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IN VITRO ANTI-SICKLING EFFECT OF CRUDE AND PARTIALLY PURIFIED FRACTIONS OF METHANOLIC EXTRACT OF *STECULIA SETIGERA* LEAF ON HUMAN SICKLED RED BLOOD CELLS

Baraka Abdullahi

Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria

Author's Email Address: barakaabdullahi@gmail.com

ABSTRACT
Sickle cell anaemia is a very important genetic disease affecting populace of the sub Saharan Africa. There is a strong need for discovery of cheap and readily available remedies for management of this disease. This research work assesses the ability of the crude methanolic extract, ethylacetate, n-hexane, butanol, and aqueous fractions of *Sterculia setigera* to reverse sickled human red blood cells *in vitro* after inducing hypoxia in erythrocytes using sodium metabisulphite. Generally, the results showed that anti-sickling activity was both concentration- and time-dependent, except for some concentrations and time intervals where the percentage of unsickled erythrocytes were abnormally higher or lower than expected. These exceptions were reflected in crude methanolic extract at 0.3 mg/ml, n-hexane fraction at 0.1 mg/ml, ethylacetate fraction at 0.2 mg/ml, butanol fraction at 0.3 mg/ml, aqueous fraction at 0.3 mg/ml with percentage unsickled erythrocytes of 29.68±1.62 %, 15.39±2.81 %, 13.01±1.62 %, 21.73±1.4 %, and 8.68±1.83 %, respectively. At the lowest concentration of 0.1mg/ml, n-hexane fraction showed the highest number of percentage unsickled erythrocytes of 76.04±1.48 %. However, at 0.3 mg/ml, ethylacetate fraction seems to have the highest anti-sickling activity of 86.01±1.69 %. Qualitative phytochemical screening revealed the presence of important phytochemicals such as alkaloids, flavonoids, steroids, tannins, saponins and cardiac glycosides. However, anthraquinones were consistently absent in the extract and all the fractions. These results goes to show that *S. Setigera* leaf extract possess anti-sickling properties and hence validates the usefulness of this plant in the management of sickle cell anaemia by traditional healers.

Keywords: *S. setigera,* Sickle cell anaemia, Erythrocyte, phytochemical screening, Antisickling effect.

INTRODUCTION
Sickle cell disease (SCD) is a potentially devastating condition that is caused by an autosomal recessive inherited hemoglobinopathy (Satarupa and Subha, 2013). This results in serious complications due to vasoocclusive phenomena and hemolysis (Omoti, 2006). The genetic abnormality is due to a substitution of the amino acid valine for glutamic acid at the sixth position on the beta globin chain, and was first described over one hundred years ago (Glitz, 2006). Hemoglobin S (HbS), the hemoglobin that is produced as a result of this defect, is a hemoglobin tetramer (α2/β2) that is poorly soluble and polymerizes when deoxygenated (Bunn, 1997).
Sickle cell anemia is particularly common among people whose ancestors come from sub-Saharan Africa, India, Saudi Arabia and Mediterranean countries. In some areas of sub-Saharan Africa, up to 2% of all children are born with the condition (WHO, 2006). In broad terms, the prevalence of the sickle-cell trait (healthy carriers who have inherited the mutant gene from only one parent) ranges between 10% and 40% across equatorial Africa and decreases to between 1% and 2% on the north African coast and <1% in South Africa (WHO, 2006). Frequencies of the carrier state determine the prevalence of sickle-cell anemia at birth. For example, in Nigeria, by far the most populous country in the subregion, 24% of the population are carriers of the mutant gene and the prevalence of sickle-cell anemia is about 20 per 1000 births, indicating about 150 000 children are born annually with sickle cell anemia in Nigerias (WHO, 2006).
Many challenges have stood in the way of the management of the sickle cell anemia. The compounds that are intended to inhibit the polymerization of hemoglobin S by increasing the concentration of hemoglobin F (hydroxyurea) have been reported to be toxic, especially when used over a long time (Baum *et al*., 1987; Iyamu *et al*., 2002; Buchanan *et al*., 2004). Bone marrow transplanting and gene therapy that offer a great promise for the actual treatment of sickle cell desease are very expensive and unaffordable.[31] Hence, identification of an indigenous plant that has antisickling activity will provide an affordable medication that can improve the quality of life sickle cell patients in Nigeria. This study on the methanolic extract of *S. setigera* was design to scientifically validate the claim of usefullness of this plant for management of sickle cell anaemia by some traditional healers in parts of northern Nigeria.
S. setigera (Del) belongs to the family Sterculiaceae. The tree is of average height, 16m tall, but can grow as high as 35m, and yields white gum from the stem. Sterculia species are well distributed in tropical Africa in areas such as the West African region and some east African countries like Sudan (Dalziel, 1956). *S. setigera* is a multipurpose savanna tree with socio-economic importance due to its gum and cultural importance in sub-Saharan Africa. It is also used in human nutrition, traditional medicine and cosmetics (Atakpama *et al*., 2012). The wood is used for non-timber forest products (NTFP) as baskets, local trays, etc. It is also used as insulation material in carpentry. The gum produced by the tree is tapped and used in cooking as an emulsifier, stabilizer and viscosifier and also in the preparation of soap for the treatment of dermatosis (Mann *et al*., 2008) and laxative, diuretic, and tranquillizer, cold decoction of the stem bark is used for the treatment of bronchitis, wound, diarrhea, gingivitis sore and abscess. Decoction of leaves is used as pain killers (Lawal *et al*., 2010). The root bark infusion of *S. setigera* is used

