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Effects of Aqueous Extract of *Terminalia catappa* L. on Membrane Stability of Sickle Erythrocytes

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Authors' contributions

This work was carried out in collaboration among all authors. Author EKC conducted the laboratory work involving blood and plant sample collection, preparations, analysis and manuscript writing. Author PCC supervised the conduct of the research. Author PCA involved in the laboratory procedures and manuscript development. Authors JA and HCN involved in manuscript development. All authors read and approved the final manuscript.

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ABSTRACT

Background: The effects of 800 mg% aqueous extract of *T. catappa* on membrane stability of human erythrocytes of HbSS genotype were investigated using *in vitro* studies.
Aim: The aim of the study was to determine the membrane stabilizing potential of aqueous extract of *T. catappa* on Sickle erythrocytes.
Study Design: Laboratory Experimental Design was used in this study.
Place and Duration: Department of Biochemistry, Imo State University, Owerri, Nigeria. The study was carried out between November, 2010 and May, 2011.
Materials and Methods: Spectrophotometric method was employed in determining the osmotic fragility index of HbSS erythrocytes. The mean corpuscular fragility (MCF) of the control was 0.422 ± 0.80 g/100 ml, whereas that of the test sample was 0.36 ± 0.014 g/100 ml. The percentage

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stabilization for the HbSS erythrocytes, which was calculated using the MCF values, was 14.28%. The MCF values of the control and the test showed significant difference ($p < 0.05$).

Results: The result showed that aqueous extract of *T. catappa* exhibited an increasing capacity to stabilize HbSS erythrocytes membrane.

Conclusion: The present study showed that aqueous extract of *T. catappa* L. exhibited membrane stability potential on Sickle erythrocytes.

Keywords: Sickle erythrocytes; mean corpuscular fragility; genotypes; osmotic fragility; Terminalia catappa.

1. INTRODUCTION

Sickle cell disease (SCD) is an umbrella term that defines a group of inherited diseases, namely; sickle cell anaemia (SCA), HbSC and HbSβ-thalassaemia, which is characterized by mutations in the gene encoding the hemoglobin β-subunit [1]. The mutation causes a single amino acid substitution of glutamic acid for valine at the sixth position on the β-globin chain to produce hemoglobin S (HbS), which polymerizes in hypoxic conditions to give rise to abnormal sickle shaped erythrocytes [2]. The disease is inherited in an autosomal recessive disorder. Homozygous patients are often affected by hypoxia, vaso-occlusive pain crises and strokes because of the aggregation of sickle erythrocytes in the microcirculation [3].

Hemoglobin is the main component of the erythrocytes with the primary function of oxygen transport [4]. The most common adult form of hemoglobin is the Hemoglobin A (HbA), but many variations of hemoglobin have been described [5]. Normal erythrocytes have a flexible biconcave disk-like shape that permits for unhindered channel through microvasculature with an estimated 120-day life span [6]. The rigidity of sickle erythrocytes membrane renders it fragile and prone to osmotic lysis. A normal erythrocyte is flexible and elastic, which enables it to move through narrow blood vessels [7]. Thus, sickle erythrocytes are described as being rigid and distorted because their resistance to hemolysis is reduced [8]. A rigid erythrocyte cannot expand, which implies that it is not flexible and therefore cannot easily move along the narrow human blood vessels. When the erythrocyte osmotic fragility decreases, the resistance increases and vice versa [9]. Therefore, the reduction in osmotic fragility by antisickling agents is an advantage in that it increases the erythrocytes' resistance to lysis. In other words, a rigid and distorted erythrocyte with low elasticity can be fragile and may undergo lysis under little stress [10].

