RESEARCH ARTICLE

Interaction of Some Antisickling Agents with Human Erythrocytes Calcium Pump

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ABSTRACT

Sickle cell disease is a red blood cells disorder, which affects millions of people in the world. One of its clinical manifestations is cation fluxes. Although the antisickling potentials of hydroxyurea (HU), *Cajanus cajan* seed (CCS) and *Zanthoxylum zanthoxyloides* (ZZL) have been reported, their interactions with human erythrocytes calcium pump have not been elucidated. The calcium pumps catalyses the hydrolysis of adenosine triphosphate and transport calcium out of the cell thereby maintaining low calcium concentration required by the cells.
Investigation of the interaction of HU, CCS and ZZL with calcium adenosine triphosphatase (Ca^{2+}-ATPase) in human sickle (HbSS) and normal (HbAA) erythrocyte membranes was carried out using in vitro approach.
The results indicated that the catalytic efficiency of Ca^{2+}-ATPase in the absence of antisickling agents in HbAA (36.34 mM^{-1}) was higher than in HbSS (33.23 mM^{-1}). Thus, in the presence of HU, the catalytic efficiency in HbAA and HbSS was 51.59 (mM^{-1}) and 41.14 (mM^{-1}) respectively. In the presence of C. cajan seed in HbAA and HbSS, the efficiency of the enzyme was 5098.33 (mM^{-1}) and 537.083 (mM^{-1}) respectively. However, there were no changes in the catalytic efficiency of the enzyme in the presence of *Z. zanthoxyloides* leaf in both genotypes.
The interaction of the antisickling agents with Ca^{2+}-ATPase indicates a mechanism of antisickling of the agents. This suggests that the agents prevent the red blood cells dehydration by increasing the efficiency of the pump. The plants could be explored in the development of antisickling drugs.

Key words: Sickle Cell Disease, Antisickling agents, Calcium Pump

Introduction

Human red blood cells (RBCs) are very important cells responsible for the transportation of oxygen to all parts of the body. Sickle cell disease (SCD) is a genetic disorder, which affects RBCs. It is characterised by human erythrocytes having abnormal beta-haemoglobin. The abnormality in the haemoglobin results from a mutation in the beta chain haemoglobin amino acid sequence where valine replaces glutamic acid at position six (Ingram, 1957). This point mutation leads to various clinical complications, including haemoglobin polymerisation that leads to vaso-occlusion and ion fluxes that lead to RBCs dehydration. The presence of valine, which is hydrophobic in an aqueous environment, makes the molecule possess a sickle-like structure, and it is referred to as sickle haemoglobin. For decades, SCD has been recognised as a clinical burden that causes the death of millions of people. The disease is common in sub-Saharan African, Asia, India, America and Saudi Arabia. The disease occurs in three ways. Firstly, the sickle cells are stalked at the junction of tiny blood capillaries thereby blocking the flow of blood causing pain and inflammation commonly referred to as vaso-occlusion. Secondly, the sickled cells lysis occur frequently without a commensurate replacement by new cell leading to anaemia and loss of vital cations such as Ca^{2+}, Na^+, K^+ and Cl^-. Thirdly, the red blood cells accumulate in the spleen causing disruption of its functions most especially in children.

One of the biochemical and pathophysiology problems of sickle cell disease is derangement in the ion gradient in the total cellular homoeostasis (Joiner, 1990; Etzion et al., 1996; Lew et al., 2005). It has been reported that intracellular calcium concentration increases in sickle cell disease (Bewaji et al., 1985; Tiffert & Lew, 2011). Also, there is a loss of potassium, chloride and water with a concomitant gain in sodium concentration (Bennekou et al., 2001; Brugnara, 2003; Lew & Bookchin, 2005). This membrane permeability otherwise known as Psickle is a major consequence of sickle cell disease (Bookchin & Lew, 2002; Ellory et al., 2007) which must be taken care of alongside the management of the disease.

