

In Vitro Antisickling Potentials of Ethanol Extract of *Annona Muricata*, *Delonix Regia* and *Senna Alata*

Onyegeme-Okerenta B M*, Essien E B

Senior Lecturer, Department of Biochemistry, Faculty of Science, University of Port Harcourt, Rivers State, Nigeria

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Corresponding Author:

E-mail Id: blessing.onyegeme-okerenta@uniport.edu.ng

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ABSTRACT

Sickle cell anemia (SCA) is a genetic condition caused by defective hemoglobin molecules. The in vitro antisickling effects of different concentrations of ethanol extracts of *Annona muricata*, *Senna alata* and *Delonix Regia* on homozygous sickle cell erythrocyte was investigated. Standard antisickling tests evaluated include: Osmotic fragility, antioxidants (Superoxide dismutase, catalase and Glutathione), and polymerization of the sickled red blood cell. The experiment was in five groups Group 1 (Normal RBC), Group 2 (Sickle RBC – experimental control group), Group 3 (Sickle RBC + *A. muricata*), Group 4 (Sickle RBC + *S. alata*) and Group 5 (Sickle RBC + *D. Regia*). Fresh blood was collected from a sickle cell patient and normal individual which were used for the study. Results obtained from these investigations show that the three extracts significantly ($p < 0.05$) increased glutathione and catalase activities in the sickled red blood cells. *A. muricata* and *S. alata* exhibited a significant ($p < 0.05$) decrease against superoxide dismutase activity while SOD activity in sickle RBC treated with *D. regia* was not significant. Results of Osmotic fragility test showed an increase in membrane stability of sickle blood when the extracts were added. Antisickling test showed the ratio at which sickle blood cells were reduced with respect to time. The results of this study indicate that the extracts have the potential to reduce the sickling activity of sickle cell blood. The extracts significantly ($p < 0.05$) reduced the polymerization of sickle blood cells at a very high concentration (0.2mg/100ml) and increased its polymerization at low concentration (0.8mg/100ml). In conclusion, findings show that ethanol extracts of *A. muricata*, *S. alata* and *D. regia* may be helpful in the management of patients with sickle cell anemia.

Keywords: *Annona muricata*, antisickling, *delonix regia*, erythrocyte fragility, membrane-stability, *senna alata*, sickle cell anemia

INTRODUCTION

Sickle cell disease is a group of genetic disorders that affects hemoglobin, the molecule in red blood cells that delivers oxygen to cells throughout the body. Sickling decreases the red blood cells flexibility leading to sickle cell anemia and results in a risk of various [1]. The inherited genetic mutation stems from a single nucleotide substitution, amino acid valine with glutamic acid, in the β -globulin gene and produces an abnormal type of hemoglobin (Hb), namely, hemoglobin S (HbS). The HbS results in sickle-shaped red blood cells (RBCs), as opposed to normal biconcave disks. The sickle-shaped RBCs stick to the vascular endothelium and block the small and large vessels. Once, the vessels become blocked, attacks of pain, known as a vaso-occlusive crisis, begin [2-4]. Sickle cell disease affects millions of people worldwide. It is most common among people whose ancestors come from Africa; Mediterranean countries such as Greece, Turkey, and Italy; the Arabian Peninsula; India; and Spanish-speaking regions in South America, Central America, and parts of the Caribbean. Sickle cell disease is the most common inherited blood disorder in the United States, affecting 70,000 to 80,000 Americans. The disease is estimated to occur in 1 in 500 African Americans and 1 in 1,000 to 1,400 Hispanic American [1]. Nearly, 80% of children affected by this anemia die before they are five years old if they are not followed medically [5]. The most common manifestations in sickle cell disease are painful vaso-occlusive crises with pain especially in the limbs which result from obstruction of the microvasculature by sickled red blood cells causing ischemia and pain, and also hemolytic anemia and end organ damage caused by vasculopathy and tissue ischemia. Sickle cell anemia has no cure but however, treatments can help to relieve symptoms and reduce the complications. Orthodox (synthetic)

