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Effects of aqueous extracts of *Zanthoxylum macrophylla* roots on membrane stability of human erythrocytes of different genotypes

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Abstract

The effects of aqueous extract of *Zanthoxylum macrophylla* (formerly *Fagara xanthoxyloides*) roots on the membrane stabilities of human erythrocyte from HbAA, HbAS and HbSS blood were investigated. The determination of mean corpuscular fragility (MCF), which is the concentration of saline causing 50% haemolysis of the erythrocytes, showed that the aqueous plant extract increased the MCF values of the various erythrocytes. There was no significant difference ($p>0.05$) between the MCF values of HbAA and HbAS erythrocytes. However, significant difference ($p<0.05$) was noted between HbAA and HbSS erythrocytes. Percentage stabilization, calculated from the MCF values showed stabilization by the plant extract of 11.11, 10.30 and 14.39 % for the erythrocytes from HbAA, HbAS and HbSS, respectively. At a concentration of 400 µM, phenylalanine, a known anti-sickling compound, produced a stabilization of 17.85% for HbSS erythrocyte under the same experimental conditions used for the aqueous plant extract. Blood viscosity studies indicated a decrease in viscosity of the HbSS blood by the aqueous extract and this reduction was significantly different ($p<0.05$) from that for HbAA and HbAS bloods. The plant extract also showed pronounced reversion of 2% sodium metabisulphite-induced sickling, an effect also observed with phenyl alanine (400 µM). These findings suggest that the plant extract has some role in stabilizing the erythrocyte membranes, thus providing possible molecular basis for earlier reports on the anti-sickling effects of *Zanthoxylum macrophylla* and its use in the management of the sickle cell disease.

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INTRODUCTION

Several reports indicate that the membranes of human erythrocytes from HbAA, HbAS and HbSS blood have varying stabilities as determined from the mean corpuscular fragility (1, 2). It has also been suggested that pharmacological agents that alter membrane stability could be applied in the control of sickling process of erythrocytes (3), which is a major physiological manifestation of the sickle cell disease.

A number of plant products have been described that could serve as agents that alter membrane stability. Indeed, some have been implicated in the management of several human ailments including sickling and sickle cell disease (4, 5, 6, 7, 8, and 9). The role of crude aqueous extracts of *Z. macrophylla* roots in the control of sickling was established by Soforowa *et al* (7). These workers isolated and identified 2-hydroxymethylbenzoic acid as an anti-sickling agent from the root of this plant. However, the possible molecular mechanism for this action was not determined. Recently, Elekwa *et al* (2) reported their findings of the positive role of aqueous extract of *Garcinia kola* (Henckel) seeds, a popularly consumed seed known as 'bitter kola' in Nigeria, in stabilizing erythrocyte membranes of different genotypes.

The present study focuses on providing further clues at the molecular level by investigating the role of the plant extract in erythrocyte membrane stability as determined by mean corpuscular fragility and viscosity studies. This is to further contribute to knowledge on the use of *Z. macrophylla* roots product in sickle cell therapy.

MATERIALS AND METHODS

Phenylalanine was purchased from Merck, W. Germany, while heparin was from Sigma Chemical Co., St. Louis, U. S. A. All other reagents used were of analytical grades. The roots of *Z. macrophylla* were purchased from the herbal line at Ogbete main market in Enugu, Nigeria, and were authenticated at the National Root Crops Research Institute, Umudike.

Blood sample collection

Blood samples were collected from consenting volunteers who visited the University of Port Harcourt Teaching Hospital and Braithwaith Memorial Hospital in Port Harcourt, by veni-puncture into lithium heparinized sterile tubes. The different blood samples were genotyped using standard electrophoretic procedure. The blood samples were stored at about $4^{o}C$ and used within 24 hours of collection.