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Due to its genetic nature and several unsuccessful attempts in providing a total and lasting solution, the disease appears to be incurable. The only current acceptable management is the application of therapeutic agents. The most widely used and clinically acceptable drug for disease management is hydroxyurea (Brawley et al., 2008; Platt, 2008; Ware, 2010). It increases foetal haemoglobin concentration in the red blood cells through a series of biochemical reactions (Fibach et al., 1993). The more the concentration of foetal haemoglobin, the less dense the sickle cells and the less the clinical complications of the disease (Platt et al., 1984). Although, there are several other agents such as cetiedil, bepridil, potassium tellurite, carbamyl phosphate, sodium cyanate, alkylureas, nicosan (from plant extracts), most of them are not yet clinically acceptable (Oyewole et al., 2008; Oyewole & Malomo, 2009; Imaga, 2010). In an attempt to find safe, inexpensive and readily available therapy for the disease, several types of researches have been conducted on indigenous ethnomedicinal plants. Among the commonly used in Nigeria are the seed of Cajanus cajan and the leaf of Zanthoxylum zanthoxyloides (Nurain et al., 2017).

The disruption of the asymmetric distribution of the cation gradient has been implicated in SCD (Lew & Bookchin, 2005). The consequence of polymerisation of haemoglobin in sickle red blood cells is not only manifested in rigidity and shape of RBCs but also results in membrane damage and therefore an uncontrolled membrane permeability to cations and other molecules. This permeability ultimately leads to a disturbed cellular homoeostasis (Spillman et al., 2013). Investigators have reported that the total Ca^{2+} content of sickle cells is raised above normal compared with that of normal erythrocytes (Eaton & Hofrichter, 1990). Concentrations of cations such as Mg^{2+}, Na^{+} and K^{+} were also reportedly affected by the sickling condition (Spillman et al., 2013). It has earlier been reported that the normal concentration of Na^{+}, K^{+}, and Ca^{2+} across cell membranes is maintained by some of the ATP-dependent cation pumps called adenosine triphosphatases (ATPases) (Carafoli, 1991; Palmgren & Nissen, 2011). These enzymes responsible for keeping these constant asymmetric cation concentrations across cell membranes use the energy from the hydrolysis of ATP. Moreover, Na^{+}/ K^{+}-ATPase and Ca^{2+}-ATPase have been shown to be present in human erythrocyte membranes to maintain these cations' gradient (Elekwa et al., 2005; Niggli & Sigel, 2008). The investigation of the level of activity of these ATPases in different human genotypes depicted that Na^{+}/K^{+}- and Ca^{2+}- ATPase activities were significantly lowest in erythrocytes with haemoglobin S and highest in haemoglobin A (Elekwa et al., 2005).

Materials and Methods

Human Blood Samples

Red Blood Cells were obtained from residual clinical samples submitted to the Clinical Chemistry Laboratory of the University of Michigan Health System for haemoglobin electrophoresis. Samples from patients who had been transfused in the prior three months had received an allogeneic bone marrow transplant, or were taking antisickling drugs were excluded. The phenotype of each sample was confirmed by electrophoretic analysis. Ethical approval was obtained from the University of Michigan Medical School Institutional Review Board (IRBMED ID: HUM00105257).

Plant Materials and Collection

The plant materials used were *Cajanus cajan* seed and *Zanthoxylum zanthoxyloides* leaf. The collection of the plants were carried out in Ilorin, Kwara State, Nigeria. The Plant Authentication was done in the Department of Plant Biology, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria. The vouchers numbers (*C. cajan*: UIH002/189 and *Z. zanthoxyloides*: UIH001/110) were issued in respect of each plant and deposited in the Herbarium of the Department.

Extraction of Plant Samples

After being air-dried in the laboratory and ground into powder using a clean electric grinder, 100 g of each plant sample was extracted in 1 L distilled water for 48 hours, filtered and freeze-dried using LAB-KIT freeze-drying machine. The percentage yields were 7.61% and 4.24% (w/w) for C. cajan seed and Z. zanthoxyloides leaf respectively. The resulting extracts were stored in the freezer at -20°C.