treatments have been used in the treatment of this anemia, for example, the use of antibiotics to prevent infections in infants diagnosed with sickle cell anemia, gene therapy and blood and marrow stem cell transplants may offer a cure for small fortunate patients. The health care cost of the management of sickle cell anemia patients is disproportionately high to compare to the number of people afflicted by the anemia. The individuals affected by this disease are mostly below the poverty line and unable to afford the high cost of treatment [6-9]. Current treatment of sickle cell disease involves blood transfusion and bone marrow transplant. After early diagnosis, the goal is health maintenance to prevent complications and medicines and treatments to manage complications, including chronic pain. Complications can include severe infections, seizures, and other clinical problems. About 5 percent of people who have received such transplants have died. Sometimes transplanted cells attack the recipient's organs. This is called graft-versus-host [9]. The use of herbal remedies is not a strange phenomenon in Africa and it has been estimated that 80% of Africans (Falodun, 2010) [10] and about 27 million South Africans are herbal remedy users [11]. Traditional medicine practitioners in Nigeria use herbal medicines to treat numerous health conditions such as mental disorder, fractures, insomnia and infertility [12]. Several studies have dealt with the use of medicinal plants to treat Sickle cell disease [13-16]. Findings have shown certain medicinal plants used to treat Sickle cell disease like *Garcinia kola*, *Annona senegalensis*, *Alcornea cordifolia*, *Fagara zanthoxyloides*, *Eugenia caryophyllata*, *Pterocarpus osun*, *Morinda lucida*, *Ceiba pentandra*, *Dacryodes edulis*, *Piper guineensis* *Allium sativum*, *Hymenocardia acida*, *Vigna unguiculata*, *Bridelia ferruginea*, *Carica papaya* and *Sorghum bicolor* and leaves of *Terminalia catapa*. Others include *Cajanus cajan* seeds,

unripe fruit of *Carica papaya*, leaves of *Parquetina nigrescens*, *Citrus sinensis*, leaves of *Persia americana*, *Ocimum basilicum* (Lamiaceae) and *Zanthoxylum zanthoxyloides* [17-23]. These plants have been demonstrated to have antisickling properties and are been used today for managing sickle cell disease. With population increase especially in the third world countries like Nigeria, there has been an increase in the incidence of sickle cell disease with a corresponding increase in the demand for its management. According to the World Health Organization (WHO) in 2007 [24], alternative strategies in the management of sickle cell disease should include the use of traditional medicine. *Annona muricata*, *Senna alata* and *Denolix regia* are medicinal plants used in the treatment of various ailments in Nigeria. Due to the increasing need for more potent therapeutic plants for the treatment of sickle cell disease, ethanol extracts of these medicinal plants were investigated for potential antisickling properties.

MATERIALS AND METHODS

Plant Extraction

Aqueous Infusion

This research project was conducted at the Department of Biochemistry, the University of Port Harcourt. The leaves of *S. alata*, *A. muricata* and *D. Regia* were obtained, authenticated and deposited at different herbariums as respectively described by Onyegeme-Okerenta and Anacleto 2017; [25-27]. They were air-dried at room temperature ($29 \pm 1^\circ\text{C}$) for 3 weeks and then pulverized with the aid of Marlex Excellent grinder (Mumbai, India). The powdered samples were kept in an airtight container until required. About 100 g of the powdered leaves were macerated in 500 mL of aqueous ethanol (70:30) and mixed intermittently for 72 h. The vacuum pump was used to filter the mixtures. The ethanol plant filtrates were dried over a water bath at 40°C and the resulting

extracts were kept in the refrigerator at -4°C .

Blood Samples

Ethical approval was obtained from the department of Biochemistry research ethics committee with the reference number UPH/BCHREC/2018/001. A total of 2.5ml of fresh blood samples were collected from each volunteer by venipuncture into an EDTA anticoagulating bottles and used for the experiment. Blood samples were all collected at lively stones medical diagnostic laboratory, Choba Rivers State, Nigeria.

