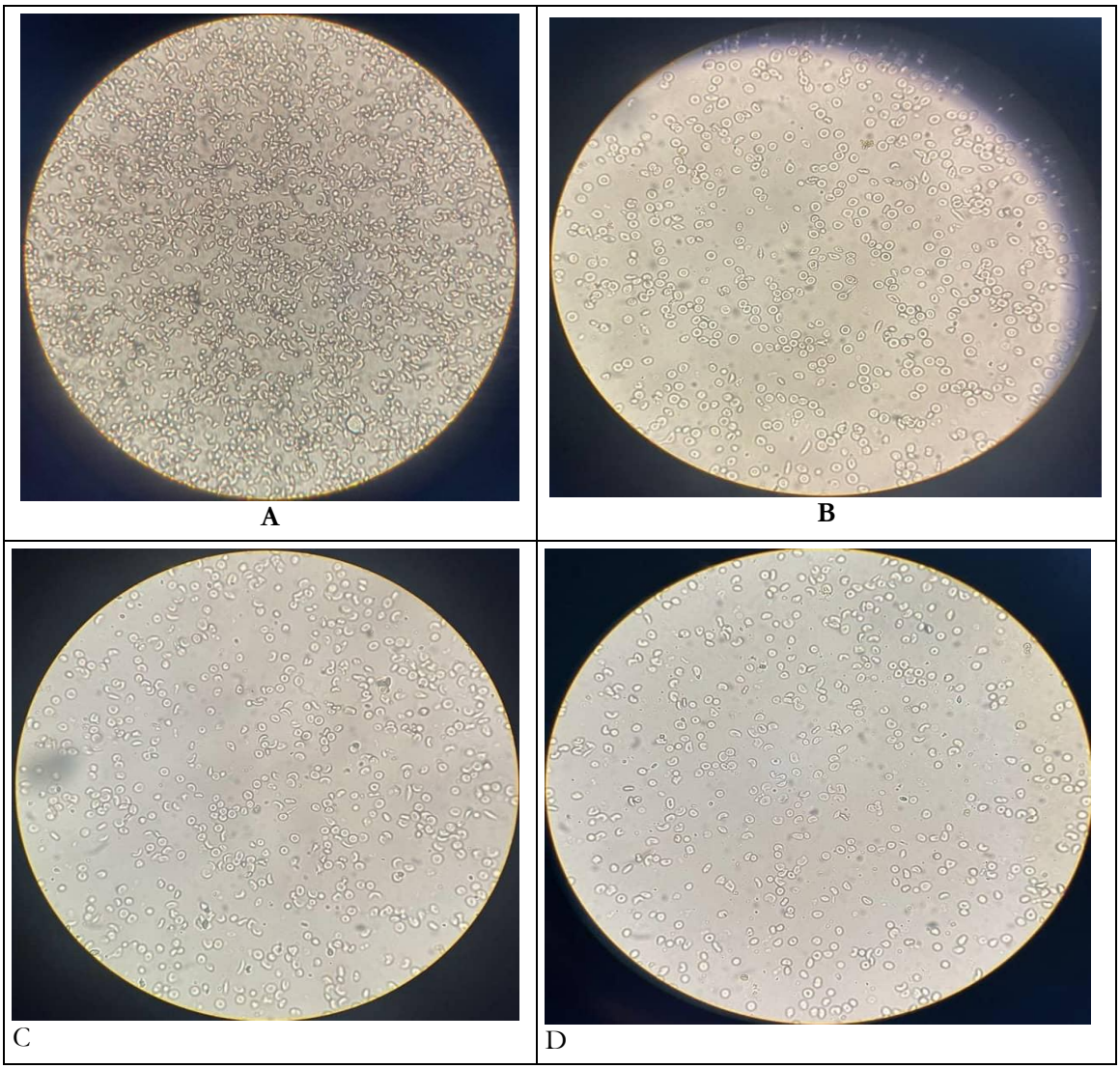


Figure 3: Microscopy of 1000 µg/mL of ethanol extract of the leaf of *Ocimum gratissimum* on SS red blood cells at 0, 20 and 30 minutes.

