Effect of *Heterotis rotundifolia* leaf extract on Hemoglobin-S (HbS) Polymerization, Osmotic fragility and Fe^{2+}/Fe^{3+} ratio.

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ABSTRACT

This study investigated the potential effect of *Heterotis rotundifolia* leaf extract on Human haemoglobin-S (HbS) erythrocyte on three criteria: gelation/polymerization rate, osmotic fragility and Fe^{2+}/Fe^{3+} ratio. Stock solution concentrations of 10.0, 5.0, 3.3, 2.5 and 2.0 percent of the crude leaf extract of *Heterotis rotundifolia* were utilized for polymerization, osmotic fragility and Fe^{2+}/Fe^{3+} ratio experiment. Spectrophotometric technique was used to measure the rate of gelation, level of osmotic fragility and Fe^{2+}/Fe^{3+} ratio. Results show that the presence of increasing concentrations of *Heterotis rotundifolia* extract recorded a progressive decrease in HbS polymerization when compared to the control. Similarly, from the results of the osmotic fragility test, a reduction in HbS erythrocyte fragility was observed in the presence of *Heterotis rotundifolia* leaf extract. The Fe^{2+}/Fe^{3+} investigation recorded no impact on the oxygen affinity of the sickled erythrocyte. The leaf extract of *Heterotis rotundifolia* demonstrated potency in inhibiting the polymerization of sickle cell hemoglobin and reduction in erythrocyte osmotic fragility. These results therefore indicate the feasibility of *Heterotis rotundifolia* as an attractive potential herb for SCD therapy.

1 INTRODUCTION

The most common form of hemoglobinopathy is Sickle cell disease (SCD) and it is known to be one of the most prevalent morbidity and mortality diseases in Africa and Middle-East but has a low incidence in Europe. Abere *et al*., 2015 reported that the homozygous (HbSS) form of Sickle cell disease that is Sickle cell anaemia (SCA) is the most common type of SCD and about 25% of Nigerian population are "carriers" of the sickle cell trait. Sickle cell disease occurs due to point mutation in the coding sequence of the β-globin gene and chain of hemoglobin and its associated with painful crisis. Studies have shown that sickle haemoglobin occurs when the red blood cells are deprived of oxygen (deoxygenated state), they undergo sickling into elongated crescent shape, a change from the normal biconcave disc which results in polymerization into crystals. "[1], [2]".

Reports have shown that *in vitro* investigations of some medicinal plant extracts have demonstrated reduction in polymerization of HbSS molecules and are potential therapeutic herbs in treatment and management of sickle cell anaemia "[3], [4], [1]", aqueous extracts of Cajanus cajan leaf and seed, Zanthoxylum zanthoxyloides leaf, and Carica papaya leaf"[5]", antisickling potential of the ethanol seed extract of *Vigna unguiculata (E1)* and *Vigna subterranean (E2)* "[6]", aqueous leaf extracts of *Basella alba*"[7]". The mechanism of action of these agents is aimed at interacting directly with the membrane architectural components and cellular processes required for erythrocyte functional and structural integrity"[7]". Hence the proponent of this study.

Heterotis rotundifolia (Family: Melastomatacea) commonly known as pink lady is a versatile perennial slender creeping herb with prostrate or ascending stems up to 40cm high, rooting at the nodes and producing from seeds and stolons. Different parts of the plant are utilized traditionally for medicinal purposes of which some have been investigated clinically "[8]".(Abere *et al.,* 2009). Phytochemical studies on the leaves revealed the presence of alkaloids, flavanoids, phenols, polyphenols, tannins, cyanogenic glycosides, anthocyanins, saponins, sapoginin cardiac glycosides and anthraqunone"[9],[10]". The leaves are used in the treatment of diarrhea"[10]", dysentery in Cameroun"[11]", the whole plant for trypanosomiasis by Nupe people of Nigeria"[12]". A literature survey showed that *Heterotis rotundifolia* has antioxidant, anti-ulcer and antibacterial potential "[13]". Offor, "[14]" revealed that the leaves are high in retinol, cholecalciferol, tocopherol and thiamine and low in pyridoxine, niacin, riboflavin, ascorbic acid, phylloqinone and cobalamin.

However, no studies have yet been carried out to investigate the antisickling property of the various parts of this plant. The purpose of this study is to investigate the effect of *Heterotis rotundiolia* crude leaf extract on the HbS erythrocyte gelation rate, osmotic fragility, Fe^{2+}/Fe^{3+} ratio and its possible role in Sickle cell disease management.

2 MATERIALS AND METHODS

2.1 Sample Collection and Identification

Heterotis rotundifolia leaves were obtained from University of Port Harcourt environment, identified and authenticated at the Department of Plant Science and Biotechnology, University of Port Harcourt Herbarium with voucher number UPH/V/1323.