Catalase Activity Assay

For determination of catalase activity, 0.2ml of sample (equivalent to 50-100mg protein) was added to 50mM of phosphate buffer (pH 7.4) containing 100mM (v/v) of H_2O_2 (Sigma, St Louis, USA) in a total of 1ml. The mixture was incubated for 2min at 37°C and the rate of change in absorbance at 240nm was recorded, which indicated the decomposition of H_2O_2 . Activities were calculated using the molar extinction coefficient of H_2O_2 at 240nm, 43.59L/mol cm. One unit of catalase activity equals the amount of protein that converts 1mmol H_2O_2 /min. All samples were measured in quadruplicates [28].

Superoxide Dismutase Activity Assay

An aliquot of supernatant from 12000g centrifugation of tissues homogenate was diluted with distilled water to make a 1:10 dilution. A 200 μl of the diluted sample was added to 2.5ml of 0.05M carbonate buffer (pH10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3ml of freshly prepared 0.3mM epinephrine to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5ml buffer, 0.3ml of the substrate (epinephrine) and 0.2ml of distilled water. The increase in absorbance at 480nm was monitored every 30s for 2.5min [29].

Glutathione-S-transferase Activity Assay

The medium for the estimation was prepared and the reaction was allowed to run for 60 s each time before the absorbance was read against the blank at 340nm. The absorbance was measured using UNICAM spectrophotometer [29].

Polymerization

The polymerization inhibition test was carried out following the method of [31]. The level of polymerization was ascertained by increasing absorbance of the assay mixture. A portion of 0.1 ml of the erythrocyte hemolysate containing HbS was introduced into a test tube followed by addition of 0.5 ml of Phosphate buffered saline (PBS) solution, pH = 7.4 {NaCl (9.0 g)/Na₂HPO₄.2H₂O (1.71 g)/ NaH₂PO₄.2H₂PO₄.2H₂O (2.43 g) per 1litre of distilled water} and 1.0ml of distilled water. The sample was transferred into a cuvette and HbS polymerization was induced by adding 3.4 ml of 2% sodium metabisulphite solution. The change in absorbance of the assay mixture was recorded at a regular interval of 30 s for 180 s at λ_{max} = 700 nm. To determine the anti-sickling property of the three antimalarial drugs, the procedure above was repeated by substituting 1.0 ml of distilled water with separate 2 mg% of the three antimalarial drugs.

In vitro Antisickling Assay

Evaluation of the ability of the leaf extracts to reverse the sickling state of the RBCs was performed by a previously described procedure [30]. Five groups labeled A, B, C, D and E were set up. Group A: contained sickle cell untreated (control) Group B: sickle cell + *A. muricata*, Group C: sickle cell + *S. alata*, Group D: sickle cell + *D. regia* Group E: sickle cell + Sodium metabisulphite. For the assay 100 μ L of SS-RBC pre-

incubated with 2% Na₂S₂O₅ was added to 100 μ L of the solution of different extracts. Each mixture was incubated at 37°C and antisickling activities observed at time intervals of 20, 40, and 60mins respectively. After incubation, 10 μ L of the mixture was diluted 100 times. A drop of each sample was examined under the oil immersion light microscope and both sickled cells and total cells were counted from five different fields of view across the slide. For the experimental control, the solution containing the extract was replaced by the saline solution. The percentage of sickling was calculated using the formula: % of sickling = number of sickling cells $\times$ 100/total cells.

Osmotic Fragility Test

The osmotic fragility test was carried out following a modification of the previously described method by [28]. The stock solution of sodium chloride was diluted in the ratio of 1:10 with distilled water to obtain at 1% solution. Nine tubes were taken to prepare dilutions. 0.05ml of the blood was then to each of the tubes and mix immediately by gently inverting several times. A sample from a normal individual was also run in parallel as a control. It was allowed to stand at room temperature (18-20°C) for 30 minutes. The amount of lysis in each tube is determined calorimetrically using a green filter, at 540nm. The first tube in the series serves as a blank (0% lysis) as it contains 0.9% isotonic saline. The 12th tube containing 0.1% saline serves as 100% lysis since this gives complete lysis.