Preparation of Erythrocyte Ghost Membrane

The procedure for the solution of erythrocyte ghost membrane as described by Bewaji et al. (1985) was followed. The human whole blood sample was collected in phlebotomy bottles. The blood was centrifuged at 5.800 x g for 10 minutes using Beckman Coulter (Allegra X-14R) Refrigerated Centrifuge and the plasma removed by aspiration. The resulting erythrocytes were washed two times with a buffer solution containing 130 mM KCl and 20 mM Tris (pH 7.4) by centrifugation at 5,800 x g for 10 minutes. The supernatant was removed after each washing. The packed RBCs were haemolysed in 1 mM EDTA buffered with 10 mM Tris, pH 7.4. This step was carried out by centrifugation for 20 minutes at 18,000 x g and repeated five times or until the content of the tube become whitish depending on the volume of haemolysing buffer used. The white haemoglobin-free RBC membranes were then rewashed twice in a solution containing 10 mM HEPES (pH 7.4). The haemoglobin- free ghost membranes were finally resuspended in a medium containing 130 mM KCl, 20 mM

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HEPES, pH 7.4, 500 µM $MgCl_2$ and 50 µM $CaCl_2$ $2H_2O$ and stored at -20°C. The protein concentration of the prepared erythrocyte membranes was determined by the procedure of Lowry et al., using bovine serum albumin as standard (Lowry et al., 1951).

Assay for the Ca2+-ATPase Activities

For the assay of the activity of Ca^{2+}-ATPase, 500 µL of buffer solution (240 mM KCl, 4 mM $MgCl_2.6H_2O$ and 40 mM Tris buffer), 20 µL of 10 mM $CaCl_2$, 20 µL of drugs/extracts and 10 µL of erythrocyte membrane were pipetted into a set of test tube arranged in triplicates and labelled 1 to 8. The mixtures were incubated for 5 minutes 5oC to equilibrate the temperature of the reaction medium before the reaction starts. Then, 0.0, 10, 20, 30, 40, 50, 60, 80 and 100 µL of 10 mM ATP were added to all the test tubes at 10 seconds interval so that the reaction in all the test tubes have equal incubation time. The volume of reaction medium was made up to 1 mL with distilled water. The reaction was incubated with BOEKEL Scientific incubator for 30 minutes. At the end of the 30th minute, the reactions were terminated by the addition of 200 µL of 5% SDS (w/v) at 10 seconds interval as did for starting the reaction. When the reaction stopped, it was left for 10 minutes with thorough shaking before the addition of freshly prepared 2 mL of mixture of acidified ammonium molybdate and ascorbic acid (Fiske & Subbarow, 1925) for colour development and left for 30 minutes. After 30 minutes, the absorbance of the content of each test tube was read at 820 nm. Control and blank experiments contained all the components of the reaction medium mentioned above except erythrocyte membranes and ATP respectively. The phosphate released during the hydrolysis of ATP by the Ca^{2+}-ATPase was determined spectrophotometrically according to the procedure of Fiske and Subbarow (1925). The principle is based on the reaction of a phosphate group (from ATP) with molybdate (from acidified ammonium molybdate) to form phosphomolybdic acid. The yellow colour of molybdate turned to blue. Hyperbolic Michaelis-Menten and Lineweaver-Burk graphs of the enzyme activity against ATP concentrations were plotted and Km, Vmax and catalytic efficiency of Ca^{2+}-ATPase were determined in each case.

Statistical Analysis

For the statistical analysis, SPSS Version 16 (SPSS Inc, Chicago Illinois, USA 2006 edition) and the data values were expressed as Means ± S.E.M. GraphPad Prism 6 was used for kinetic graphs. The results were analysis using analysed of variance (ANOVA), and a statistically significant association was taken at $P < 0.05$.