A is the control (2% Sodium metabisulphite + SS red blood cells), % Sickled red blood cells = 100. B showed 1000 µg/mL of ethanol leaf extract of *Ocimum gratissimum*+ 2 % Sodium metabisulphite + SS red blood cells at 0 minute, % reversed sickled red blood

cells = 71.49. C showed 1000 µg/mL of *Ocimum gratissimum*+ 2 % Sodium metabisulphite + SS red blood cells at 20 minutes, % reversed sickled red blood cells = 75.92. D showed 1000 µg/mL of ethanol leaf extract of *Ocimum gratissimum*+ 2 % Sodium metabisulphite + SS red blood cells at 30 minutes, % reversed sickled red blood cells = 87.70.

Effect of the ethanol extract of *Ocimum gratissimum* leaf on the Oxidation of AA haemoglobin.

UV-visible spectroscopic scans of 100 µl of AA haemoglobin mixed with 900 µl of potassium phosphate buffer (baseline) and with three different concentrations of ethanol extract of the leaves of *Ocimum gratissimum* (250 µg/mL, 500 µg/mL and 1000 µg/mL) at different time intervals (0 min, 10 mins, 20 mins).

The oxidative studies of *Ocimum gratissimum* ethanol leaf extract on haemoglobin AA showed a minimal oxidative effect with a slight reduction of the haemoglobin concentration from 0.07 mM to 0.06 mM and a slight decrease in the absorbance maxima of the ratio of the β-peak (576 nm) to the α-peak (540 nm) from 1.04 to 0.95. There was also a slight increase in the 630 nm band of Fe^{3+} from 0.08 to 1.23 due to oxidation. Also, the absorbance at 650 nm which is clearly distinct from the absorbance of the 630 nm band is attributed to chlorophyll.