to treat jaundice (El-Kamali & El-Khalifa, 1999). Industrially, the gum is used as adhesives (El-Kamali & El- Khalifa, 1999).
In northern Nigerian traditional medicine, the leaf of the plant is used for treating malaria, fever while the bark and root bark are used for treating cold, bronchitis, and sickle cell anaemia respectively (Igoli *et al*., 2005). Hence the crude methanolic extract as well as the methanolic extracts of n-hexane, ethylacetate, butanol, and aqueous fractions therefore were assessed for their antisickling activity

MATERIALS AND METHODS

Plant Sample Collection, Identification, and Extraction

Fresh leaf of *S. setigera* were collected from Dam Madami village in Zaria Local 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 particles 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 h. Exactly 500 g of the powdered plant material was weighed and then loaded into a soxhlex extractor. The sample was then defatted with petroleum ether (40-60) for 8 h and then subsequently extracted with methanol. The resulting solvent was evaporated at room temperature.

Blood Sample Preparation

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 (sickle cell haemoglobin) adult patients who visited Ahmadu Bello University Teaching Hospital. About 3 ml blood sample was collected by venipunture into anticuagulated EDTA tubes where about 5 ml of normal saline was added and then spinned in a centrifuge (Labofuge 300, Heraeus, Kendro laboratory, Newton, U.S.A) at 3000 rmp for about 5 minutes after which the supernatant was discarded. This was repeated twice, and the washed erythrocytes were reconstituted in about 5ml of normal saline and kept in the fridge for use.

Experimental Protocols

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* (93 g) 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 a sampled bottles and stored in a refrigerator at 4 ºC 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: Out of 9 ml of the 1 mg/ml stock, 0.1 ml, 0.2 ml, and 0.3 ml were measured into Ependolff tubes, and 0.9 ml, 0.8 ml, and 0.7 ml of normal saline was sequentially added to the tubes to obtain extract and fraction concentrations of 0.1, 0.2, and 0.3 mg/ml, respectively. These were used immediately for determination of the anti-sickling properties of the test samples.

Determination of Anti Sickling Properties.