Results

The results of this work indicated that the activity of Ca^{2+}-ATPase was inhibited by hydroxyurea and increased by the aqueous plant extracts of *C. cajan* seed and *Z. zanthoxyloides* leaf as shown in the hyperbolic Michaelis-Menten graphs in Figures 1 to 6. The kinetic parameters were obtained from the Lineweaver-Burk plots (inserted in Michaelis-Menten plots). Hydroxyurea inhibited the activity of Ca^{2+}-ATPase in both HbAA and HbSS erythrocytes (Figures 1 and 2).

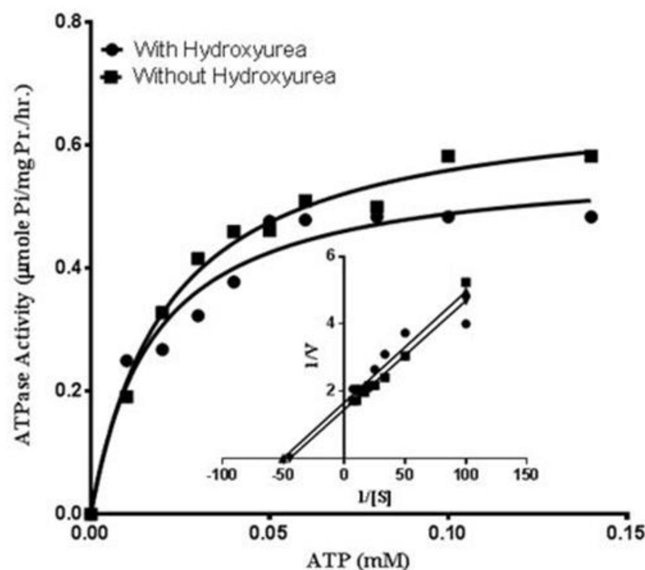

Figure 1. *Michaelis-Menten and Lineweaver-Burk Graphs of Activity of Ca^{2+}-ATPase in the Presence and Absence of Hydroxyurea in Human HbAA.*

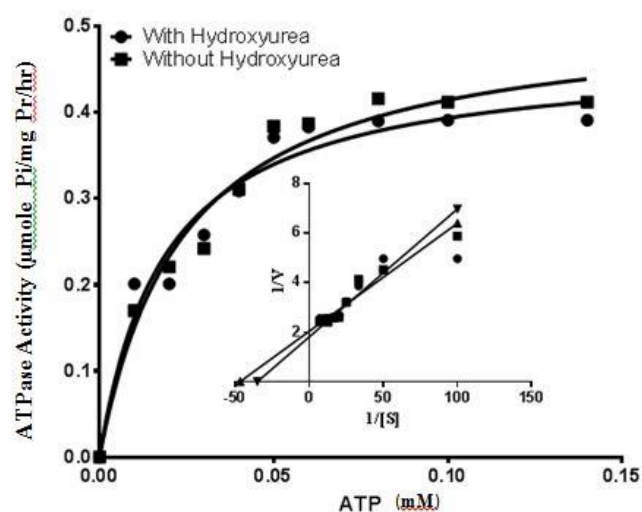

Figure 2. *Michaelis-Menten and Lineweaver-Burk Graphs of Activity of Ca^{2+}-ATPase in the Presence and Absence of Hydroxyurea in Human HbSS.*

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This is indicated by the hyperbolic curves with the symbols (■) and (●) for the presence and absence of the drug respectively. Although, the same pattern of inhibition was overserved in HbAA and HbSS, the activity in HbAA was higher than in HbSS as indicated by the Vmax of the enzyme. However, the activity of the enzyme in the presence of *C. cajan* seed extract was higher than in the absence of the extract as indicated by (■) and (●) respectively (Figures 3 and 4). The same effect was observed in the presence of the aqueous extract of *Z. zanthoxyloides* leaf (Figures 5 and 6).

