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In *vitro* Effects of *Chrysophyllumalbidum* Leaf Extract on Transport Atpase in Sickling

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ABSTRACT

Effects of ethanolic leaf extract of *Chrysophyllumalbidium* on erythrocyte ATPases, using phenylalanine model was examined. Mg^{2+}-, Ca^{2+}- and Na^{+}-, K^{+}-ATPases activities expressed as µmole pi/mg protein/hr $x10^{-3}$ were assayed in the AA and SS erythrocyte membrane preparations using varied concentrations of extract. Results revealed significantly higher activity ($p<0.05$) of Mg^{2+}-ATPases (250.8 ± 4.7) for SS erythrocyte membrane when compared to that of AA (108±2.32) in the absence of extract. In the presence of phenylalanine and extract, a dose-dependent significant decrease in activity ($P<0.05$) was obtained. Ca^{2+}-ATPase, activity, in the absence of extract, were 178±0.99 for SS and 231.50±28.77 for AA erythrocyte membrane preparations. Phenylalanine and the extract increased this value significantly ($P<0.05$). Na^{+}-, K^{+}-ATPase activity, in absence of the extract, was significantly lower ($P<0.05$) in SS (184.60 ± 2.3) when compared to AA (220.6 ±7.7) erythrocyte membranes. Phenylalanine showed a dose-dependent significant increase ($P<0.05$) in the activity of Na^{+}, K^{+}ATPase when compared to that of the control. The extract showed significant decrease ($P< 0.05$) in the activity of Na^{+}, K^{+} at low concentration but showed higher activity at higher concentrations. This study suggests that mode of action of extract of *C. albidium* could be the ability of the phytochemicals to interact with transport ATPasesand so correct the SS erythrocyte's faulty ion pump and enhance its cellular volume.

Keywords: *Chrysophyllumalbidium*, *transport ATPase*, Sickle cell disease, leaf extract

INTRODUCTION

Sickling is the development of sickle cells in the blood.Sickle-cell disease (SCD) or drepanocytosis is an autosomal recessive hereditary blood disorder with over dominance, characterized by red blood cells that assume an abnormal, rigid, sickle shape.Sickling decreases the cells' flexibility and results in a risk of various complications. The sickling occurs because of an alteration in the hemoglobin gene [9]. The precise difference between normal and sickle-cell haemoglobin is identified from amino acid substitutions in protein. In sickle-cell haemoglobin, a single amino acid in the beta chains is altered [28]. A single amino acid substitution (Glutamic acid to Valine) at the sixth position of homozygous state results in sickle-cell anaemia (termed HbSS); whereas in the heterozygous state a mixture of normal (HbA) and abnormal (HbS) beta chains is produced resulting in a sickle- cell trait,(termed HbAS) with only one chromosome carrying the abnormal gene.

Sickle cell disease is known to be one of the diseases ravaging most world populations cutting across nations and ethnic divide. Each year, over 300,000 children born are affected by drepanocytosis and half of them die before the age of five years [7,21]. In Africa, 20-25% of the Sub-Saharan population suffers from or transmits sickle celldisease, with Nigeria having the highest incidence [2, 30]. A number of deficiencies are caused by abnormal haemoglobin, for instance HbS. Under hypoxic conditions, deoxy-HbS molecules polymerize, forming rigid, sickle cells; leading to the deformation of the normal disc biconcave RBC [20]. This polymerization of the sickled-cells causes the red cell membrane to lose its functional abilities resulting in loss of K^{+} and water and a corresponding gain of Na^{+}. More so, during sickling, increased intracellular free Ca^{2+} occurs, resulting in loss of K^{+} with accompanying movements of Cl^{-} and water. Consequently, small blood vessels are blocked by the clumping of sickled RBC's, preventing blood supply [1, 25].

ATPases, in general, are membrane transporters with intrinsic catalytic activity. Transport ATPases are ion motive proteins that couple the transport of ions such as H^{+}, H^{+}/K^{+}, Ca^{2+},Na^{+}/K^{+}, Mg^{2+} across membranes with the hydrolysis of ATP. The unidirectional active transport of ions such as Na^{+}, K^{+} and Ca^{2+}, has been reported to play major role in maintaining the stability of the erythrocyte membranes [24]. The presence of three different Adenosine triphosphate, Na^{+}, K^{+} - ATPase, Ca^{2+} - ATPase, and Mg^{2+} - ATPase in human erythrocyte membranes has been reported [10,18]. The determination of the activity levels of these ATPases in different human genotype has shown that Na^{+} -, K^{+}- and Ca^{2+}- ATPases are significantly lower in HbSS erythrocytes than HbAA one, while Mg^{2+}-