Statistical Analysis

Each test was performed in triplicate and the results were expressed as mean $\pm$ standard deviation. Statistical analysis was done using t-test. Values were compared and regarded significant at $p < 0.05$.

RESULTS

Table 1: Antioxidant properties of ethanol extracts of *A. muricata*, *D. regia* and *S. alata*.

	Glutathione (Unit/ml)	Catalase ($\mu\text{mol/ml}$)	SOD (Unit/ml)
Normal RBC	3.53 $\pm$ 0.01	3.77 $\pm$ 0.01	0.340 $\pm$ 02
Sickle RBC	3.42 $\pm$ 0.02 ^a	8.77 $\pm$ 02 ^b	0.17 $\pm$ 002 ^c
Sickle RBC + <i>A. muricata</i>	4.73 $\pm$ 0.01 ^a	20.31 $\pm$ 0.01 ^b	0.110 $\pm$ 0.01 ^c
Sickle RBC + <i>S. alata</i>	5.03 $\pm$ 0.01 ^a	22.51 $\pm$ 0.01 ^b	0.13 $\pm$ 0.01 ^c
Sickle RBC + <i>D. regia</i>	3.77 $\pm$ 0.10 ^a	19.50 $\pm$ 0.10 ^b	0.28 $\pm$ 0.01 ^c

Note: Data are reported in Mean $\pm$ Standard Deviation, values with the same superscript shows Statistical Significance at $p < 0.05$.

Percentage inhibition of sickling of red blood cells by ethanol leaf extracts of *A. muricata*, *D. regia* and *S. alata* at various time intervals are presented in Fig. 1. The

results obtained for the three leave extracts tested showed that the percentage of sickling cells significantly decreased ($p < 0.05$) with the increase in time.

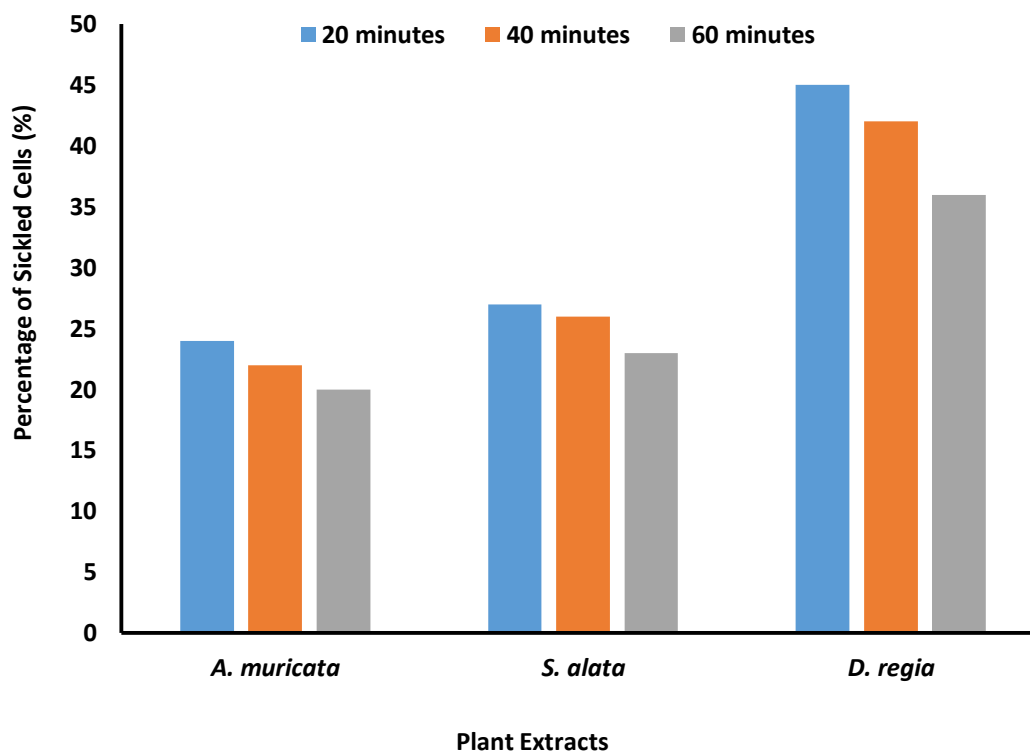


Figure 1: Inhibition of sickling of RBCs by ethanol extracts of *A. muricata*, *D. regia* and *S. alata*.