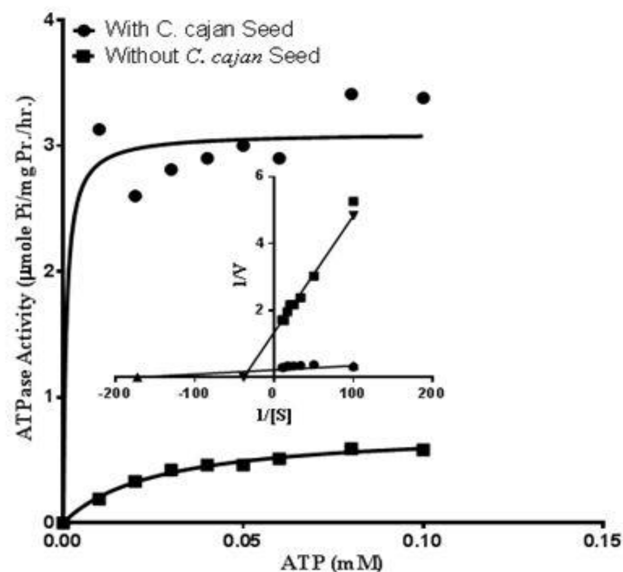

Figure 3. *Michaelis-Menten and Lineweaver-Burk Graphs of Activity of Ca^{2+}-ATPase in the Presence and Absence of C. cajan Seed Extract in Human HbAA.*

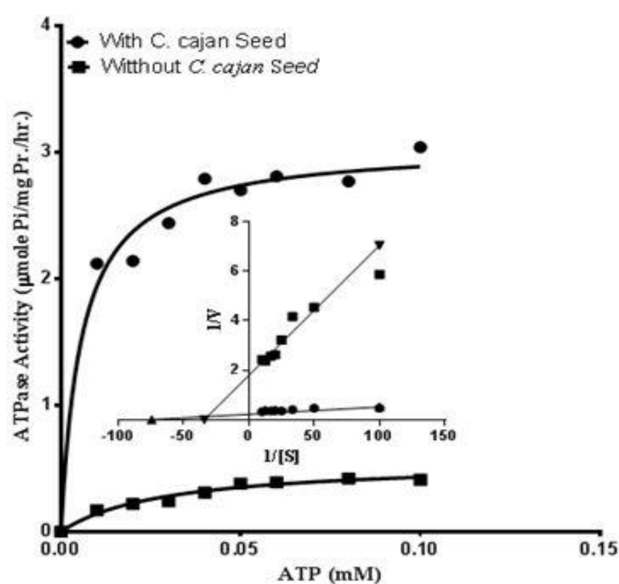

Figure 4. *Michaelis-Menten and Lineweaver-Burk Graphs of Activity of Ca^{2+}-ATPase in the Presence and Absence of C. cajan Seed Extract in Human HbSS.*

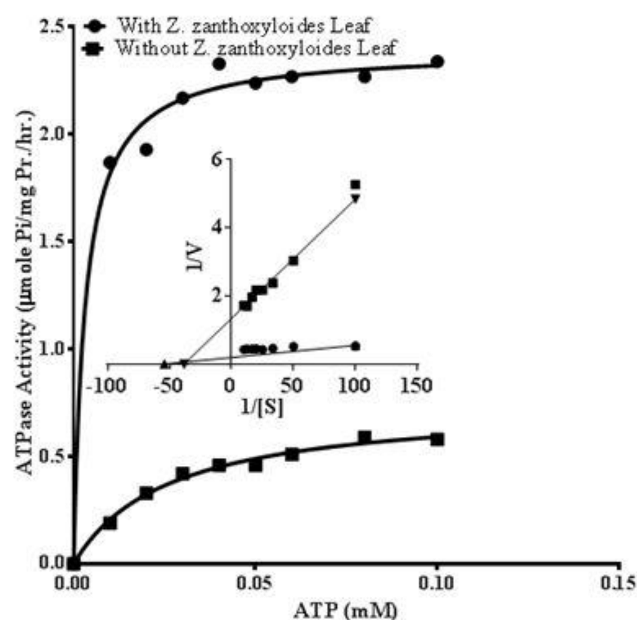

Figure 5. *Michaelis-Menten and Lineweaver-Burk Graphs of Activity of Ca^{2+}-ATPase in the Presence and Absence of Z. zanthoxyloides Leaf Extract in Human HbAA.*