The ability of the plants extract to reverse the sodium metabisulphate induced sickling was estimated using sodium metabisulphite test as described by Imaga *et al*., 2009 (Imaga *et al*., 2009). In this test, 2 % sodium metabisulphite was used to induce sickling in washed erythrocytes (0.5 ml). A drop of the induced erythrocyte was placed on a microscopic slide and a thin blood film was made which was then stained with Giemsa stain and examined under the oil immersion light microscope (X100). The 0.1 mg/ml, 0.2 mg/ml, and 0.3 mg/ml of the methanolic extract and fractions were tested by adding 0.5 ml to the sickled erythrocytes, and the mixture was incubated for 120 min at 37 ^{0}C in a water bath (Grant JB series B and T, Keison international Ltd, Chelmsford, Essex CM1, England). Samples were then taken and then smeared on microcope slide after 0 min, 30 min, 60 min, 90 min and 120 min as earlier described. Each sample smear was fixed with 98 % methanol, dried and stained with Giemsa stain for about 45 minutes, and each sample was examined under the oil immersion light microscope (Herius, Kendro laboratory, Newton, U.S.A) by focusing at x 1000 the slide and counting at least 500 red blood cells in each sample from five different fields of view across the slide, while recording the total number of sicked and unsickled red blood cells. Positive control contained p-hydroxy benzoic acid (5 mg/ml). The percentage of unsickled cells was determined using the formula;

$$\% \text{ of unsickled cells} = \frac{\text{Mean number of sickled cells at 0 min- Mean number of sickled cell at time(t) x 100}}{\text{Mean number of sickled cell at 0 min}}$$

Phytochemical Analysis:

Qualitative phytochemical analysis was conducted on both the crude methanolic extract and partially purified fractions of the extract using standard methods described by other authors (Akindakun, 2005; Jaliwala *et al.,*2011; Maras, 2011).

Statistical Analysis

The data obtained are expressed as mean ± SEM of two, 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

In vitro Antisickling Activity

Antisickling Activity of Crude Methanolic Extract

Fig.1 shows the results of the effect of various concentrations of the crude methanolic extract of the leaf of *S. setigera* on percentage number of unsickled human red blood cells at different time intervals. Here, a significant ($p<0.05$) difference was observed in the percentage number of unsickled red blood cells treated with the extract at all concentrations when compared with p-hydroxy benzoic acid and normal saline controls, except for 0.1 mg/ml and 0.3 mg/ml at 30 min with an activity of 28.27±1.26 %, and 29.68±1.62 %, respectively.

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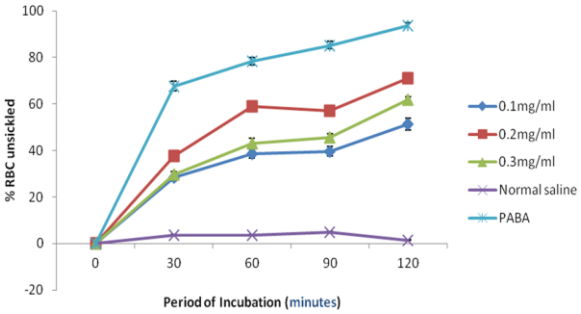

Figure 1: Comparism of the antisickling effect of crude methanolic extract of *S. setigera* leaf at various concentrations and time intervals.

Antisickling Activity of n-Hexane Fraction

Table 2 shows the percentage number of unsickled human red blood cells after treatment with various concentrations (0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml) of the n-hexane fraction of the leaf methanolic extract of *S. setigera* at different time intervals. From the result, it was observed that There was a significantly ($p<0.05$) lower percentage number of unsickled red blood cells treated with the fraction at all concentrations when compared with p-hydroxy benzoic acid except for 0.1mg/ml at 30 and 60min. as well as normal saline treated blood cells except for 0.1mg/ml at 90min with an activity of 15.39±2.81 %, 0.2 mg/ml at 30 min with an activity of 5.27±1.00 %, and 0.3 mg/ml at 30 min with an activity of 5.83±1.68 %.