Osmotic fragilogram of ethanol leaf extracts of *A. muricata*, *D. regia* and *S. alata* on the human Red Blood Cells is shown in Fig. 2. Result indicated that there was a significant increase ($p < 0.05$)

in membrane-stability of Sickle RBC when ethanol leaf extracts of *A. muricata*, *D. regia* and *S. alata* were added to the Sickle RBC respectively.

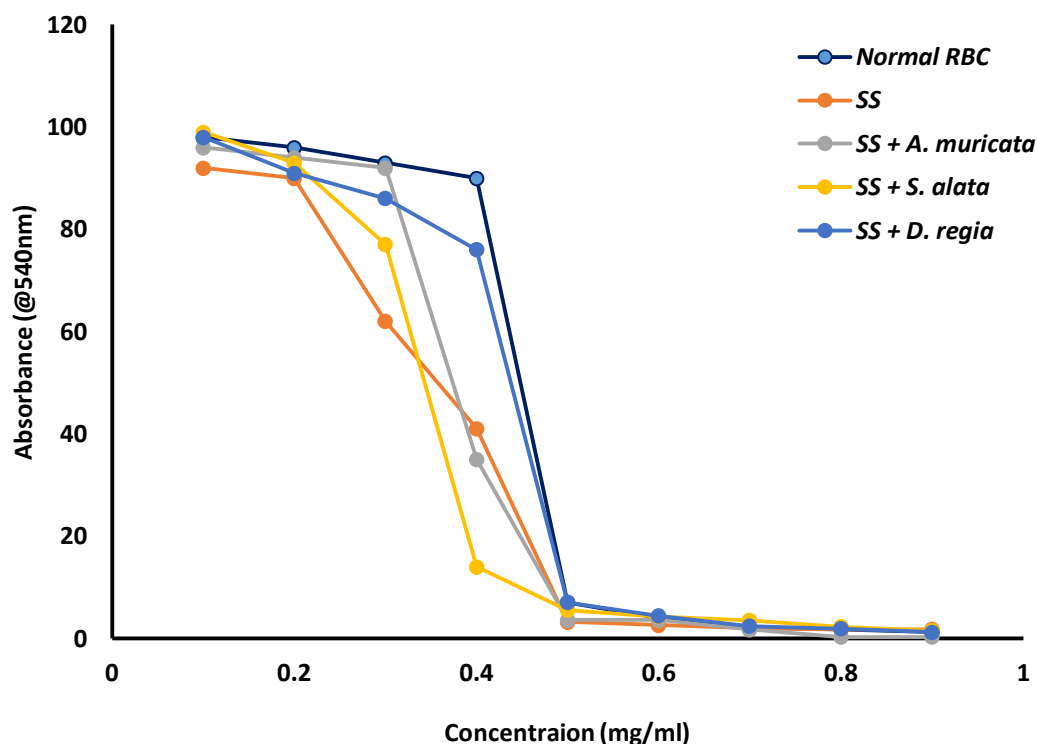


Figure 2: Osmotic fragilogram of ethanol leaf extracts of *A. muricata*, *D. regia* and *S. alata* on the human red blood cells.

Inhibition of Red Blood Cells polymerization by ethanol leaf extracts of *A. muricata*, *D. regia* and *S. alata* are presented in Fig. 3–5 respectively. Results obtained, showed that there were increases

in the delay of polymerization at various concentrations (0.5, 1.0 and 2.0mg/100ml) of *A. muricata*, *D. regia* and *S. alata* ethanol leaf extracts at varied time intervals.