2.2 Preparation of Extract

The leaves were washed under continuous current of water, crushed into pulp with mortar and pestle to extract the juice (undiluted). The extract was kept at 4°C in a refrigerator for at least 24 hours before subsequent tests. Stock solution concentrations of 10.0, 5.0, 3.3, 2.5 and 2.0 percent of the crude leaf extract of *Heterotis rotundifolia* were utilized for polymerization, osmotic fragility and Fe^{2+}/Fe^{3+} ratio experiment.

2.3 Preparation of erythrocyte sample

5ml of blood was drawn from a volunteer through vein puncture who expressed the HbS genotype. The blood sample was collected into EDTA anti-coagulant bottles. Erythrocytes were washed 3 times by centrifuging with

normal saline at 3000rpm for 10mins, and the test carried out with the washed and intact erythrocytes.

2.4 Polymerization Experiment

Sodium meta-bisulfite induced polymerization of molecules of HbS was determined as reported by Iwu *et al*. "[15]" with little modification according to Chikezie *et al*."[16]". The underlying principle is that when molecules of HbS are deprived of oxygen, they experience gelation, transforming to deoxyHbS molecules. Exactly 0.1 ml of HbS hemolysate was added into a test tube and then 0.5 ml of phosphate buffer saline solution and 1 ml of distilled water was introduced afterwards. The mixture was reassigned into a beaker and 3.4 ml of 2g/ml aqueous solution of sodium meta-bisulfite was introduced. Using a spectrophotometer, the absorbance of the mixture was recorded at every thirty seconds for one hundred and eighty seconds (control test). This procedure was repeated by replacing distilled water with one point zero percent of six increasing concentrations (2.0, 2.5, 3.3, 5.0 and 10.0 %) of *Heterotis rotundifolia* extracts (test specimen) respectively. Sodium meta-bisulfite was utilized specifically as a reductant. The intensity of polymerization was deduced by documenting changes in absorbance of the test mixture with succession of time.

Calculations

Percentage polymerization was determined mathematically as proposed by Chikezie *et al*. thus;

% Polymerization=

Where

At/c =Absorbance of test/control assay at time = t (s).

Ac180thsecs = Absorbance of control assay at the 180ths.

2.5 Determination of Erythrocyte Osmotic Fragility

Osmotic fragility of erythrocyte in all samples was determined by a measure of haemoglobin released from red blood cells when placed in an environment containing serial dilutions of Phosphate Buffer Saline (PBS) solution as described by Oyewale "[17]", with minor modifications. 0.1ml of washed red blood cells were suspended in 1.0 ml buffer solution: pH = 7.4 and added to 5ml of the different concentrations of saline i.e. 0.0, 1.0, 3.0, 4.5, 5.0, 7.0, and 9.0g/L of NaCl in different centrifuge tubes. The fifth centrifuge tube contained distilled water. The tubes were then inverted several times to mix its content. The centrifuge tubes were incubated for 30 min at body temperature (37 °C). Subsequently, the contents of test tubes were centrifuged at 1200rpm for 10 min. The supernatant was decanted and haemoglobin content determined spectrophotometrically at λmax = 540 nm using PBS (0.0 g/L) solution as blank. Haemolysis in each test tube was expressed as a percentage, taken as 100% the maximum value of absorbance of the test tube that contained erythrocytes suspended in distilled water (0.0 g/100 ml).

Evaluation of Percentage Erythrocyte Hemolysis.

The percentage of hemolysis was calculated as follows;

% of hemolysis = Absorbance reading of test supernatant÷Absorbance reading of 100% hemolysis, multiplied by 100.

2.6 Determination of Fe^{2+}/Fe^{3+} ratio

The Fe^{2+}/Fe^{3+} ratio was determined by the methods of Davidson and Henry"[18]". The oxygen affinity of hemoglobin and methemoglobin were measured at 540 nm and 630nm respectively. The approach employs lyzing 0.02 ml whole blood in 5.0 ml distilled water and 0.02 ml normal control. To determine the Fe^{2+}/Fe^{3+} ratio, 0.02 ml of the anti-sickling agent was added to 5.0 ml distilled water and 0.02 ml of blood and incubated for 1 hr in a test tube. The absorbances of Hb and metHb were measured according to the method above.

3 RESULTS

The results of all analyses are shown in Tables 1-3. Table 1 shows the effect of the extract on HbS erythrocyte gelation (polymerization) rate. Table 2 depicts the effect of extract on human HbS Fe^{2+}/Fe^{3+} ratio. Table 3 shows the effects of the extracts on the osmotic fragility of human HbS erythrocyte. Results are expressed in percentages (%).