Table 2: The Percentage Level of Human Sickled Red Blood Cells Unsickled Following Treatment with Leaf n-Hexane Fraction of Methanolic Extract of *S. setigera* at Various Concentrations and Time Intervals

Incubation Time (min)	Normal saline (Control)	Concentrations of Fractions / Standard			
		0.1mg/ml	0.2mg/ml	0.3mg/ml	PABA(5mg/ml)
30	8.04±1.46^{a}	76.04±1.48^{d}	5.27±7.00^{a}	5.83± 1.68^{a}	50.0±0.01^{c}
60	8.07±1.43^{a}	76.31±1.26^{d}	21.91±1.48^{b}	29.42±1.05^{b}	58.6± 2.89^{c}
90	10.25±2.81^{a}	15.39±2.81^{a}	21.07±2.89^{b}	33.58±2.82^{b}	75.0± 2.80^{d}
120	10.85±1.82^{a}	38.44±1.40^{b}	22.61±1.86^{b}	49.76±1.49^{c}	91.6±1.40^{e}

The values in the Table are the Mean±SD. Values with different superscripts verticallyt are significantly different at $p<0.05$.

Antisickling Activity of Ethylacetate Fraction

Fig 3 shows the percentageof unsickledhuman red blood cells following treatment with different concentrations of the ethylacetate fraction of the methanolic extract of *S. setigera* leaf at different time intervals. Incubation of the sickled cells with the fraction showed that the anti sickling effect of the fraction had a significant ($p<0.05$) decrease in the percentage number of unsickled red blood cells treated with the fraction at all concentrations when compared with p-hydroxy benzoic acid treated blood cells except for 0.3 mg/ml at 30 min with an activity of 86.01±5.69 %.

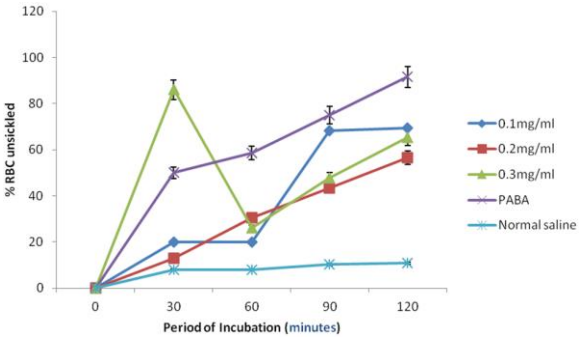

Figure 3: Comparism of the antisickling effect of crude and Ethylacetate fraction of *Sterculiasetigera* leaf at various concentrations and time intervals

Antisickling Acitivity of Butanolic Fraction

The percentage level of unsickled human red blood cells after treatment with different concentrations of the butanolic fraction of the leaf methanolic extract of *S. setigera* at different time intervals is presented in Fig 4. It was observed that there was a significant ($p<0.05$) decrease in the percentage of unsickled cells after treatment with the extract at all concentrations when compared with the positive control, p-hydroxy benzoic acid, conversely, a significant ($p<0.05$) decrease in the percentage of unsickled cells after treatment with the extract at all concentrations was observed when compared with normal saline treated blood cells except for 0.1 mg/ml, and 0.3 mg/ml at 30 min with an activity of 28.27±1.26 % and 29.68±5.62 % respectively (Fig 4).

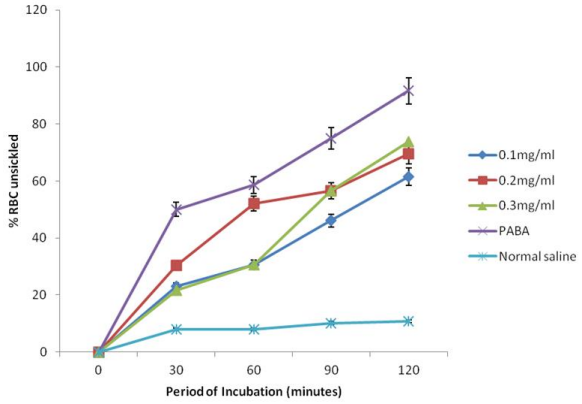

Figure 4: Comparism of the antisickling effect of crude and Butanolic fraction of *Sterculiasetigera* leaf at various concentrations and time intervals.

