PRELIMINARY EVALUATION OF THE ANTI-SICKLING AND POLYMERIZATION TIME RATE OF AQUEOUS EXTRACTS OF *BETA VULGARIS* (BEETROOT) ON SICKLE CELL DISORDER (*IN-VITRO* MODEL)

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Abstract

Sickle cell disease is an autosomal recessive genetic haematological disorder. Therapeutic approaches involve symptomatic management using conventional pharmacologic agents and substances of plant origin. This work was aimed at evaluating the *in-vitro* anti-sickling and polymerisation time rate effects of aqueous extracts of *Beta vulgaris*(beetroot) on sickle cell disease. Aqueous extraction of the beetroot was carried out. Phytochemical tests were conducted. Blood samples were collected from sickle cell patients. Osmotic fragility test and percentage haemolysis in different concentrations of NaCl (0 to 0.9 %w/v) were evaluated. Anti-sickling evaluation was done by inducing sickling in cells using 2 % sodium metabisulphite, while polymerization rate was determined. Phytochemical tests results revealed the presence of terpenoids, saponins and carbohydrate. The beetroot extract showed a significant reduction of the sickled cells from 89.1 to 35.6 % by the 5 %w/v beetroot and from 89.1 to 36.47 % by 2.5 %w/v beetroot, while the chemical standard (para hydroxybenzoic acid) reduced the sickled cells from 89.1 to 30.9 %. Polymerization rate was reduced from 1.52 to 1.17 % by the 5 % w/v beetroot and from 1.66 to 1.22 % by 2.5 %w/v beetroot over 30 min, while para hydroxybenzoic acid reduced the polymerization rate from 1.66 to 1.19 %. Beetroot aqueous extract reduced the percentage of sickled cells, rate of haemoglobin polymerization, and osmotic fragility of sickled red blood cells with results comparable to standard treatment agents. Aqueous extract of beetroot may be used as alternative agent to para hydroxybenzoic acid in sickle cell disease management.

Keywords: Beetroot; sickle cell reversal; osmotic fragility; polymerization rate.

INTRODUCTION

Sickle cell disease (SCD), also known as sickle cell anaemia is an autosomal recessive genetic haematological disorder that involves the mutation of the genes encoding for blood formation. Numerous clinical manifestations of sickle cell disease include pain, vaso-occlusive crisis, splenic sequestration, acute chest syndrome, aplastic anaemia, haemolytic anaemia, and stroke. Also, sickled erythrocytes tend to block capillaries *in vivo*, causing stasis and thereby starving organs of both nutrient and oxygen leading to organ damage (Onyegeme-Ekerenta *et al*., 2019; Iyamu *et al.*, 2002).

Sickle cell disease affects the shape and flexibility of RBCs in such a way that it prevents their smooth movement through small human blood vessels. Normal red blood cells are biconcave and flexible, a property that enables them to move freely and smoothly through narrow blood vessels. It also enables them to live longer to about 120 days. One of the motives for anti-sickling drug design is to have a drug that can prevent or reverse the sickle shape

phenotype of the RBCs (Nurain *et al.*, 2017).

Sickle cell anaemia is a life-threatening disease to individuals who suffer from it. The life expectancy for people with SCD in the United States is 58 years as at 2014 (Thein and Thein, 2016). Africa being a developing continent coupled with its inadequate health facilities, the life expectancy is lower. In Nigeria, about 150, 000 children are born with sickle cell disease annually (Muoghalu, 2018).

In Africa, there is high prevalence of sickle cell anaemia which cuts across all ethnic groups, gender, and income class. Management of sickle cell anaemia have proven difficult owing to low availability and unaffordability of the drugs such as Hydroxyurea, Niprisan® used in the management of sickle cell crisis. In addition, these drugs have been associated with side effects e.g., Hydroxyurea causes impotence in male.

Due to the high prevalence of sickle cell anaemia in Africa, there is need to develop drugs with anti-sickling effect for more medical interventions to ameliorate the crisis, decrease the mortality rate and increase life expectancy. This pressing need for safe, effective, and inexpensive therapeutic agents can be achieved using ethnomedicines from indigenous plants.