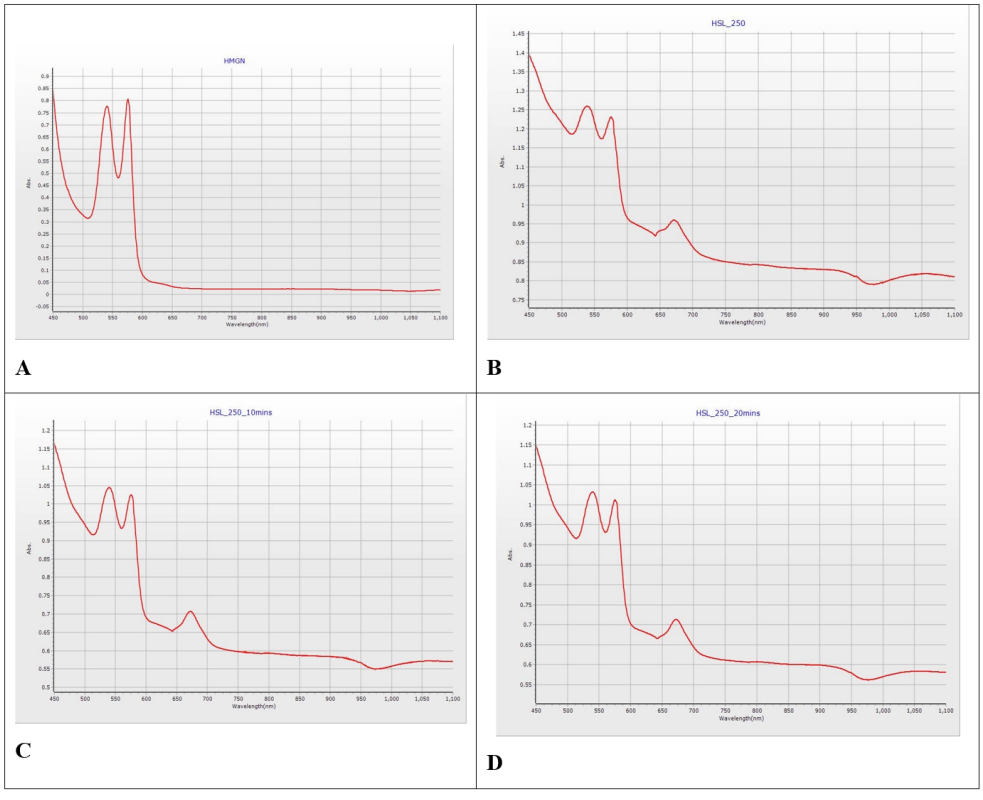


Figure 4: Effect of 250 µg/mL of ethanol extract of leaves of *Ocimum gratissimum* on oxidation of haemoglobin AA.

A is the Spectrum of oxy-haemoglobin A and 900 µl of potassium phosphate buffer (Control). Concentration of haemoglobin is 0.07mM. Haemoglobin purity = 1.04, absorbance at 630 nm wavelength = 0.08. B showed a UV-visible Spectroscopy of AA haemoglobin and 250 µg/mL of ethanol leaf extract of *Ocimum gratissimum* at 0 minutes (Concentration of haemoglobin is 0.07mM). Haemoglobin purity= 1.07. Absorbance at 630 nm = 0.93. C showed a UV-visible Spectroscopy of AA Haemoglobin and 250 µg/mL of ethanol leaf extract of *Ocimum gratissimum*at 10 minutes (Concentration of haemoglobin is 0.07mM). Haemoglobin purity= 0.98. Asorbance at 630 nm band = 0.66. D showed a UV-visible Spectroscopy of AA haemoglobin and 250 µg/mL of ethanol leaf extract of *Ocimum gratissimum*at 20 minutes (Concentration of haemoglobin is 0.07mM). Haemoglobin purity = 0.98. Absorbance at 630 nm = 0.68.