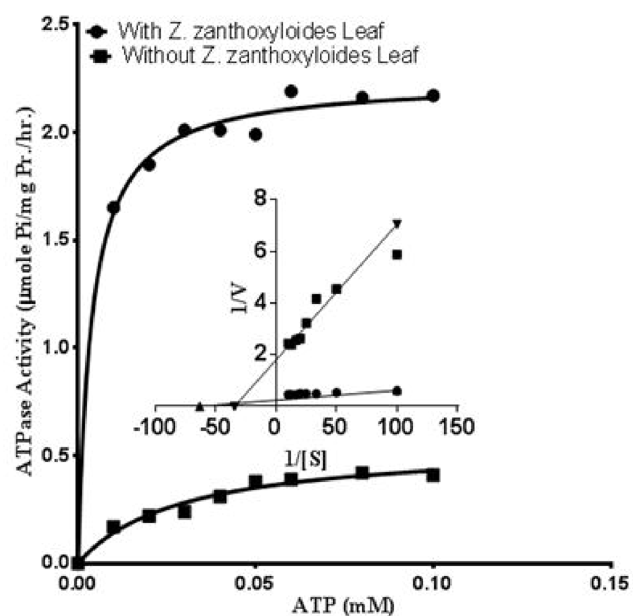

Figure 6. *Michaelis-Menten and Lineweaver-Burk Graphs of Activity of Ca^{2+}-ATPase in the Presence and Absence of Z. zanthoxyloides Leaf Extract in Human HbSS.*

Some kinetic parameters of Ca^{2+}-ATPase were determined to know the effects of administration of hydroxyurea and the plant extracts on them. The parameters determined were Km, Vmax, Kcat and catalytic efficiency of the enzymes.

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The Km of Ca^{2+}-ATPase in human normal erythrocytes (HbAA) in the absence of antisickling agents (0.041±0.01 mM) was significantly higher (P<0.05) than in the presence of hydroxyurea (0.022±0.00 mM), *C. cajan* seed (0.001±0.00 mM) and *Z. zanthoxyloides* leaf (0.010±0.00 mM) the plant extracts (Table 1). This is indicated by the superscript letters a, b, c, and d. However, while the Vmax of Ca^{2+}-ATPase in the presence of hydroxyurea (0.681±0.04 µmole Pi/mg protein/hour) was significantly lower, it was significantly higher in the presence of *C. cajan* seed (3.059±0.12 µmole Pi/mg protein/hour) and *Z. zanthoxyloides* leaf (2.914±0.11 µmole Pi/mg protein/hour) when compared to the velocity in the absence of the agents (0.894±0.11 µmole Pi/mg protein/hour) (Table 1). The rend of observation was recorded for HbSS (Table 2). The catalytic efficiency of an enzyme is a measure of the actual performance of the enzyme per unit time in the presence of its substrate. It is expressed as a ratio of Kcat to Km (Kcat/Km). The results in this work indicated that the catalytic efficiency of Ca^{2+}-ATPase was highest in the presence of *C. cajan* seed followed by *Z. zanthoxyloides* and hydroxyurea. The efficiency of the enzyme was lowest when there were no antisickling agents (Tables 1 and 2).