Several researchers have investigated the anti-sickling potential of medicinal plants with promising results (Nurain *et al.*, 2018; Nwaoguikpe and Braide, 2012; Onyegeme-Okerenta *et al.*, 2019; Onyegeme-Okerenta and Essien, 2019; Ogwutum *et al.*, 2018)

Beetroot botanically named *Beta vulgaris* (L) belongs to the family-Chenopodiaceae, which includes approximately 1400 species, divided into 105 genera. Members of this family are dicotyledonous (Chawla *et al.*, 2016). Beetroot is a rich source of phytochemical compounds that includes ascorbic acid, carotenoids, phenolic acids, and flavonoids (Georgiev *et al.*, 2010; Kujala *et al.*, 2000; Wootton-Beardet *et al.*, 2011;).

There have been several folkloric uses of this plant as a haematinic and in the management of sickle cell crisis in sickle cell patients with promising results. This work was therefore aimed at evaluating the *in-vitro* anti-sickling and polymerisation time rate effects of aqueous extracts of *Beta vulgaris* on sickle cell disorder.

METHODS

Extraction

Bulb of *Beta vulgaris* was bought and washed properly. The skin was peeled, and the succulent red part cut to pieces, blended, and sieved with a 1 mm sieve.

Phytochemical Screening of Plant Extracts.

Qualitative phytochemical screening was carried out according to the method of Harbourne *et al.* (1998).

Blood sample collection

An ethical approval was obtained prior to commencing this study from Chukwuemeka Odumegwu Ojukwu University Teaching Hospital, Amaku, Awka, Anambra State.

Fresh blood samples were collected with full informed consent from the sickle cell individuals in the steady state of the disease, aged between 10 and 25 years of both sexes, who had not taken any herbal medication for SCD within the past six months.

Preparation of Sickle Cell Blood

Venous blood samples (5 ml) were collected in sodium EDTA bottles. Erythrocytes were isolated from whole blood by centrifuging at 1500 x g for 15 minutes. The erythrocyte sedimented while the plasma was siphoned out carefully using a Pasteur pipette. By repeated inversion, the erythrocytes were suspended in a volume of isotonic saline equivalent to

the siphoned plasma. The suspended erythrocytes were used for the anti-sickling test while some quantity was freeze-thawed in a freezer to release a hemolysate. The hemolysate was used for the haemoglobin polymerization experiment (Nwaoguikpe *et al*., 2012).

Osmotic fragility tests

The osmotic fragility of erythrocytes measures the membrane stabilizing effect of the extracts in osmotic stress/ hypotonic lysis after an incubation period. The protocol by Onyegeme-Okerenta *et al.* (2019) was used for the analysis. To 10 ml reaction vessels containing 4 ml of different concentrations (0.00 - 0.90 %) of buffered saline pH 7.4, 1 ml of a range of concentrations of extract (5 mg/ml to 1.25 mg/ml) and 0.05 ml Hbss blood were added. The mixture was left to incubate at room temperature for 30 min and then centrifuged at 2000 rpm for 15 min. The supernatant collected was read at 560 nm against blank (0.90 % saline concentration). The percentage haemolysis was calculated using the formula:

$$\frac{Absorbance\ of\ supernatant\ in\ tube}{Absorbance\ of\ supernatant\ in\ tube\ with\ 0\ \%\ NaCl}\ X\ \frac{100}{1} \quad \ldots\ldots 1$$

***In vitro* anti-sickling activity of the extracts (Sickling reversal)**

A serial dilution of different concentrations of aqueous extracts of *Beta vulgaris* was prepared in the saline solution. For the assay, 100 µL of SS-RBC was pre-incubated with 100 µL of 2 % sodium metabisulphate and 100 µL each of a solution of the different concentrations of the extracts for different concentrations of 1.25, 2.5 and 5 % w/v. Each mixture was incubated at 37 °C for 2 h (time necessary to obtain maximum sickling). After incubation, 10 µL of the mixture was diluted 100-fold. A drop of each sample was examined microscopically (Amscope, USA) using a magnification of X 100. Both sickled cells and total cells were counted from five different fields of view across the slide. For the negative control, the solution containing the extract was replaced by saline solution.

The percentage of sickling was calculated using the formula:

$$\text{Percentage of sickling} = \frac{number\ of\ sickled\ cells}{total\ cells} \times 100 \quad \ldots\ldots 2$$

The experiment was repeated using para-hydroxybenzoic acid (chemical standard) as mentioned above and the percentage of sickling calculated (Nwaoguikpe *et al*., 2012).