Antisickling activity of Aqueous Fraction

Fig 5 presents the percentage of unsickled human red blood cells following treatment with different concentrations of the aqueous fraction of the leaf methanolic extract of *S. setigera* at different time intervals.There was a significant ($p<0.05$) decrease in the percentage of unsickled red blood cells after treatment with the fraction at all concentrations when compared with p-hydroxy benzoic acid treated red blood cells. However, only the 0.3 mg/ml solution at 120 min, and 0.1 mg/ml after 90 min and 120 min

caused significant ($p<0.05$) difference when compared with normal saline.

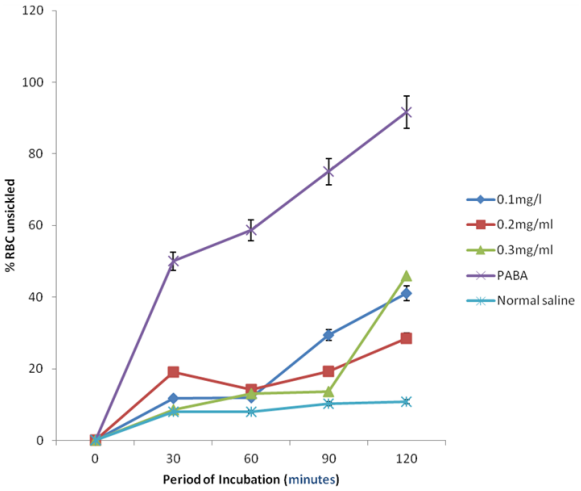

Figure 5: Comparism of the antisickling effect of crude and Aqeous fraction of *Sterculia setigera* leaf at various concentrations and time intervals.

Antisickling Activity of crude extract and fractions at 0.1mg/ml Concentration.

Fig 6 shows a comparative antisickling activity of the methanolic extract and all the partially purified fractions using concentration of 0.1 mg/ml of both the extract and fractions at different time intervals. After 30 mins of treatment with extract and fractions, n-hexane fraction had the highest activity of 76.04±1.48 % while the least activity was observed in the aqueous fraction with an activity of 11.76±2.10 %. However, at 120 min time interval, Ethylacetate fraction showed the highest antisickling activity of 69.58±1.45 %.

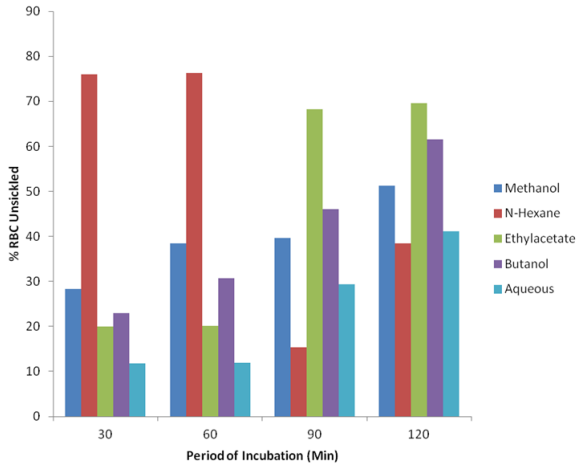

Fig 6: Comparism of the antisickling effect of crude and solvent fractions of *S. setigera* leaf (0.1 mg/ml) after different time intervals.

Antisickling Activity of crude Extract and Fractions at 0.2 mg/ml concentration

Fig 7 shows a comparative antisickling activity of the methanolic extract and all the partially purified fractions of *S. setigera* using concentration of 0.2 mg/ml of both the extract and fractions at different time intervals. After 30 mins of treatment with extract and fractions, the crude methanolic extract had the highest activity of 37.65±1.61 % while the least activity was observed in the n-hexane fraction with an activity of 5.27±2.01 %. Again, at 120 min time interval, methanolic extract showed the highest antisickling activity of 70.97±0.38 %.