Chikezie et al. [11] asserted that the erythrocyte osmotic fragility of is a composite index of their shape, hydration, and ranged within certain limits. Osmotic fragility is a measure of the resistance of the erythrocytes to undergo lysis under osmotic stress [12]. The test involves exposing erythrocyte to decreasing concentrations of isotonic phosphate buffered saline and measuring the level of haemolysis spectrophotometrically at room temperature. The test is generally useful to ascertain the level of stability and functionality of plasma membrane [13]. Compounds that can significantly promote membrane integrity or destabilization act by direct chemical contact with biomolecules that constitute the architectural structure of plasma membrane [14]. Other compounds may act in such a way that the activities of certain erythrocyte redox enzymes, such as glutathione reductase [15,16], glutathione peroxidase [17] and glucose-6-phosphate dehydrogenase [18], that are required for membrane integrity are compromised. The aqueous extract of *T. catappa* L. showed antioxidant activity as evidenced in its ability to inhibit Fe^{2+}/H_2O_2 induced lipid peroxidation [19]. Hence, the present study aimed to investigate the membrane stability potential of aqueous extract of *T. catappa* on sickle erythrocytes.

2. MATERIALS AND METHODS

2.1 Collection/ Preparation of Plant Specimen

Fresh leaves of *T. catappa* were harvested within the environment of Imo State University Owerri, Nigeria. The plant specimen was identified and authenticated by Professor F. N. Mbagwu at the herbarium, Department of Plant Science and Biotechnology. A voucher specimen was deposited at the herbarium for reference purpose.

The fresh leaves of *T. catappa* were washed under continuous current of distilled water for 15

min. The leaves were crushed and ground with ceramic mortar and pestle. Two grams (2.0 g) of the pulverized specimen was suspended in 100 ml of distilled water and allowed to stand for 6 h at 37°C. The aqueous extract (2.0 g%; w/v) of *T. catappa* was obtained by simple filtration method with Whatman No. 2 filter paper. The filtrates were centrifuged at 1200 xg for 5 min to remove tissue debris. The supernatant was carefully harvested with Pasteur pipette into sterile test tubes.

2.2 Collection of Blood Samples/ Preparation of Erythrocyte Haemolysate

Five millilitres (5.0 ml) of human venous blood samples of HbSS genotype were collected by venipuncture. Blood was collected and stored in EDTA anticoagulant tubes from donor who had been screened for glucose-6- phosphate dehydrogenase deficiency. The blood samples were collected in November, 2010, from hospitalized patients between the age brackets of 18-25 yrs attending clinic at Federal Medical Centre, Owerri.

The erythrocytes were washed by methods as described by [20]. Within 2 h of collection of the blood samples, portions of 1.0 ml of the samples were introduced into centrifuge test tubes containing 3.0 ml of buffer solution (pH = 7.4). The erythrocyte suspension was mixed and centrifuged at 1200 xg for 10 min to separate the erythrocytes from liquid phase. After centrifugation, the supernatant was carefully withdrawn with Pasteur pipette and decanted. The sediment constituted the harvested erythrocyte. The erythrocytes were re-suspended in the buffer and washed three times by similar centrifugation technique. The erythrocytes were finally suspended in 2.0 ml of isotonic phosphate buffered saline and used for analyses.

2.3 Determination of Erythrocytes Osmotic Fragility

Osmotic fragility of human erythrocyte genotype HbSS was determined by a measure of hemoglobin released from erythrocytes suspended in serial dilutions of isotonic phosphate buffered saline. Determination of red blood cells osmotic fragility was carried out based on the method described by [21] with minor modifications as reported by [22]. The fraction of the red blood cells lysed when suspended in isotonic phosphate buffered saline of varying concentrations was investigated by spectrophotometric method. A stock of isotonic phosphate buffered saline, osmotically equivalent to 9.0 g/L NaCl was prepared as follows: NaCl (9.0 g), $Na_2HPO_4.2H_2O$ (1.71 g) and $NaHPO_4.2H_2O$ (2.43 g) were dissolved in 1 litre of distilled water. Dilution of equivalents of 0.9, 0.7, 0.5, 0.3, and 0.2 g/ 100 ml NaCl were prepared respectively. Each dilution had a final volume 5.0 ml [23].