Preparation of *Z. macrophylla* roots extract

The roots were washed with distilled water and air-dried before being ground into fine powder with an electrical grinder. The powdered root sample was extracted in distilled water (100g / 250 ml) for 24 hours after which the resulting mixture was filtered through a Whatman #1 filter paper. The clear filtrate was stored at $4^{o}C$ and used as stock solution in the experiments reported in this study.

Determination of osmotic fragility

This followed the procedure developed by Parpart *et al* (10) as modified by Elekwa *et al* (2). A 10 g/l solution was made from 100 g/l NaCl stock buffered at pH 7.4 with 150 mM phosphate. Dilutions equivalent to 9.0, 7.0, 6.0, 5.5, 5.0, 4.0, 3.0 2.0 and 1.0 g/l NaCl were made to 50 ml (final volume).

A 0.05ml aliquot of blood sample was added to 5 ml of the various hypotonic solutions, and immediately mixed by inverting several times. The tubes were allowed to stand for 30 minutes at room temperature. The contents were re-mixed and centrifuged for 5 minutes at 1500 g. The absorbance of the supernatant was read at 540 nm using 9.0 g/l NaCl tube as blank. This procedure was repeated for the different blood genotypes of HbAA, HbAS and HbSS, with five samples per genotype. Each blood sample was used thrice and the average taken.

For the effect of the plant extract, 0.5 ml extract (50g/250ml) was added to 4.5 ml of hypotonic solution. The final NaCl concentrations were maintained as with the controls (no addition) by using appropriate stock NaCl solutions. A 0.05 ml aliquot of blood sample was added and the mixture treated as described earlier. The total volume was 5.05 ml, as with the control.

The mean corpuscular fragility (which is the concentration of saline causing 50% haemolysis of the erythrocytes) was obtained from a plot of % lysis against NaCl concentration (g/l).

Determination of blood viscosity

The measurement of blood viscosity was done using an Ostwald viscometer. Three milliliters of distilled water was mixed with 2 ml normal saline, and the mixture was introduced into the viscometer. Using a stopwatch, the time taken for the meniscus of the liquid to fall from the top to the bottom mark on the wall of the viscometer was recorded. The test blood samples replaced the distilled water for the test experiments. All readings were carried out at laboratory temperature of 30^{o} C.

Effects of aqueous extracts and phenylalanine on blood viscosity

This was as reported by Elekwa *et al* (2). The reaction mixture contained 0.25 ml of the aqueous plant extract (100g / 250 ml), 3.0 ml of blood sample and 1.75 ml of buffered saline. The mixture was incubated at 37^{o}C, and the viscosity determined at 30-, 60- and 90-minute intervals using the procedure described earlier. A 0.2 ml of 10 mM phenylalanine solution replaced the extract for the determination of the effect of phenylalanine.

Determination of *in vitro* sickling / reversal of sickling

The determination was as outlined by Elekwa *et al* (2). Three milliliters of HbSS blood was diluted with 0.15 M phosphate buffer (pH 7.4) and mixed with 3 ml of 2% sodium meta-bisulphite. A drop from the mixture was spotted on a microscope slide and covered with a cover slip. Petroleum jelly was applied to seal the edges of the cover completely to exclude air.

Under the microscope, four hundred cells were counted at every 10-minute interval for 60 minutes. At each count, the percentage of sickled cells was noted and the percentage sickled cells calculated. For the reversal of sickling, a 1:20 dilution of artificially sickled cells was made. A 0.55 ml aliquot of the aqueous plant extract (50g/ 250 ml) was added to 5 ml of the diluted, artificially sickled blood. A drop of the mixture was fixed on a glass slide and observed in the microscope as detailed earlier. Two hundred cells were counted at 60-minute intervals for 3 hours. The percentage of sickled cells was determined on a time-dependent basis.

Statistical analysis

The student t-test was applied at 5% confidence level.