Sickle Cell Haemoglobin Polymerization Inhibition Test

The original methods of Noguchi *et al.* (1989) as reported by Nwaoguikpe *et al.*, (2012) was used for the HbSS polymerization experiment. HbSS polymerization was assessed by the turbidity of the polymerizing mixture at 700 nm using 2 % solution of Sodium metabisulphite as a reductant or deoxygenating agent. A 4.4 ml volume of 2 % Sodium metabisulphite, 0.5 ml of normal saline (0.9 % NaCl) and 0.1 ml of haemoglobin were pipetted into a cuvette, shaken and the absorbance read in a Spectrophotometer at 700 nm for 30 min at 5 min intervals. This served as control while distilled water was used as blank for all assays.

For the test assay, 0.5 ml normal saline was replaced with 0.5 ml anti-sickling agent (extract of beta vulgaris) and readings taken at 5 min interval for a period of 30 mins. The rates of polymerization were calculated from the formula below:

For the polymerization test:

$$Rp = \frac{pf - pi}{t} \quad \ldots\ldots\ldots\

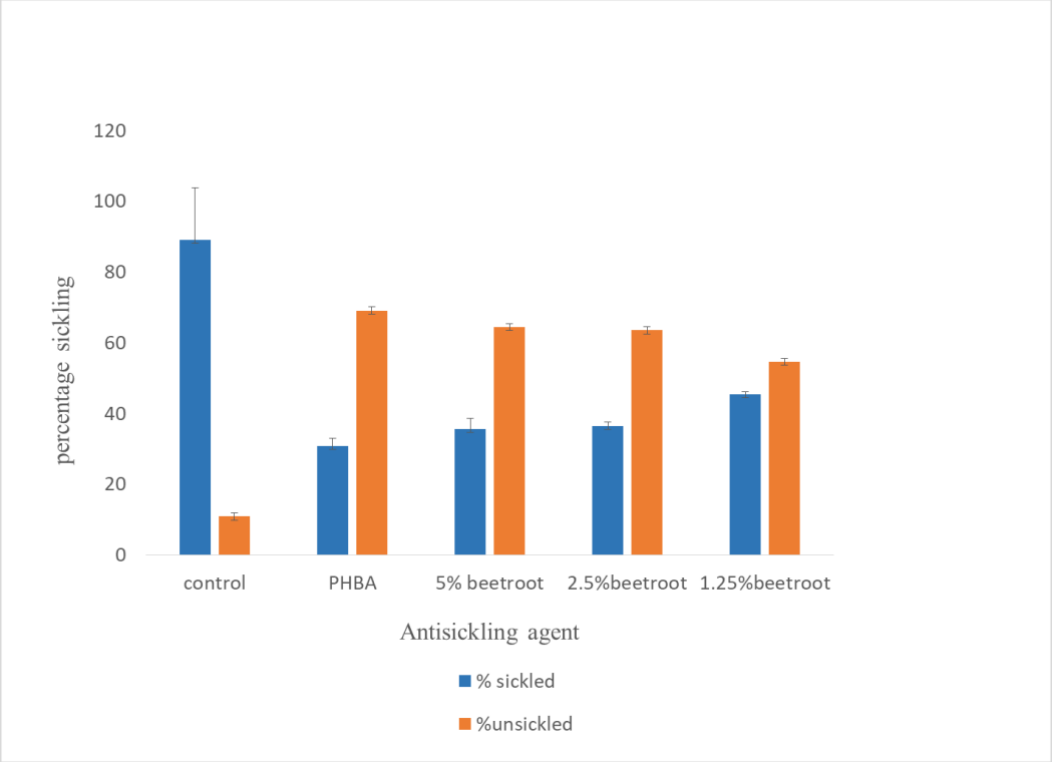

Fig 1: Inhibition of sickling of RBCs by aqueous extracts of Beetroot and Parahydroxybenzoic acid