Five millilitres (5.0 ml) of each isotonic phosphate buffered saline (0.9 - 0.2 g/100 ml) was introduced into corresponding test tubes while 5 ml of distilled water was added to the sixth test tubes. A 0.5 ml aqueous extract of leaves of 80 mg% *T. catappa* was delivered into each of the given test tubes (1 - 6). To each test tube, 0.05 ml of red blood cell suspension was added and mixed thoroughly by inverting the test tubes several times. For the control experiment, the same procedure was repeated but devoid of aqueous extract of leaves of *T. catappa*. The suspensions were allowed to stand for 30 min at room temperature after which the contents were centrifuged at 1200 xg for 10 min. The relative amount of hemoglobin released into the supernatant was determined with the use of a spectrophotometer at the maximum wavelength (λ_{max}) of 540 nm. The isotonic phosphate buffered saline and distilled water served as blank and 100% lysis point, respectively.

2.4 Evaluation of Percentage Haemolysis and Stabilization of Erythrocytes

The quotient of absorbance of the content of each test tube (1st - 6th) and the seventh test tube was multiplied by a factor of 100 (Equation 1). The range of values represented the percentage of erythrocyte lysis at each corresponding isotonic phosphate buffered saline concentration (0.9 - 0.2 g/100 ml).

$$\text{Percentage hemolysis (\%)} = \frac{OD_A}{OD_B} \times 100 \qquad (1)$$

Where,

$O.D_A$ = Absorbance of test tube (1st - 6th) supernatant
$O.D_B$ = Absorbance of 7th test tube supernatant

The corresponding concentration of isotonic phosphate buffered saline that caused 50% lysis of erythrocyte defined the mean corpuscular fragility (MCF) [21,24]. The MCF values were interpolated from cumulative erythrocyte osmotic fragility curves obtained by plotting the percentage lysis against concentrations of isotonic phosphate buffered saline.

The relative capacity of the aqueous extract of leaves of *T. catappa* to stabilize or disrupt erythrocyte membrane was evaluated as percentage of the difference between the MCF values of the test and control samples (Equation 2) [25]. Thus;

$$\% \text{ Stability} = \frac{MCF_{TEST} - MCF_{CONTROL}}{MCF_{CONTROL}} \times 100 \quad (2)$$

2.5 Statistical Analyses

The data were analysed by Student's t-test as described by Pearson and Hartley (1996). Mean ($p = 0.05$) was considered significant.

3. RESULTS

3.1 Determination of Osmotic Fragility Index of Human HbSS Erythrocytes (Control)

The MCF of sickle erythrocytes in the absence of aqueous extract of *T. catappa* at various isotonic phosphate buffered saline concentrations and their percentage hemolysis is presented in Table 1.

Table 1. Percentage hemolysis in the presence of isotonic phosphate buffered saline concentrations

Saline Conc. (g/100 ml)	Hemolysis (%)
0.9	1.88 ± 0.80
0.7	3.53 ± 0.99
0.5	39.34 ± 5.77
0.3	67.75 ± 6.52
0.2	76.34 ± 5.68
0.0	100 ± 0.00
MCF = 0.422 ± 0.80 g/100 ml	

The osmotic fragility of HbSS erythrocytes was 0.422 ± 0.80 g/100 ml, i.e. the reference or control MCF index

3.2 Determination of Osmotic Fragility Index of Human HbSS Erythrocyte (Test)

The MCF of sickle erythrocytes in the presence of 800 mg% aqueous extract of *T. catappa* is presented in Table 2. The various isotonic phosphate buffered saline concentrations and percentage hemolysis of the various tests is also presented in Table 2.

3.3 Determination of Mean Corpuscular Fragility and Percentage Stabilization

The MCF of the HbSS erythrocytes in the presence of the 800 mg% of aqueous extract of *T. catappa* is presented on Table 3 in addition to the percentage stabilization and the MCF values of the individual test.

