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*IN VITRO*METHAEMOGLOBIN REDUCING POTENTIAL OF CRUDE METHANOLIC EXTRACT AND FRACTIONS OF *STERCULIASETIGERA* LEAF ON HUMAN SICKLED RED BLOOD CELLS

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ABSTRACT
Sickle cell anaemia is a hereditary disease affecting the red blood cells as a result of acquisition of a mutant β-globin gene, one from each parent. One of the pathophysiology of sickle cell anaemia is abnormally high concentration of methaemoglobin in the circulating red blood cells. Treatments for sickle cell anaemia are complex and expensive, Hence, cheap alternative remedies have to be identified. This study evaluated the percentage methaemoglobin concentration of sodium metabisulphite sickled erythrocytes in the presence of methanolic extract and fractions of Sterculia setigera leaf at concentrations of 0.2mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8mg/ml, and 1mg/ml which showed that a significant (p<0.05) difference existed between the methanolic extract and fractions except for n-hexane and butanolic fractions and the control. The results obtained from this study show the capacity of Sterculia setigera in preventing haemoglobin oxidation to form methaemoglobin, hense its usefulness in the management of sickle cell anaemia by some traditional doctors in northern Nigeria.
Keywords: Methaemoglobin, Sterculiasetigera, Anaemia, Pathophysiology, Haemoglobin S.

INTRODUCTION

Sickle cell disease is a group of related heamoglobin disorders prominent amongst them is sickle cell anaemia, also known as depranocytosis. Sickle cell anaemia is a genetic disorder in which the SS individual possesses an abnormal beta globin gene resulting from a single base substitution in the gene encoding the human B-globin subunit (Imaga *et al*., 2010). Under deoxygenated condition, this substitution causes a drastic reduction in the solubility of sickle cell haemoglobin as the haemoglobin molecules polymerize to form long crystalline intracellular mass of fibres causing deformation of the normal bioconcave shape of erythrocyte into a sickle shape (Bunn, 1997). Sickle cell anaemia patients suffer from painful crisis, acute chest syndrome and malfunctioning of organs including the spleen, heart and brain as well as from degeneration of the bone (Written and Bertles, 1989). HbSS leads to poor quality of life and reduced life span with an average life expectancy of 40 to 50 years (Platt *et al*, 1994).

Globally, more than 50 million people are actually affected by sickle cell anaemia (Diop *et al*, 2000). Nigeria remain the most affected by this disorder with more than 3 % of its population is affected (Ibrahim *et al*, 2007).

sterculia setigera (Del) belongs to the family sterculiaceae. It is an average height with a thick trunk and deciduous leaves. sterculia setigera is a multipurpose savannah trees vastly distributed throughout west and east African region (El-bashir *et al*., 2015). various African communities use the plant for various medicinal illnesses.

Most of the proposed treatments for sickle cell anaemia (SCA) appear to be fruitile over the years. The focus of this research is to determine the efficacy of the use of *sterculia setigera* to tackle methaemoglobin formation which is one of the challenges of SCA.

MATERIALS AND METHODS

Plant Sample Collection

Fresh leaf of *S. setigera* were collected from Dan Madami village in ZariaLocal Government Area in Kaduna State of Nigeria. It was authenticated at the Herbarium Unit of the Department of Biological Sciences, Ahmadu Bello University Zaria, with voucher number 365. Sand on the leaves were removed by rapid rinsing under running tap water, after which they were spread on a metallic tray and allowed to dry under shade except the day it was pounded, when it was dried under the sun for about 2 hours.

Blood Sample

Ethical clearance was obtained from the Health Research Ethical Committee (HREC), Ahmadu Bello University Teaching Hospital, Shika Nigeria via an approval Ref.No. ABUTH/HREC/TRG/36 dated 3rd October, 2013. Blood samples were collected from confirmed HbSS adult patients who visited Ahmadu Bello University Teaching Hospital. Blood sample (3ml) was collected by venipunture into anticuagulated EDTAtubes.

Chemicals and Reagents

Methanol, petroleum ether, chloroform, ethylacetate, n-heaxane, sulphuric acid were of analytical grade and were purchased from Sigma-Aldrich (Toyochem specialty chemicals, Birmingham, United Kingdom). Distilled water, Ependolff tubes.