Discussion

Drepanocytosis, popularly known as sickle cell disease affects ion transport in humans. The disease is characterised by red blood cell, which assumes an abnormal sickled rigid non-elastic or nonflexible shape, which result in various clinical complications (Tosteson, 1955; Tosteson et al., 1955). One of the clinical manifestations of SCD is cellular dehydration, which results in the loss of K^+ and water with a concomitant gain in Na^+. Also, there is an intracellular influx of Ca^{2+} resulting in the increase in the concentration. Adenosine Triphosphatases are a group of enzymes that perform the roles of transporting ions with intrinsic activity to catalyse biochemical reactions. They are also known as transport ATPases. Their activity coupled the transport of ions like Ca^{2+}, Na^+, K^+, H^+ and Mg^{2+} with the hydrolysis of ATP into ADP and Pi. The movement of these ions by the enzymes has been reported to play a vital role in maintaining erythrocyte membrane stability and integrity (Jinks et al., 1978; Hoffmann & Dunham, 1995). Therefore, the interaction of some antisickling agents is investigated on these transport enzymes to know their mechanism of antisickling action and whether they maintain the membrane integrity as part of their antisickling properties.

The inhibition of the activity of calcium pump as observed in this work could be attributed to several reasons which include its cytotoxicity effect on the enzyme. Sometimes also the hyperactivity of the pump leads to a reduction in the concentration of intracellular calcium, which could affects cell functions. So, the inhibition by hydroxyurea prevent this as part of it antisickling properties. It was also recorded that the activity of the enzyme in HbAA was higher

Table 1: *Effect of Some Antisickling Agents on Some Kinetic Parameters of Ca2+-ATPase in Human Erythrocytes with HbAA*

Antisickling Agents	Km (mM)	Vmax (µmole Pi/mg Pr./hr.)	K cat (µmolePi/mg Pr./hr.)	K cat/Km (mM-1)
Without Agent	0.041±0.01[a]	0.894±0.11[a]	1.490	36.341
Hydroxyurea	0.022±0.00[b]	0.681±0.04[b]	1.135	51.591
Cajanus Cajan Seed	0.001±0.00[c]	3.059 ± 0.12[c]	5.098	5098.333
Zanthoxylum zanthoxyloides Leaf	0.010 ±.00[d]	2.914 ± 0.11[d]	4.857	485.667

Each experiment was repeated at least three times. Data represent Mean ± S.E.M for triplicate determinations. Values with a, b, c, and d down the column are significantly different (P<0.05).

Table 2: *Effect of Some Antisickling Agents on Some Kinetic Parameters of Ca2+-ATPase in Human Erythrocytes with HbSS*

Antisickling Agents	Km(mM)	Vmax (µmole Pi/mg	K cat (µmole Pi/mg Pr./hr.)	K cat/Km (mM^{-1})
Without Agent	0.031±0.00[a]	0.618±0.14[a]	1.030	33.226
Hydroxyurea	0.022±0.01[b]	0.543 ± 0.11[b]	0.905	41.136
Cajanus cajan Seed	0.012±0.00[c]	3.867±0.13[c]	6.445	537.0.83
Zanthoxylum zanthoxyloides Leaf	0.010±0.00[d]	2.914±0.01[d]	4.857	485.667

Each experiment was repeated at least three times. Data represent Mean ± S.E.M for triplicate determinations. Values with a, b, c, and d down the column are significantly different (P<0.05).

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than in HbSS. There are several interactions of Ca^{2+}-ATPase with many different proteins such as Protein Kinase C, calmodulin and the membrane phospholipids that normally present in the erythrocytes membrane and act as activators of the enzyme. This observation could also be due to the higher intracellular concentrations of Ca^{2+} in sickle cells, which cause severe problems in such a way that it causes changes in the morphology of the erythrocytes. So, inhibition of the activity of the enzyme is necessary to normalise this higher concentration. It may also cause an increase in the efflux of K^+ or it may lead to inhibition of Na^+/K^+- ATPase (Palma et al., 1994). The increase in the intracellular concentration Ca^{2+} has been implicated in activation of Ca^{2+} dependent K^+ channel (Meech, 1978; Latorre et al., 1989). In reversing the dehydration of sickling cell, the rate of Cl^-, K^+ and water loss must be inhibited. Thus, hydroxyurea is observed in this research to inhibit the activity of Ca^{2+}-ATPase to prevent the dehydration due to loss of water and K^+.