RESULTS

The results of the MCF (the concentration of saline causing 50% haemolysis of the erythrocytes) determinations are presented in Table 1. The aqueous extract of *Z. macrophylla* roots increased the MCF values for the three genotypes from 3.60 $\pm$ 0.1 to 4.00 $\pm$ 0.01 for HbAA; from 3.40 $\pm$ 0.10 to 3.75 $\pm$ 0.15 for HbAS; and from 2.80 $\pm$ 0.10 to 3.20 $\pm$ 0.10 for HbSS bloods. There was no significant difference ($p>0.05$) between the MCF values of HbAA and HbAS erythrocytes. However, significant difference ($p<0.05$) was noted between HbAA and HbSS erythrocytes.

Table 1: Median Corpuscular Fragility (MCF) values and % stabilization for HbAA, HbAS and HbSS erythrocytes in the without or with Phenylalanine (400 µ and aqueous extract of *Z. macrophylla* roots [2.0 % (w/v)].

	HbAA	HbAS	HbSS
	(MCF expressed as [NaCl] g/l)		
Control	3.60 $\pm$ 0.10	3.40 $\pm$ 0.10	2.80$\pm$ 0.10*
+ Phe	4.30 $\pm$ 0.12	3.80 $\pm$ 0.14	3.30$\pm$ 0.10*
+ extract	4.00 $\pm$ 0.14	3.75 $\pm$ 0.15	3.20 $\pm$ 0.1*
	(% stabilization)		
+ Phe	19.44	11.77	17.85
+ extract	11.11	10.30	14.39

*(Data represent mean $\pm$ SD of five triplicate determinations; Phe = phenylalanine; *statistically different from HbAA value)*

Phenylalanine, a known anti-sickling agent also increased the MCF values from 3.60 $\pm$ 0.1 to 4.30 $\pm$ 0.12 for HbAA; from 3.40 $\pm$ 0.10 to 3.80 $\pm$ 0.14 for HbAS; and from 2.80 $\pm$ 0.1 to 3.30 $\pm$ 0.10 for HbSS bloods. The % stabilizations, calculated from the MCF values, for the effects of both phenylalanine and the aqueous plant extract showed increased stabilizations.

Table 2: Effects of Phenylalanine (400 μM) and aqueous extract of *Z. macrophylla* roots [2.0 % (wt/vol.)] on the viscosity of blood samples of different genotypes.

	Viscosity ($x10^{-3}$ Pa-S)						
	+ Phenylalanine				+ *Z. macrophylla* extract		
Incubation time (minutes)	0	30	60	90	30	60	90
HbAA	1.7	1.6	1.4	1.4	1.6	1.5	1.5
HbAS	2.1	1.8	2.0	2.0	2.0	2.0	2.0
HbSS	3.4	2.6	1.8	1.3	2.7	2.4	1.9

Phenylalanine caused increased stabilizations of 19.44% (HbAA), 11.77% (HbAS) and 17.85 %(HbSS) while those for the plant extract were 11.11% (HbAA), 10.30%(HbAS) and 14.39% (HbSS) (Table 1).

Blood viscosity determinations are shown in Table 2. At zero minute, the viscosity ($x10^{-3}$ Pa-S) values were 1.7 for HbAA, 2.1 for HbAS and 3.4 for HbSS. In the presence of the aqueous extract of *Z. macrophylla* roots the observed viscosities at 30 minutes following addition of the extract to blood were similar to the control for HbAA and HbAS bloods but decreased from 3.4 ($x10^{-3}$ Pa-S) to 2.7 ($x10^{-3}$ Pa-S) for HbSS blood.

The results of the reversion of metabisulphite-induced sickling by the aqueous extract of *Z. macrophylla* roots are shown in Figure 1. The percentage of sickled cells dropped from an initial value of 80% at zero-time to 44% at 30-minute incubation with the extract. The drop progressed during the subsequent 30-minute samplings times and was 38% after 6 hours incubation with the extract.