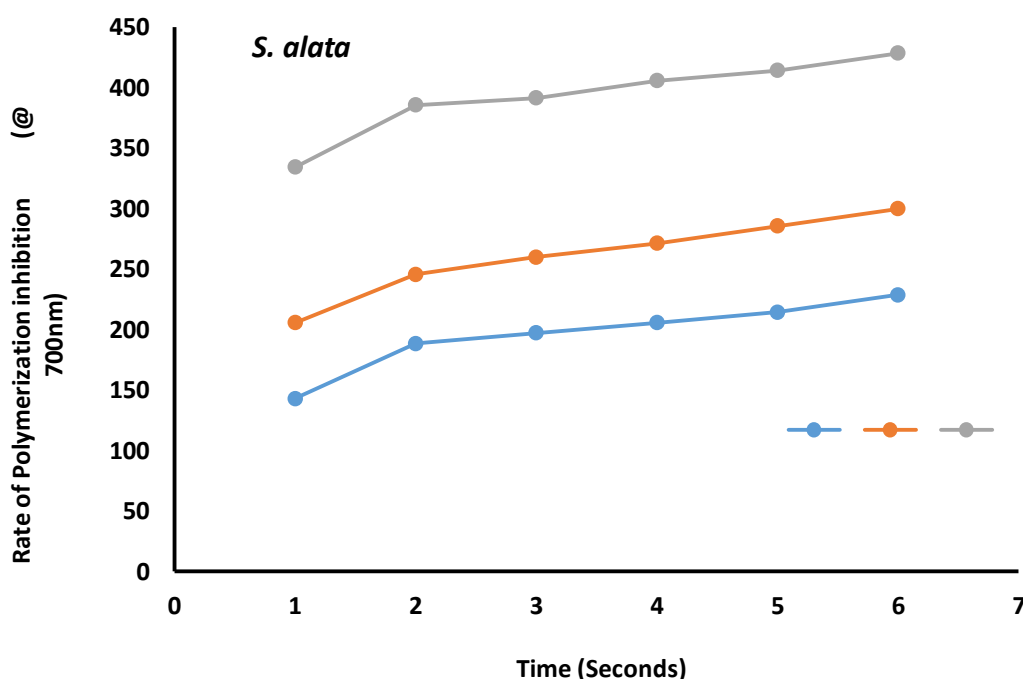


Figure 3: RBCs polymerization inhibition by ethanol extracts of *S. alata*.

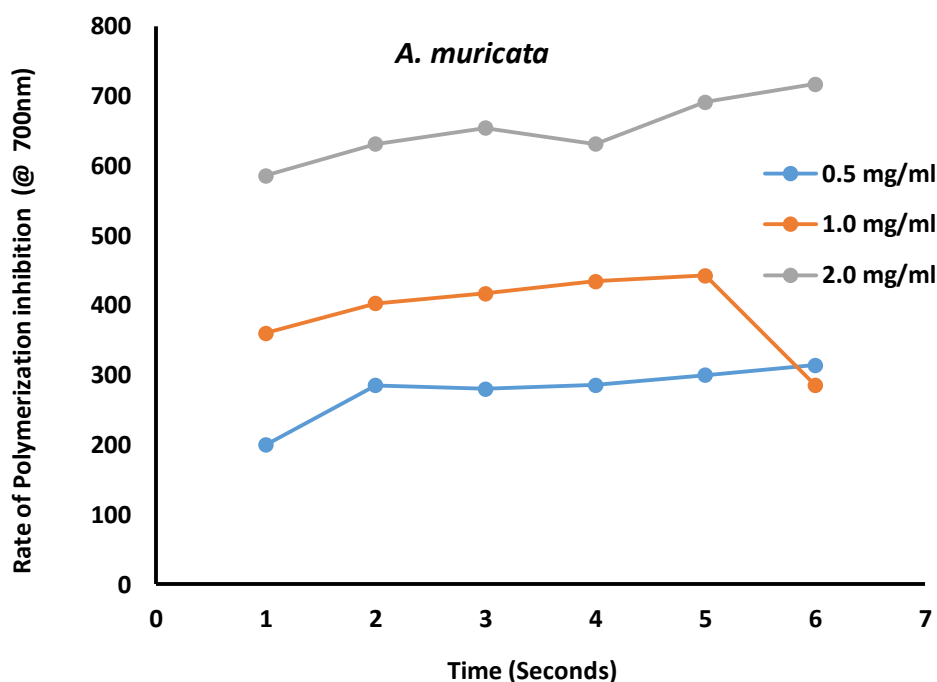


Figure 4: RBCs polymerization inhibition by ethanol extracts of *A. muricata*.