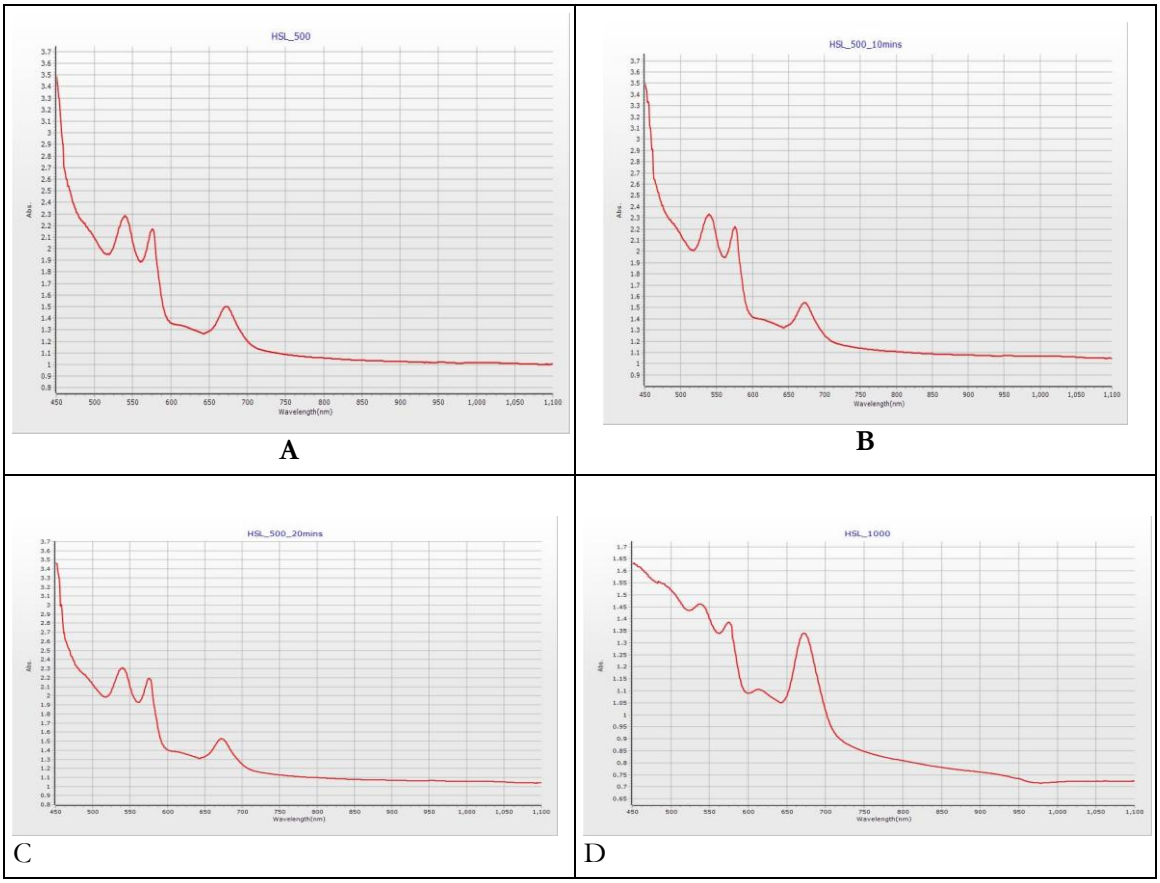
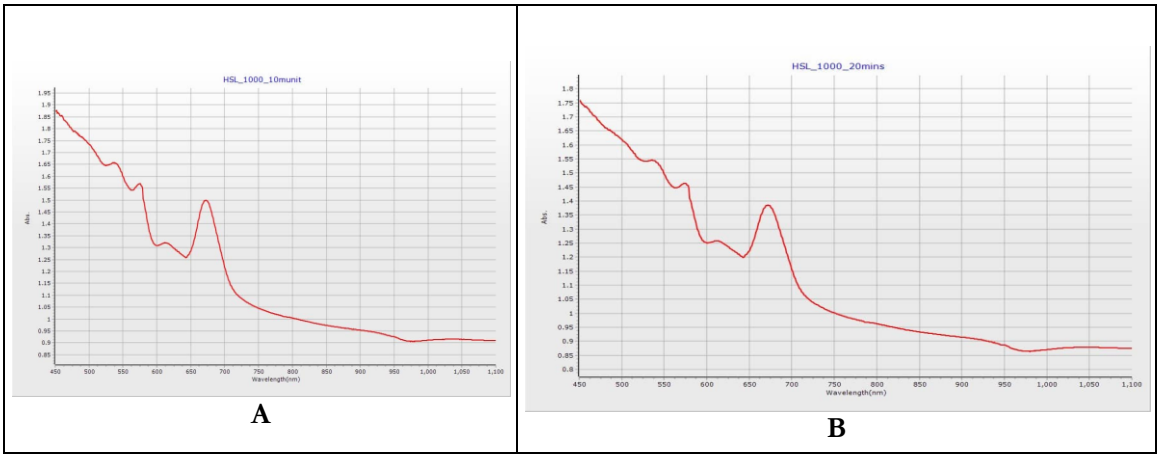


Figure 5: Effect of 500 µg/mL of ethanol extract of leaves of *Ocimum gratissimum* on oxidation of haemoglobin AA.

A showed a UV-visible Spectroscopy of AA Haemoglobin and 500 µg/mL of ethanol leaf extract of *Ocimum gratissimum* at 0 minutes (Concentration of haemoglobin is 0.06mM). Haemoglobin purity = 0.95. Absorbance at 630 nm band = 1.30. B showed a UV-visible Spectroscopy of AA Haemoglobin and 500 µg/mL of ethanol leaf extract of *Ocimum gratissimum* at 10 minutes (Concentration of haemoglobin is 0.06mM). Haemoglobin purity = 0.95. Absorbance at 630 nm = 1.36. C showed a UV-visible Spectroscopy of AA Haemoglobin and 500 µg/mL of ethanol leaf extract of *Ocimum gratissimum* at 20 minutes (Concentration of haemoglobin is 0.06mM). Haemoglobin purity = 0.95. Absorbance at 630 nm band = 1.34. D showed a UV-visible Spectroscopy of AA Haemoglobin and 1000 µg/mL of ethanol leaf extract of *Ocimum gratissimum* at 0 minutes (Concentration of haemoglobin is 0.06mM). Haemoglobin purity = 0.95. Absorbance at



A **B**

630 nm band = 1.07.