DISCUSSION

The osmotic fragility of cells reflect their ability to take up water without lysis and the ability of the normal human erythrocyte (HbAA genotype) to withstand hypotonicity results from its biconcave shape which allows the cell to increase its volume by about 70% before the surface membrane is stretched (10). This osmotic fragility is experimentally denoted by the MCF value, and in this study, the MCF of the HbAA and HbAS erythrocytes were significantly ($p<0.05$) higher than that of the HbSS. A plausible explanation is that the biconcave shape of the HbAA and possibly HbAS erythrocytes allow for more volume for water in a hypotonic solution before 'stretching' of the membrane that progresses to lysis.

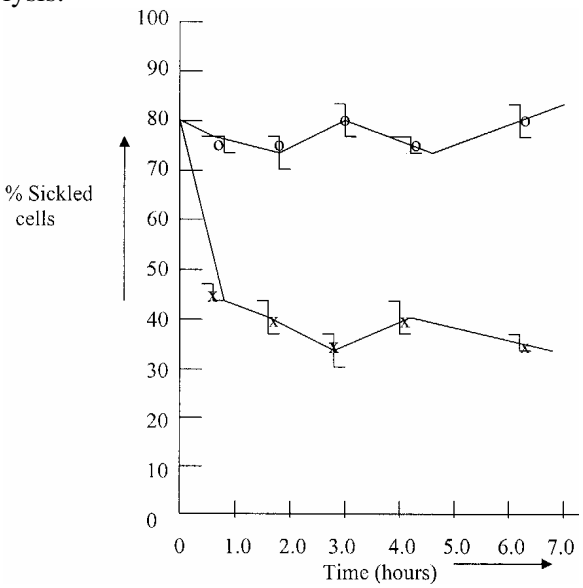

Figure 1. The reversion of sickling induced by 2% sodium meta-bisulphite by aqueous extract of *Z. macrophylla* roots. Data represent mean ± SD for triplicate determinations (o-o, control; x-x, with extract).

Both the aqueous plant extract and phenylalanine produced stabilization of the erythrocytes membranes, reflected in the

increases in MCF values for the different blood genotypes. The stabilization was greatest for the HbSS erythrocyte. It is possible to explain this stabilization effect by noting that these agents made the HbSS erythrocyte to withstand higher concentrations of NaCl by increasing its volume, reverting the shape of the sickling to produce some biconcave shape, and thereby maintain membrane integrity. Such effects have been reported for homoserine that inhibits *in vitro* sickling of HbSS erythrocytes in hypotonic solutions (11). The observed increased stabilization of HbSS erythrocyte by the plant extract suggests beneficial effect to HbSS individuals.

It was observed in the present study that the stabilization by the aqueous extract of *Z. macrophylla* roots was lower than for phenylalanine. This finding differs from the observation of Elekwa *et al* (2) where the stabilization by aqueous extracts of *Garcinia kola* (Henkel), a popularly consumed seed in Nigeria, was higher than that produced by phenylalanine. This suggests that the extract from *G. kola* (Henkel) could be a better agent in the stabilization of human erythrocytes than that from *Z. macrophylla* roots.

The above findings in the present study suggest increase in stability of the membranes by both phenylalanine and the aqueous extract of *Z. macrophylla* roots, and also suggest that the HbSS erythrocyte is the least stable, an observation supported by the reports of Ibeh *et al* (1).

The results of the reversion of metabisulphite-induced sickling by the aqueous extract of *Z. macrophylla* roots (Figure 1) is in agreement with the observations of anti-sickling effects for this plant product (5, 7) commonly used as a trado-medical plant in Nigeria. It is notable that the various effects seen in this study, when compared to the report of Elekwa *et al* (2) on *G. kola*, showed less positive effects by the extracts from *Z. macrophylla* on the parameters investigated.

Nonetheless, the findings of conferring increased stability to the erythrocyte membranes as well as the reversion of sodium metabisulphite-induced sickling provide possible molecular explanation for the earlier reports on the role of the aqueous extracts of *Z. macropylla* roots in the reversion of sickling, hence supporting the use of the extracts in the management of the sickle cell disease.

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