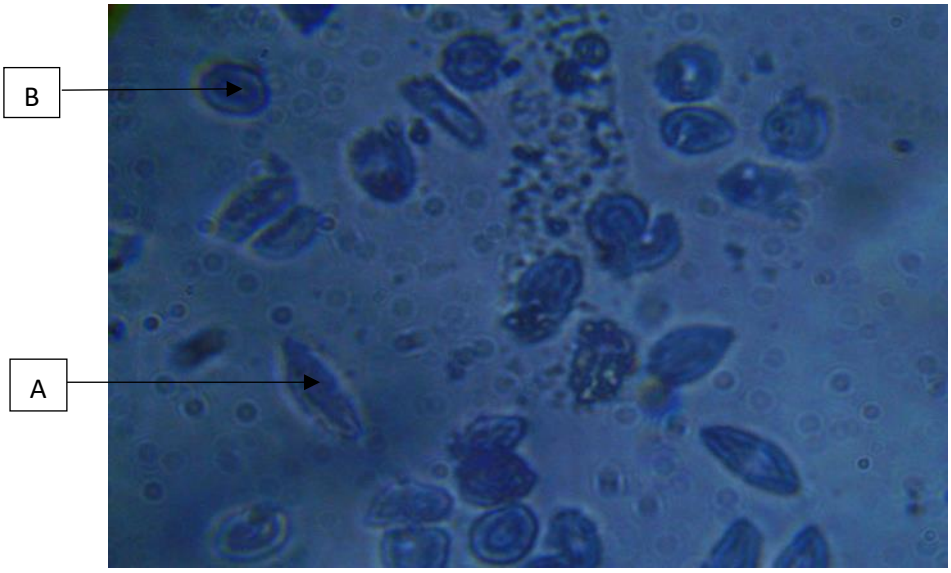

Fig. 2: Structural shape of sickled (A) and normal (B) red blood cells when viewed under microscope at X 100 magnification.

Osmotic fragility testing

The result of this experiment as presented in Fig. 3 shows the effects of para hydroxy benzoic acid and the plant extract on the resistance of RBCs to haemolysis at different salt concentrations. This is a test aimed at establishing the ability of an anti-sickling agent to enhance the red blood cells resistance to lysis arising from hypotonicity. Hypotonic lysis occurs when water molecules move into the cells through osmosis against a solute

concentration gradient. It has been reported that an increase in surface-to-volume ratio can increase the resistance of RBCs to haemolysis (i.e., decrease the osmotic fragility). Examples include cases in iron-deficient anaemia, thalassemia, sickle cell anaemia, and liver disease. On the other hand, a decrease in surface-to-volume ratio can decrease the resistance of RBCs to haemolysis (i.e., increase the osmotic fragility), with examples in the haemolytic anaemia and hereditary spherocytosis (Cooper *et.al*., 1968)

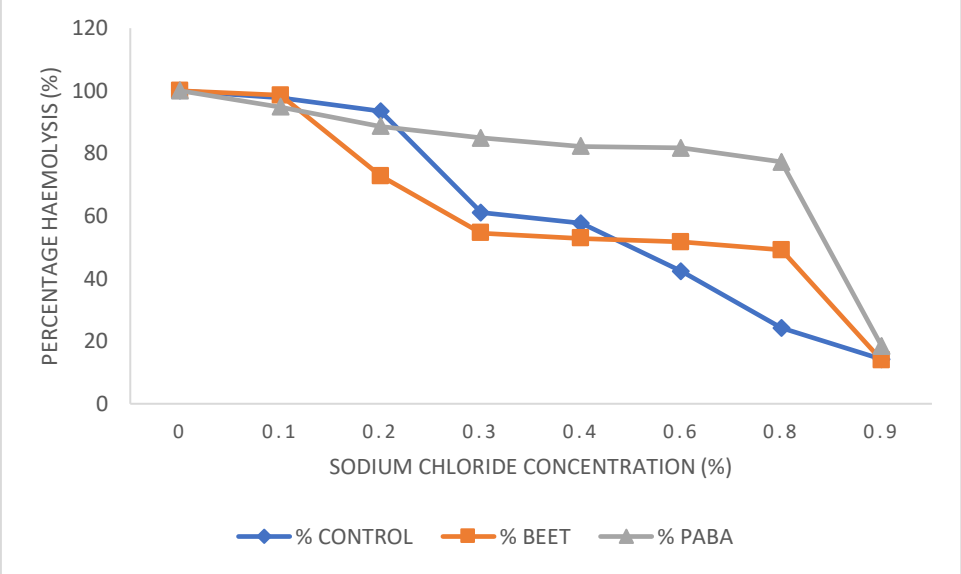

Fig. 3: Osmotic Fragility test

The osmotic fragility of red blood cell for the aqueous extract of beetroot was fully observed between 0.30 % and 0.10 % NaCl concentration, representing the onset and the completion of the haemolysis with increasing hypotonicity. The Beetroot showed a better resistance to haemolysis as was evident in the results obtained when compared with parahydroxybenzoic acid and the control.