Table 1. Effect of *Heterotis rotundifolia* extract of on human HbS gelation rate

Conc of extract (%)	0sec	30sec	60sec	90sec	120sec	150sec	180sec	ΔOD/3mins	% gelation
Control	1.513	1.522	1.524	1.527	1.527	1.530	1.531	0.018	100
2.0	1.550	1.556	1.560	1.560	1.561	1.562	1.566	0.016	88.89
2.5	1.542	1.548	1.550	1.552	1.553	1.554	1.557	0.016	88.89
3.3	1.538	1.548	1.550	1.551	1.552	1.553	1.553	0.015	83.33
5.0	1.538	1.544	1.548	1.550	1.551	1.551	1.551	0.013	72.22
10.0	1.538	1.542	1.545	1.545	1.547	1.547	1.548	0.011	61

Table 2. Effect of *Heterotis rotundifolia* extract of on human HbS Fe^{2+}/Fe^{3+} ratio

Conc of crude extract (%)	Fe^{2+} OD 540nm	Fe^{3+} OD 630nm	Fe^{2+} / Fe^{3+}
Control	0.274	0.078	3.513
2.0	0.255	0.065	3.923
2.5	0.275	0.076	3.618
3.3	0.264	0.081	3.259
5.0	0.237	0.064	3.703
10.0	0.302	0.095	3.179

Table 3. Effect of *Heterotis rotundifolia* extract of on human HbS Osmotic fragility

Saline concentration %)	D1	% Haemolysis	D2	% Haemolysis	D3	% Haemolysis	D4	% Haemolysis	D5	% Haemolysis
0.0	0.330	100	0.385	100	0.357	100	0.354	100	0.358	100
1.0	0.081	24.55	0.060	15.58	0.052	14.57	0.050	14.12	0.049	13.69
3.0	0.076	23.03	0.063	16.36	0.050	14.00	0.048	13.56	0.046	12.85
4.5	0.066	20.0	0.053	13.77	0.044	12.32	0.071	20.06	0.065	18.16
5.0	0.084	25.45	0.056	14.55	0.040	11.20	0.063	17.80	0.048	13.41
7.0	0.073	22.12	0.061	15.84	0.048	13.45	0.046	12.99	0.046	12.85
9.0	0.079	23.94	0.057	14.81	0.055	15.41	0.102	28.81	0.067	18.723

4 DISCUSSION

The presence of increasing concentration of the extract (Table 1) elicited a progressive decrease in the rate of gelation of HbS erythrocyte with respect to the control. This observation is an obvious reflection of the extract possessing anti-sickling capability to bind and shield the contact points of HbS monomers required for polymerization "[19]". The activity of the extract to decrease sickling could be due to the presence of some bioactive compounds they possess "[20]". The study of Aja et al. "[9]" to evaluate the phytochemical constituents of *Dissotis rotundifolia* leaf and root revealed the presence of the following bioactive compounds; alkaloids, phenols, polyphenols, tannins, flavonoids, cyanogenic glycosides, anthraquinones, anthocyanin, and saponins. The antisickling activity could be lined to their ability either to inhibit *in vitro* polymerization of hemoglobin or some structural modification linked to the environment of hemoglobin by the extracts "[21]").

Patients with sickle cell anaemia are prone to oxidative stress mediated by free radicals which is associated with repeated polymerization to sickled hemoglobin leading to painful episodes or crisis. Clinical investigations have demonstrated that antioxidant molecules act as potent polymerization inhibitors and enhancers of the oxidant status of sickle erythrocyte "[22]". This effect could be attributed to different concentrations of antioxidant molecules such as flavonoids, phenols and polyphenols which have been identified in the leaves and roots of this plant "[20], [9]".

Osmotic fragility index is a measure of the capacity of erythrocytes to withstand osmotic stress "[23]". From the data in Table 2, it can be deduced that the extract reduced the osmotic fragility of sickled erythrocyte when compared to the control, thereby increasing the resistance of erythrocytes to hypotonic lysis. This investigation reveals the ability of the crude extract of *Heterotis rotundifolia* to maintain a stable erythrocyte integrity and inhibitory action on the hemolysis of erythrocyte in induced hypotonic stress state. From the study it can also be deduced that the decreased osmotic fragility indicated the presence of flattened red cells in which the volume-to-surface area ratio was decreased.

Furthermore, Fe^{2+}/Fe^{3+} ratio is an essential parameter for assessing the oxygen affinity of erythrocytes "[24]". Therefore under hypoxic conditions, this ratio decreases resulting in sickle cell disease and a decrease in

oxygen affinity as well. The ability of plant extract to improve Fe^{2+}/Fe^{3+} ratio indicates an increase in the oxygen affinity of the red blood cells. The result of the experiment revealed that the crude extract of *Heterotis rotundifolia* had no remarkable impact on the Fe^{2+}/Fe^{3+} ratio.

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ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Mr Batombari and Miss Akpan Bridget for their assistance during this research.

COMPETING AND CONFLICTING INTERESTS

Authors have declared that no competing and conflicting interests exist.