Figure 6: Effect of 1000 µg/mL of ethanol extract of leaves of *Ocimum gratissimum* on oxidation of haemoglobin AA.

A indicates a UV-visible Spectroscopy of AA Haemoglobin and 1000 µg/mL of ethanol leaf extract of *Ocimum gratissimum* at 10 minutes (Concentration of haemoglobin is 0.06mM). Haemoglobin purity = 0.95. Absorbance at 630 nm band = 1.29. B showed a UV-visible Spectroscopy of AA Haemoglobin and 1000 µg/mL of ethanol leaf extract of *Ocimum gratissimum* at 20 minutes (Concentration of haemoglobin is 0.06mM). Haemoglobin purity = 0.95. Absorbance at 630 nm band = 1.2

Effect of ethanol extract of *Ocimum gratissimum* leaf on the oxidation of SS haemoglobin

The oxidative studies of *Ocimum gratissimum* ethanol leaf extract on SS haemoglobin with UV-visible spectroscopy showed a decrease in absorbance ratio of the β peak (576 nm) and the α peak (540 nm) from 1.04 to 0.94. There is also a slight decrease in concentration of haemoglobin from 0.07 mM to 0.06 mM. There was a mild increase in the 630 nm band from 0.07 to 1.08 which is due to oxidation. At the early stages, there is minimal oxidation. But when the haemoglobin is incubated with the extracts over time, oxidation begins to occur more rapidly.