Activity Guided Fractionation

The methanolic extract obtained from the powdered leaf of *S. setigera* was subjected to activity- guided fractionation, after thin layer chromatography was carried out to determine the best solvent system for the purpose. Thereafter, the methanolic extract of the leaf of *S. Setigera* (93g) was partitioned with 1 Litre each of n-hexane, ethylacetate, butanol, and distilled water using the method of De *et al.*, (2009). Fractions obtained were dried in a dish under fan, after which they were placed in sample bottles and stored in a refrigerator at 4ºC until required. The percentage yields of the fractions were calculated using the formular:

$$\text{Yield of fraction \%} = \frac{\text{Weight of fraction}}{\text{weight of methanolic extract}} \times 100$$

Reconstitution of extract and fractions: Samples (70mg) each of methanolic extract and its fractions were dissolved in 7ml of normal saline to provide a solution of 10mg/ml, from this stock solution, 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1ml were measured into Ependolff tubes and then 9.8ml, 9.6ml, 9.4ml, 9.2ml, and 9.0ml of normal saline were added to get fraction concentrations of 0.2, 0.4, 0.6, 0.8 and 1mg/ml, respectively. These were being used for the determination of the percentage methaemoglobin.

Determination of Methaemoglobin Concentration

This test was carried out to determine if the fractions can significantly reduce methaemoglobin concentration in the blood. The effect of the fractions on the methaemoglobin concentration was determined by the method of Paul *et al.*,(2009). Since methaemoglobin and heamoglobin have their absorbance peaks at 630nm and 540nm respectively, a higher absorbance at 540nm indicates reduction in methaemoglobin when compared to the control sample. In this test, 0.02ml of each specified concentration (0.2mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8mg/ml, and 1mg/ml) of each fraction solution added to a mixture of 5.0ml distilled water, and 0.02ml of whole blood that was previously mixed and allowed to stand for 60 min at room tempreature, The absorbance of the mixture was read at two different wavelength (540 nm and 630 nm). The percentage plasma methemoglobin was obtained with the formula;

$$\text{Percentage methaemoglobin} = \frac{(A\,630)^2}{(A540)^2 + (A630)^2} \times 100$$

Where A540 and A630 are absorbance at 540 nm and 630 nm respectively.

Mineral Analysis: Methanolic extract (10mg) and its fractions were digested using the method described by Ogunfowokan *et al.*, (2009) for each sample, 1g was digested in a Teflon cup with about 30ml aqua-regia (HNl:$HN0_3$,3:1) on a thermostat hotplate at 150°C for 2 hours. The Teflon cup were dismantled and allowed to simmer while adding about 5ml of hydrogen fluoride (HF) and the mixture was allowed to sit for 30 minutes and then filtered into a 50ml volumetric flask which is made up to mark with distilled deionized water. Using the filterate, chromium, copper, iron, magnesium, and zinc concentrations were determined with the atomic absorption spectrophotometer in duplicate.

Statistical Analysis

The data obtained are expressed as mean ± SEM of two measurements, and were statistically analyzed using analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used to compare different group means, while $P<0.05$ was considered significant in all cases.

RESULTS

Table 1 shows the effect of methanolic extract and various fractions of the leaf of *Sterculia setigera* at different concentrations on percentage methaemoglobin levels. For the methanolic extract and all the fractions, it was observed that there was a significant ($p>0.05$) decrease in the level of methaemoglobin following treatment with all extract concentrations when compared with the distilled water and blood control with the exceptions of n-hexane and butanolic fractions at 0.2mg/ml having a methaemoglobin concentration of 20.20±0.12%, and 21.38±0.24% respectively.

Table 1: The Effect of Methanolic Extract and Various Methanolic Extract Fractions of the Leaf of *Sterculia setigera*at Different Concentrations on Percentage Methaemoglobin Level

Extract Conc	% Methaemoglobinlevel after treatment with extract and fractions				
mg/ml	Methanol	n- hexane	Ethylacatate	Butanol	Aqueous
0.2	11.68±0.73^{a}	15.55±0.28^{ab}	20.20±0.12^{b}	21.38±0.24^{b}	17.32±0.11^{ab}
0.4	11.25±0.94^{a}	13.48±0.33^{a}	11.81±0.10^{a}	11.17±1.21^{a}	15.88.58^{ab}
0.6	10.19±0.67^{a}	11.11±0.66^{a}	15.29±0.21^{ab}	13.69±0.17^{a}	13.23±0.25^{a}
0.8	9.45±0.83^{a}	14.71±2.46^{a}	12.57±0.29^{a}	9.76±0.56^{a}	13.96±0.14^{a}
1	8.21±0.15^{a}	9.61±0.13^{a}	11.81±0.47^{a}	12.64±0.67^{a}	13.00±1.03^{a}
Dist.water	22.51±0.92^{b}	22.51±0.92^{b}	22.51±0.92^{b}	22.51±0.92^{b}	22.51±0.92^{b}

The values in the Table are the Mean±SD. Values with different superscript vertically are significantly different at p<0.05.