However, the effect of *C. cajan* seed and *Z. zanthoxyloides* leaf extracts activated the activity of calcium pump. The use of these plants in folk medicines has been reported. But the investigation of the effects of these plants on the transport enzymes as a mechanism of their action has been neglected in the sickle cell disease research. The activities were higher in erythrocyte membrane with HbAA than HbSS. The increase in the extracts concentration resulted in the increase in the activities of the enzymes. However, at higher concentration, the activity tends to remain unchanged. At this point, when an increase in concentration leads to no increase in activity, it could be suggested that all the active sites of the enzyme have been occupied. It should be noted that plant extract composed of many different phytochemicals or secondary metabolites that could be responsible for its therapeutic potential. It could be that the phytochemicals were able to modulate the activities of the enzymes. Several works have been done on the phytocomponents of *C. cajan* to elucidate the reason for its potentials as antisickling (Imaga, 2010; Mohanty et al., 2011). Thus, the mechanism of action of the plants as antisickling agents could be as a result of their phytochemical components. The ability of this plant to modulate the activity of the enzyme may be due to the presence of the phytochemicals such as alkaloids, tannins, saponins, glycosides and flavonoids (Nurain et al., 2017)

Another important known antisickling plant is *Z. zanthoxyloides*. The effect of the leaf extract of the plant on the activity of ATPases was also studied. *Z. zanthoxyloides* has been reported to contain many phytochemicals including divanilloylquinic acids and phenylalanine that could be responsible for its therapeutic potentials (Banso & Ngbede, 2006; Ouattara et al., 2009; Nurain et al., 2017). The modulatory activity of this plant extract may be due to the ability of its phytochemicals to interact with the active sites of the enzyme. The observation may be because of the morphology of the sickle cells.

Adenosine Triphosphatases are intrinsic enzymes that coupled the hydrolysis of ATP into ADP and Pi with the transport of ion from one cellular compartment to another usually from/to intracellular to/from extracellular environments. These activities together with the impermeable properties of the plasma membrane enable them to maintain the constant red blood cell homoeostasis. The activities of ATPases also keep the asymmetric ion gradient in the RBCs, a condition at which the cells function excellently. However, in the disease state, e.g., sickle cell disease, the derangement in the asymmetric gradient has been implicated. The functions of RBCs are affected by the availability of oxygen. Thus, in patients with sickle cell disease, the RBCs without adequate oxygen are defective. This oxygen is also dependent on the cell shape and cation content. This also related and influenced by the cellular calcium overload. The changes from normal biconcave to the sickled shape of the sickle cells in the intracellular environments in the absence of oxygen is due to crystallisation of insoluble sickled iron-deficient haemoglobin (Tosteson, 1955; Tosteson et al., 1955). The intracellular calcium overload, loss of K^+, Cl^- and water with the corresponding gain in sodium experienced in the RBCs would have definitely resulted from the up regulation or down regulation of the activity of the membrane-bound ion transporters, ATPases (Jason et al., 2012).

The inhibition of hydroxyurea on Ca^{2+}-ATPase is uncompetitive in HbAA (Figure 1) but mixed competitive in HbSS (Figure 2) respectively. The Km, Vmax and catalytic efficiency of Ca^{2+}-ATPase were all affected in the presence of hydroxyurea and the plant extracts. Both Km and Vmax decreased in the presence of hydroxyurea in HbAA and HbSSerythrocytes. The Km of the enzyme in the presence of antisickling agents was significantly lower than in the absence of the agents. This suggested that the agents were able to increase the affinity of the enzyme for its substrate (ATP). In terms of increasing substrate affinity of the enzyme, hydroxyurea has the highest effect. This could be as a result of its synthetic nature and that of the plant could be as a result of the presence of impurities. The same trend was observed in HbSS except that the effects were lower in HbSS. The catalytic efficiency of the enzyme in both HbAA and HbSS were also activated in the presence of all agents. This suggested that the antisickling agents perform their roles in the management of sickle cell disease by activating the efficiency of the enzyme in removing excess calcium ion from the cell.

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