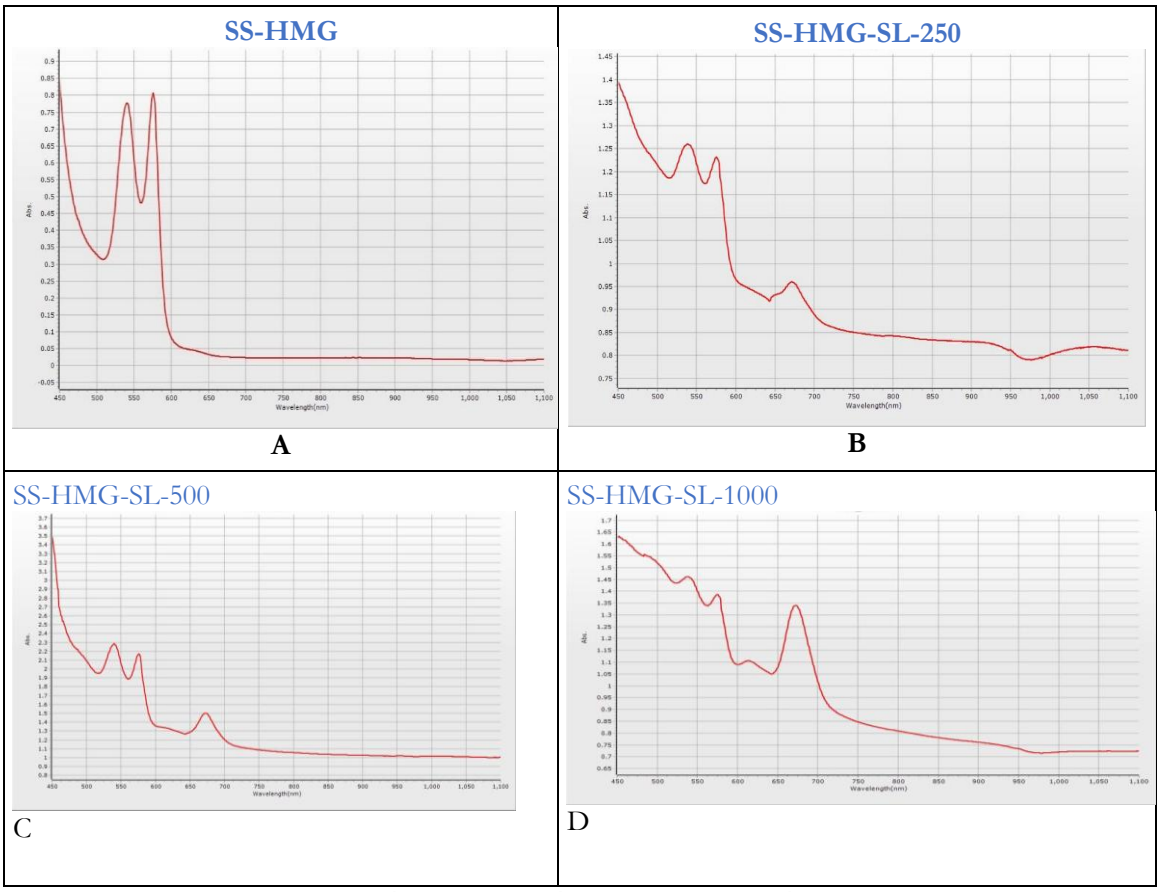


Figure 7: Effect of 250 µg/mL, 500 µg/mL and 1000 µg/mL of ethanol extract of leaves of *Ocimum gratissimum* on oxidation of haemoglobin SS.

A is the control (Spectrum of oxy-haemoglobin S and 900 µl of potassium phosphate buffer). Concentration of haemoglobin is 0.07mM. Haemoglobin purity = 1.04, absorbance at 630 nm wavelength = 0.07. B showed a UV-Visible scan of 250 µg/mL of ethanol leaf extract of *Ocimum gratissimum* mixed with 100µl of SS haemoglobin and 900 µl of Potassium phosphate buffer (baseline). Haemoglobin purity = 1.04, concentration of

haemoglobin = 0.07 mM, absorbance at 630 nm band = 0.07. C showed a UV-Visible scan of 500 µg/mL of ethanol leaf extract of *Ocimum gratissimum* mixed with 100µl of SS haemoglobin and 900 µl of Potassium phosphate buffer (baseline). Haemoglobin purity = 0.96, concentration of haemoglobin = 0.06 mM, absorbance at 630 nm band = 1.31. D showed a UV-Visible scan of 1000 µg/mL of ethanol leaf extract of *Ocimum gratissimum* mixed with 100µl of SS haemoglobin and 900 µl of Potassium phosphate buffer (baseline). Haemoglobin purity = 0.94, concentration of haemoglobin = 0.06 mM, absorbance at 630 nm band = 1.08.

Haemolytic effect of the ethanol extract of leaves of *Ocimum gratissimum* on SS red blood cells

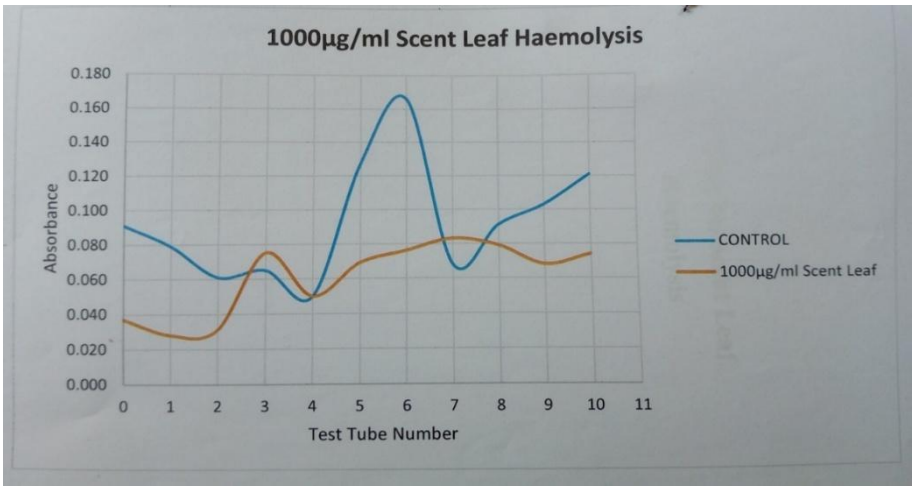


Figure 8: Haemolytic effect of ethanol extract of *Ocimum gratissimum* leaf for the 1000 µg/mL concentration.