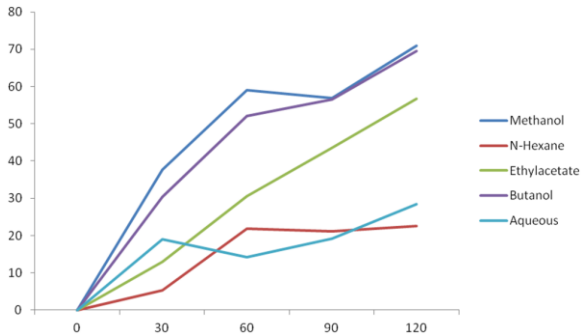

Fig 7: Comparism of the antisickling effect of crude and solvent fractions of *S. setigera* leaf (0.2mg/ml) after different time intervals.

Antisickling Activity of Crude Extract and Fractions at 0.3 mg/ml Concentration

Fig 8 shows a comparative antisickling activity of the methanolic extract and all the partially purified fractions of *S. setigera* using concentration of 0.2 mg/ml of both the extract and fractions at different time intervals. After 30 mins of treatment with extract and fractions, the crude Ethylacetate fraction had the highest activity of 86.01±1.69 % while the least activity was observed in the n-hexane fraction with an activity of 5.83±1.69 %. Again, at 120 min time interval, Ethylacetate fraction showed the highest antisickling activity of 65.29±2.86 %

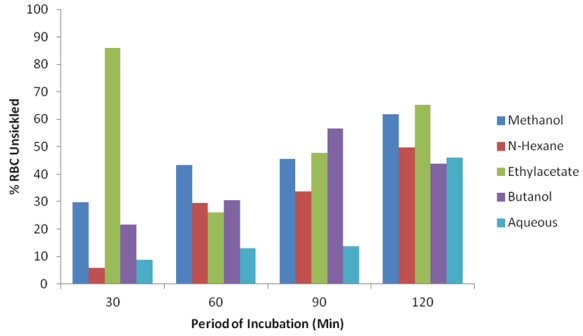

Fig 8: Comparism of the antisickling effect of crude and solvent fractions of *S. setigera* leaf (0.3 mg/ml) after different time intervals.

Qualitative Phytochemistry

Table 6 shows the result of the phytochemical screening ofthe crude methanolic extract and the various fractions of the leaf of *S. setigera*. The methanolic extract tested positive to all phytochemicals assessed for, whilehe n-hexane fraction showed the presence of all phytochemicals, except saponins. The

ethylacetate fraction showed the presence of cardiac glycosides, tannins, flavonoids, carbohydrate, steroides and triterprens, saponins and alkaloids, but the butanol fraction showed the presence of all phytochemicals except for alkaloids. The aqueous fraction contained carbohydrate, cardiac glycosides, flavonoids, tannins, steroids, alkaloids, triterpenes and saponins, although saponins were absent in froth test. Anthraquinones were consistently absent in all the fractions and the crude methanolic extract (Table 6).

Table 6: Phytochemical Constituents of Crude Methanolic Extract and Fractions of the Leaf of *Sterculia setigera*.

Phytochemical	Methanol (Crude)	n-Hexane	Butanol	Ethylacetae	Aqueous
Carbohydrate	+	+	+	+	+
Alkaloids	+	+	-	+	+
Flavonoids	+	+	+	+	+
Steroids & Triterpenes	+	+	+	+	+
Cardiac glycoside	+	+	+	+	+
Tannins	+	+	+	+	+
Saponins	+	-	+	+	-
Anthraquinone-	-	-	-	-	-