Fig 2 shows the concentration of minerals in methanolic extract and fractions. here it can be seen that butanol fraction had the highest concentration of iron, zinc, and magnesium with values of 233.60±0.00, 3657.20±0.00, and 141.52±0.00 respectively. Aqueous fraction however had the highest concentration of copper with 46.00±0.00ppm. However, copper was absent in ethylacetate, and n-hexane fraction similarly, chromium was found to be present only in both ethylacetate fractions.

Figure 2: Mineral Concentrations of Methanolic extract fraction of *Sterculia setigera*

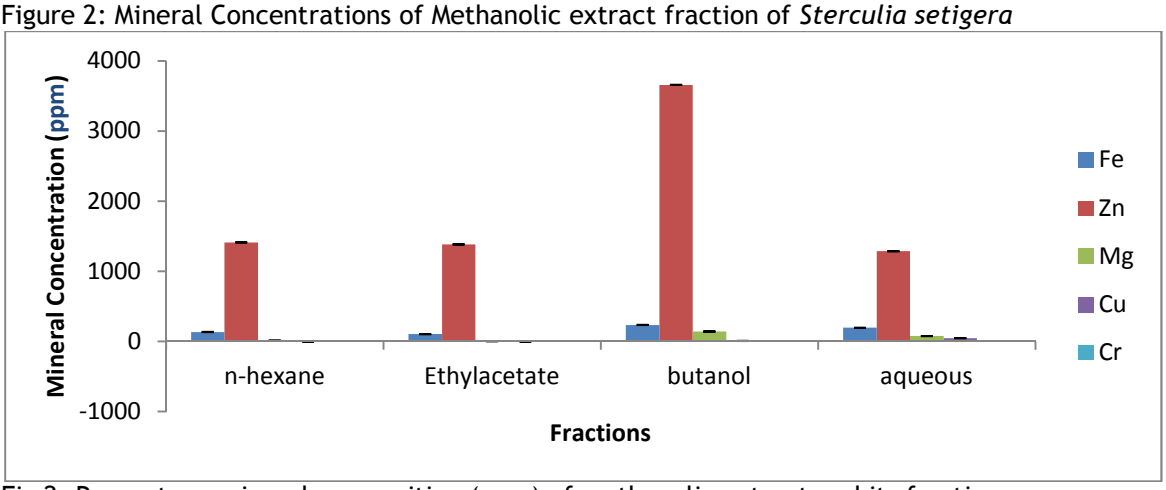

Fig 2: Percentage mineral composition (ppm) of methanolic extract and its fractions

DISCUSSION

The findings from the present study showed that, reduction of plasma methaemoglobin concentrations by the methanolic extract and methanolic extract fractions of *S setigera* leaf was dose dependent. The concentrations were significantly decreased to 8.21±0.15%, 9.61±0.13%, 11.81±0.47%, 12.64±0.67%, and 13.00±1.03% by methanol, ethylacetate, n-hexane, butanol, and aqueous fractions respectively (Table 1), thus reflecting their antioxidant effect. In normal blood, only a very small amount of methaemoglobin is present since the erythrocyte contains a system responsible for reducing Fe3+ of the heme to Fe2+. This system includes the nicotinamide adenine dinucleotide phosphate (NADPH), methaemoglobin reductase and cytochrome B5: it is known that NADPH enables the synthesis of reduced glutathione (GSH) which reduces the cytotoxic action of hydrogen peroxide, while the metabolic shunt pathway of pentose phosphate in erythrocytes is necessary for the synthesis of NADPH (reducing power) that protects hemoglobin and membrane lipids against oxidation (Roth, 1997: Keifer *et al.*, 2004). Thus, these *in vivo* antioxidant system prevents the formation of hydrogen peroxide, considered as one of the most important sources of generating oxygen free radicals that are harmful to cellular function (Nanfack *et al.*, 2013). The capacity of flavonoids to reduce methaemoglobin to haemoglobin *in vitro* have been suggested to indicate their antioxidant activity. Thus, its presence in the extracts and fractions could protect erythrocytes against premature aging and apoptosis induced by free oxygen radicals in sickle cell patients (Ibegbulem *et al.*, 2010).

The methanolic extract of this plant caused the highest reduction in methaemoglobin concentration, which is in line with previous work on *Adansonia digitata* that revealed that the high antioxidant activity of its extracts could be attributed to the high content of total phenols (Mpiana *et al.,* 2014).

CONCLUSION

The results obtained in this research reveal that the methanolic extract and fractions of *Sterculia setigera* possess abilities to prevent oxidation of haemoglobin to form methaemoglobin in a sickle cell red blood cell.

RECOMMENDATION

The reaction mechanism for the observed reduction in the formation of methaemoglobin by *Sterculia setigera* extract and fractions is not yet understood. Hence further work should be carried out to elucidate the possible mechanism.

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