The haemolysis curve was plotted for the control and the addition of the plant extract. Eleven test tubes were used for the haemolysis study. Test tube 0 to 10 comprised 0 µL to 1000 µL saline and 1000 µL to 0 µL distilled water, respectively to form a saline gradient with distilled water. The absorbance at 577 nm was plotted against each test tube number. The plant extract showed a stabilizing haemolytic effect compared to the control.

DISCUSSION

This work identifies *Ocimum gratissimum* ethanol leaf extract as a possible source of the growing number of plants with bio-active molecules that in Africa can serve as alternative drugs for the management of sickle cell disease. While other plants such as *Cajanuscajan, Carica papaya, Allium sativum* (bulb), *Aleo vera* (leaves and gel),

Fagarazanthoxyloides roots, *Piper guineensis, Percea Americana,* Garcinia kola also exhibit anti-sickling properties (Mehanna, 2001).

Ocimum gratissimum leaves are used for seasoning of various local foods such as rice, beans and plantain (Faluyi, 2020).

The data from microscopic studies was obtained by the application of 2 % sodium metabisulfite which induces the sickling of haemoglobin S red blood cells through deoxygenation (Moody *et al.,* 2003). The *Ocimum gratissimum* ethanol leaf extract had significant anti-sickling potential as it converted many of the sickled red blood cells to normal red blood cells with a maximum reversal of 87.70 % at 30 minutes compared to the control with 100 % sickled cells. The mechanism of reversal of sickled erythrocytes may result from the hydrophobic interaction of the bio-active molecules from the plant extracts to the lipid bilayer of the erythrocyte membrane (Elekwa *et al.,* 2005). This is because the erythrocyte membrane has hydrophobic molecules. Another mechanism of action could be the interaction of the aromatic or alkylic molecules from the extracts on the abnormal valine residue at the sixth position of the beta subunit on the polymerizing sickled haemoglobin. This mechanism suggests the possible interference of the bio-active compounds with the hydrophobic interactions by the abnormal valine residue at the beta-subunit leading to inhibition of the formation of haemoglobin S polymers (Franklyn *et al.,* 2021).

Both anti-sickling mechanisms require the presence of aromatic and alkylic compounds which has been shown by the phytochemical data of this work. *Ocimum gratissimum* ethanol leaf extract was screened for phytochemicals. Results of the screening revealed the presence of several bio-active compounds such as polyphenols, flavonoids, alkaloids, tannins, saponins etc. Some of these compounds are resveratrol, quercetin, kaempferol, naringenin, genistein, vannilic acid.

While *Ocimum gratissimum* is widely consumed as a regular meal in West Africa, its capacity to oxidize haemoglobin even at high concentrations of the plant extract is minimal as demonstrated from this work.

Finally, sickled red blood cells treated with ethanol leaf extracts of *Ocimum gratissimum* are stable and do not show any haemolysis.

CONCLUSION

Despite its anti-sickling and its anti-oxidative potentials, the value of *Ocimum gratissimum* can be realized by pulverizing the leaves into powdered form, bottled as solutions and tested in the social field on patients with sickle cell anaemia. Such trials can only improve the lives of patients with minimum or low cost. The search for bio-active anti-sickling and anti-oxidative bio-active compounds against sickle cell anaemia which afflict over 12 % of the global population of Africans remains a key research objective. The search for harmless anti-sickling plants for the management of sickle cell disease must be sustained pending the day that scientific development will make affordable and available to the African sicklers, the genetic engineering through the beta-globin gene transplant to stem cells of the bone marrow to correct the mutated gene into a normal globin gene that would produce normal cells and enhance the life of sickle cell patients.

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