Key; + = Present, - =Absent

DISCUSSION

The results of antisickling assay of the methanolic extract and different fractions of *S. setigera* in this study showed that it exhibited a substantial antisickling activity. The highest antisickling activity was exhibited by 0. 2 mg/ml methanolic extract which showed an activity of 70.97±0.38 % at 120 min, while among the fractions, 0.3 mg/ mlethylacetate fraction exhibited the highest antisickling, having a significant antisickling activity of 65.29±2.86 % after 120 mins incubation. This is followed by the n-hexane fraction giving 49.76±1.49 % of unsickled RBC (Red blood cells) at 0.3 mg/ml concentration. The butanol fraction showed the third highest significant antisickling activity of 46.01±1.49 % at 120 mins incubation time. However, the least activity was exhibited by aqueous fraction showing its highest significant antisickling activity of 43.92±1.59 % at 120 mins incubation time. Sodium metabisulfite is used as model in sickle cell anaemia research because it creates hypoxic conditions for red blood cells leading to the loss of the morphology, HbSS polymerization and consequently, sickling of the erythrocytes. However, treated erythrocyte was considered to be unsickled, if the cell was not in the characteristic sickle shape or in crenated holly leaf pattern (Gorecki *et al*., 1999). The polymerization of Hbss erythrocyte is a major event in the pathophysiology of sickle cell disease. Hence, the effects of anti-sickling and sickling reversal of the methanolic extract of *S. setigera* and its fractions could be considered significant in alleviating sickle cell symptoms in patients. That the effect observed was time and dose dependent agrees with the report that anti-sickling activity of the drug tellurite, thiocyanate and hydroxyurea were dose and time-dependent (Oyewole *et al*., 2008). The extracts used in this study achieved a highest significant increase ($p<0.05$) in percentage number of unsickled red blood cells with the ethylacetate fraction, probably because of the presence of phenolic compounds in this fraction (Fig 3). The use of sodium metabisulphite to induce sickling is probably a more drastic approach than what actually happens in the vascular system of humans (Egunyomi *et al*., 2009).Therefore, it is expected that the methanolic extract and its fractions may achieve more efficient sickling reversal *in vivo,* when used in the management of human patients.

A review by Ameh *et al.* (2012) revealed that antisickling herbs abound in West Africa but that the most promising ones are likely yet to be discovered. This is because, several plants possess various nutrients and antinutrients of medicinal importance, (Iba *et al*., 2014). The contributions of a number of phytochemicals to the antisickling effect of plant product have been reported by several researchers (Adejumo *et al*., 2012). For example *Carica papaya* Linn and *Sorghum bicolor* antisickling activity was suggested to be due to phytochemicals. (Cyril-olutayo *et al*., 2009) Similarly, amongst the Efik, Ibibio, Hausa, Igbo, Idoma and Yoruba people, the use of Clove(*Eugenia caryophyllata*); *Piper guineenssis*, *Aframomum melegueta*; *Pterocarpus osun* have been reported (Ahmeh & Abdul, 2004) Although the efficacy of some of these plants have been demonstrated *in vitro,* their modes of actions are still not properly understood (Dash *et al.,* 2013).

The results of phytochemical screening of the various fractions of the leaf of *S. setigera* presented in Table 6 show that the methanolic extract and all its fractions have several contains phytochemical constituents that have been reported to have the potential to act as a source of useful drugs or serve to improve the health status of consumers. Numerous reports have suggested that flavonoids, (Middleton *et al* 1986) tannins, (Harborne 1998) saponins (Beutchet 1997) have anti-inflammatory properties, and have capacity to bind cations, thereby stabilizing erythrocyte membranes (Oyedapo *et al* 2004) and reducing frequency of SCA crises. These properties can be particularly relevant in alleviation of osteoarticulars painful crisis, the most usual symptom presented by sickle cells patients. Similarly, such compounds can help the sickle cell patients to fight against the accompanying severe infections, which usually are the principal causes of the deaths.

Conclusion

Its been over a century since the dicovery of sickle cell anemia without successfully finding a readily available cure even though some remarkable acheivements have been made in the area of gene therapy, bone marrow transplantation, and now stem cells research. But then this disease is affecting mostly people of tropical africa, characterised by low income economies as well as lack of adequate health care facilities, Hence there is much need for availalability of cheap local herbs that can help alleviate this disease. The results obtained from this research revealed that the methanolic extract and its fractions possessed sickling reversal potential in sickled erythrocytes. The protective mechanisms are yet unknown, but it may be related at least, in part to the presence of bioactive components especially, antioxidants component present therein.

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