Table 2. Percentages hemolysis and MCF of test samples at various saline concentrations

	Saline concentration (%)	0.90	0.70	0.50	0.30	0.20	0.00
A_1	% Haemolysis	10.9	6.36	10.0	59.09	89.09	100.00
	MCF (g/100 ml)	0.34					
A2	% Haemolysis	10.00	5.00	10.00	71.00	86.00	100.00
	MCF (g/100 ml)	0.37					
A3	% Haemolysis	9.63	14.45	10.80	75.90	87.95	100.00
	MCF (g/100 ml)	0.37					

The MCF for the various test samples i.e. A1, A2 and A3 were 0.34 g/100 ml, 0.37 g/100 ml and 0.37 g/100 ml respectively

Table 3. MCF values of each test, MCF standard deviation and the percentage stabilization

	MCF Value (g/100 ml)	% Stabilization
A1	0.34	-19.00
A2	0.37	-11.90
A3	0.37	-11.90
X±S.D	0.36 ± 0.014	-14.26 ± 3.34

MCF and % stabilization of the HbSS erythrocyte in presence of 800 mg of T. catappa

4. DISCUSSION

There are evidences that aqueous extract of *T. catappa* leaves, possess antioxidant activity as showed in its ability to inhibit Fe^{2+}/ H_2O_2 induced lipid per oxidation as reported by [19]. Jain et al. [26] also ascertained that the ethanol extract leaf extract of *T. catappa* caused dose-dependent inhibition of osmotic induced hemolysis of human erythrocytes as well as extended the clotting time and deformability of HbSS erythrocytes. The finding implicated the role of cross-linking of spectrin, an erythrocyte membrane protein of major structural importance, in oxidative damage of sulphydryl group [27].

Therefore, build up of oxidant contributes to accelerated damage of sickle erythrocytes membrane and aging.

Previous *in vitro* studies [28,29,30], reported the capability of xenobiotics to interfere with erythrocyte membrane integrity and stability. In previous studies [31], MCF of the HbSS and HbAS erythrocytes were considerably higher than that of the HbSS erythrocytes. A plausible biochemical explanation is the biconcave shape of the HbAS erythrocytes, which allowed for more volume of water in a hypotonic solution before stretching the membrane prior to lysis. A relatively higher MCF value indicated that a lower osmotic stress was required to lyse the erythrocyte membrane [32].

The result of these study showed that MCF value tends to be (low/high), which infers that there is membrane stability or otherwise. The present findings were in agreement with previous report [33], which showed that the effect of aqueous extract of *Zanthoxylum zanthoxyloides* (Lam) roots on the membrane stabilities of human erythrocyte plays some stabilizing effects on the erythrocytes membrane and the stabilization was greatest for the HbSS erythrocytes.

Furthermore, the study, investigated the pattern of lysis of human erythrocytes when suspended in varying concentrations of isotonic phosphate buffered saline. These findings were in agreement with previous findings [34,35]. The present report showed that the MCF value of human erythrocytes suspension obtained from venous blood was 0.351 ± 0.06 g/100 ml [13]. In contrast to our findings, Dewey et al. [21] reported 0.465 g/100 ml and 0.415 g/100 ml as MCF values of heparinized blood obtained from two strains of allophenic mice. The difference in MCF values may not be unconnected with the previous reports [36], in which they noted a significant increase in the osmotic resistance of erythrocytes obtained from blood samples stored in EDTA anti-coagulant test tubes.

Likewise, previous reports [25,35] established variations in erythrocyte osmotic resistance amongst animal kingdoms and strains.

5. CONCLUSION

The present study showed that the MCF of sickle erythrocytes of the control sample was significant difference from the test samples. Therefore, 800 mg% aqueous extract of *T. catappa* confers an increased stabilizing effect on the HbSS erythrocytes membrane. The present research findings showed that the aqueous extract of *T. catappa* have membrane stability potential on Sickle erythrocytes membrane, which provides a scientific basis for the traditional use of *T. catappa* for management of SCD. We therefore recommended that further investigations to ascertain the relationship between membrane stability and cellular activities in the presence of aqueous extract of *T. catappa*.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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