When the osmotic fragility is reduced, the resistance to haemolysis is increased. The values obtained in the presence of the tested aqueous extract of beetroot also indicate a relatively higher efficacy in the reduction of osmotic fragility.

Red Blood Cell Polymerization Inhibition Test

It has been well established from research in the past that the genetic mutation in the globin chain is where the sickle cell disease originated. One of the clinical manifestations of this genetic RBC disorder is polymerization of haemoglobin in the hypoxia (Nagel *et.al*., 1979). Therefore, haemoglobin polymerization is one of the processes targeted in drug design against sickle cell disease.

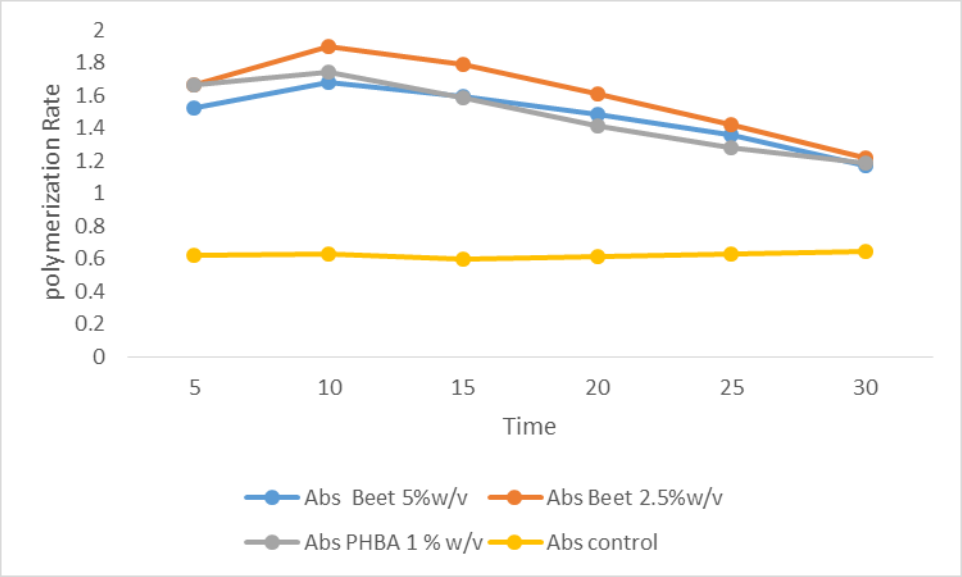

Fig. 4: RBC polymerization inhibition rates by aqueous extracts of Beetroot and Para hydroxybenzoic Acid.

Fig. 4 presented the effect of aqueous extract of beetroot and para hydroxybenzoic acid on sickle cell RBC polymerization inhibition. The initial absorbance of the polymerizing cells was measured at time zero (i.e., immediately after addition of sodium metabisulfite) and subtracted from the final absorbance taken at 30 min. The resulting value divided by 30 gives the rate of polymerization inhibition. It was observed that 2.5 %w/v aqueous extract of beetroot possessed the fastest rate in haemoglobin polymerization inhibition, followed by 5 % w/v aqueous extract of beetroot and para hydroxybenzoic acid. Rate of polymerization inhibition in the control (normal saline) is the lowest because there are no antisickling agents to prevent the polymerization reactions. There was a peak haemoglobin polymerization at 10 min for all the sample. Also, the absence of initial induction of the sickling in the control sample showed lower absorbance but a steady increase after the drop at 15 min which indicated increased polymerization.

It can be inferred that some of the bioactive components in the aqueous extract of beetroot were able to interact with haemoglobin molecules (two or more amino acid residues) or the RBCs membrane to bring about inhibition of the polymerization (Noguchi *et.al*., 1989).

CONCLUSION

The aqueous extract of *Beta vulgaris* was able to reduce the percentage of sickled cells, the rate of haemoglobin polymerization, and the osmotic fragility of human sickled RBCs. Further data analyses suggest that the ability of these natural plant extract to exhibit these properties is probably due to the presence of the identified phytochemicals. Thus, aqueous extract of beetroot may be used as an alternative agent to para benzoic acid or a precursor in ameliorating the sickling in human HbS containing RBCs.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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