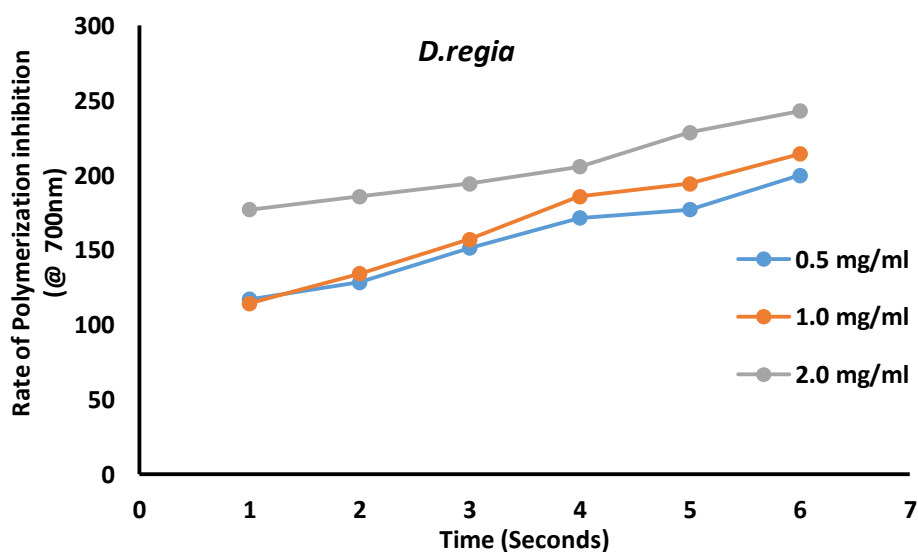


Figure 5: RBCs polymerization inhibition by ethanol extracts of *D. regia*.

DISCUSSION

Sickle cell anemia is an inherited form of anemia in which the red blood cells are not healthy enough to carry adequate oxygen throughout the body causing constant anemia and occlusion of small blood vessels leading to excruciating body pains, crisis, complications and other manifestations which may occur for no reason, or there may be a precipitating trigger factor [32]. Anti-sickling tests have been developed with the aim of controlling

the symptoms of sickle cell disease and limiting the episode of crisis. Anti-sickling agents interfere in three different stages, the sickle cell gene level, the hemoglobin S polymerization level and/or red cell membrane level. We have demonstrated experimentally, the antisickling properties of ethanol extracts of *A. muricata*, *S. alata* and *D. regia* by assaying their ability to inhibit sickle cell hemoglobin polymerization, Osmotic fragility, Gelatin and antioxidants (glutathione, catalase,

superoxide dismutase) activities. The three extracts significantly ($p < 0.05$) increased the enzymatic activities of glutathione-S-transferase and catalase in the sickled red blood cells. *A. muricata* and *S. alata* exhibited a significant ($p < 0.05$) decrease against superoxide dismutase activity while SOD activity in sickle RBC treated with *D. regia* was not significant. This indicates that *A. muricata* and *S. alata* have a more antioxidant property which enhances their ability to reduce oxidative stress that contributes to sickle cell crisis and possibly exert antisickling effect on the sickle red blood cells. These enzymatic antioxidants eliminate free radicals which are capable of causing inflammatory diseases in sickle cell patients and also help protect the body from oxidative stress during the inflammatory process [33]. It is reported that plants with more antioxidant property have a higher possible antisickling effect, [28]. Sickle cell anemic individual is prone to oxidative stress and damage when they are in crises. Therefore, ethanol extracts of these plants can be used to mop up the free radicals generated during a crisis and so contribute to the effective therapeutic management of the disease. The nature and mode of interaction between biochemical agents and contact points of deoxyhemoglobin S molecules in addition to erythrocyte membrane structural components are crucial determining factors in the capability of these agents to retard or inhibit polymerization of deoxyhemoglobin S molecules, alleviate or prevent erythrocyte sickling disorder. The present study showed that increase in the concentrations of ethanol extracts of *A. muricata*, *S. alata* and *D. regia* significantly ($p < 0.05$) inhibited the polymerization of sickle blood cells while a decrease in the concentrations of the extracts significantly ($p < 0.05$) increased its polymerization. The inhibition of polymerization of deoxyhemoglobin S molecules occurred in

a dose-dependent manner and it was an obvious reflection of the capability of the plant extracts to decrease the rapid rate of polymerization of hemoglobin S molecules in the blood sample. Studies by Chikezie (2011) and Chikezie *et al.*, (2013), [35-36] reported that the process of gelation (polymerization) of hemoglobin molecules increases the formation of sickling cells. Also, *in vitro* deoxygenation of RBC by sodium metabisulphite caused progressive aggregation and polymerization of the individual hemoglobin molecules.

Research into antisickling properties of medicinal plants has been rewarding in the management of sickle cell anemia. Antisickling test showed the ratio at which sickle blood cells were reduced with respect to time. Percentage inhibition of sickling of red blood cells by ethanol leaf extracts of *A. muricata*, *D. regia* and *S. alata* at various time intervals was reported in this study. Results obtained showed that the percentage of sickling cells significantly decreased ($p < 0.05$) with the increase in time. This is an indication that the extracts have the potential to reduce the sickling activity of sickle cell blood by increasing the oxygen affinity of the cells. Plant extracts contain bioactive constituents that may enhance the antisickling efficacy of the extract. The presence of such compounds could partially explain the pharmacological properties of these extracts. An example of such phytochemical is phenol which is richly present in *A. muricata*. Studies have also suggested the presence of essential minerals such as zinc, potassium, calcium, sodium, copper, iron and magnesium in *A. muricata*. The action of zinc and magnesium in improving the iron ratio is likened to reversing the functional state of sickle erythrocytes to their original biconcave structure, thereby increasing the oxygen affinity of the cells [37].

Studies done on normal red blood cell and

sickle red blood cell in the past showed that osmotic fragility of normal red blood cells is greater than that of sickle red blood, due to their biconcave shape which allows for more volume of water in a hypotonic solution before stretching of the membrane leading to hemolysis. Sick erythrocytes have been reported to have a distorted volume –to– surface ratio when compared to normal erythrocytes [38]. Most of the cells supplemented with ethanol extracts of *A. muricata*, *S. alata* and *D. regia* were still rounded after incubating in salt solutions which showed that the extracts have induced membrane stability on the sickle red blood cell. This observed inhibition in RBC lysis after treatment with these extracts is indicative of protective properties of the extract on the RBC membrane, thus helping to maintain membrane integrity.

CONCLUSION

Ethanol extracts of *A. muricata*, *D. regia* and *S. alata* have the potentials to reverse sickling in human sickle RBCs. This was shown in their ability to significantly decrease the rate of hemoglobin polymerization, the percentage of sickled cells, and the osmotic fragility of human sickle RBCs. Similarly, they possess antioxidant properties which protect the red cells from oxidative damage caused by free radicals. The inhibitory/reduction activities of ethanol extracts of *A. muricata*, *D. regia* and *S. alata* could be due to the synergistic properties of bioactive compounds present in these plants. Therefore, it can be concluded that they may be used for the therapeutic management of sickle cell anemia

AUTHORS' CONTRIBUTIONS

BMO and EEB conceptualized the study and reviewed the research proposal, conducted the pharmacological research and analyzed the field data, as well as drafted the manuscript. The authors participated in writing and giving feedback

on the manuscript. The authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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