ATPase was significantly higher in SS erythrocyte than in AA erythrocytes [3, 18]. The study of [5] also indicated that sickle cell membrane-bound Ca^{2+} ATPase activity is lower than that of the normal erythrocyte. The normal low calcium concentration in the human erythrocyte is assured by a powerful calcium extrusion pump. Without this, elevated intracellular calcium destroys normal red shape and plasticity, increases potassium leak transport and inhibits the Na^{+}, K^{+} pump [19, 26, 27]. The Ca^{2+} - ATPase of erythrocyte membranes has been implicated in the maintenance of red cell volume. There is a possible Ca^{2+} pump failure in HbSS erythrocytes [3, 26]. This partly explains the accumulation of Ca^{2+} by those cells. Ca^{2+} accumulation by HbSS erythrocytes leads to changes in shape which are unfavourable to the survival of the cell. It has been suggested that sickling of red cells could be reversed if excess Ca^{2+} in the red cells is pumped out. Deoxygenation increases membrane permeability to Mg^{2+} leading to a net loss of intracellular Mg^{2+} [5,25].

A number of plant species, some of which had been used in traditional-medical practice have shown promising results either as possible anti-sickling agent, or as playing useful role in sickle cell disease management. [19, 22,25]. One of such plants that are commonly used to ameliorate the sickle cell crisis in Eastern Nigeria is *Chrysophllumabidum*.*Chrysophllumabidum* is a native of many parts of tropical Africa. The tree is a representative of the family Sapotaceae and is a common feature in a closed forest, also it is frequently planted in village compounds and village squares. The tree inhabits the low forest of Nigeria and the fruit is eaten throughout the country. In the Northern Nigeria it is commonly found in the Plateau region [29]. The fruits each with five shiny seeds are greenish when unripe but pale orange in colour when ripe. The fruits which appear in July ripen between December and March. The sweet pleasantly acid fruit pulp is the edible portion. The plant is used in traditional medical practice in Southern Nigeria for the treatment of blood diseases including sickle cell.

In Nigeria and most parts of developing countries, especially among the lower socio-economic class who cannot afford the high cost of Western medicine as well as traditionalists who simply believe in their efficacy, medicinal plants capable of ameliorating the painful crisis associated with SCD are continuously being investigated with a view to contributing to the search for substances that would be effective in solving the sickle cell disease problem. This study therefore is aimed at evaluating the bioactive constituents and the effect of *Chrysophyllumalbidium* leaf extract on AA and SS erythrocyte transport ATpases using phenylalanine as a model, identifying if ion transport and hence membrane stability is involved in its mode of action as an anti-sickling agent.

MATERIALS AND METHODS

Plant collection and preparation

The plant leaves were collected in December 2008 from the forest of Nkari Community in AkwaIbom State, Nigeria. A specimen of the collected leaves was identified by the taxonomist in the Department of Botany, University of Nigeria, Nsukka; where a voucher specimen was deposited for further reference. The leaves were dried in air and ground into powder in a mixer. An amount of 200g of the finely powdered leaves were macerated with 95% ethanol at ambient temperature (3 x 4L x 48 h) and concentrated using a rotary evaporator. The leaves extract was stored in a clean, well-labeled container for use in the subsequent experiments. Preliminary phytochemical analysis of the extract was performed based on procedures, outlined by [15, 28]. All the reagents used were of analytical grade.

Blood Collection

4-5 ml of blood samples from volunteer sickle-cell patients were collected by venipuncture into lithium heparinized sterile tubes at the University of Nigeria Nsukka Medical Centre. The blood samples obtained were from sickle- cell patients who were not transfused for the past five months. The volunteers, who aged between 17-25 yrs. and of both sexes, were in reasonably good health. The genotypes of the patients were confirmed by cellulose acetate paper electrophoresis. All the experiments were carried out with fresh blood samples.

Red Cell Membrane Preparation

The procedure of [16] was adopted. The freshly collected whole blood sample was centrifuged at 5, 000 g^{-1} for 10 minutes at room temperature. The resultant precipitate was washed four times with 0.15M NaCl (pH7.4). The final precipitate was lysed by swirling in 5mM $NaH_2PO_4.2H_2O$ (pH7.7) and was centrifuged at, 5000 g^{-1} for 10 mins. The resultant precipitate was washed with 10mM tris-HCI (pH7.7) and suspended in 3ml distilled water. The isolated membranes obtained were stored in the refrigerator (4^0C). It was used within 24 hours after collection of blood samples. The determination of protein concentration of the membrane preparations was carried out by the Biuret method as outlined by Gornall*et al* [15] using bovine serum albumin (BSA) as standard.

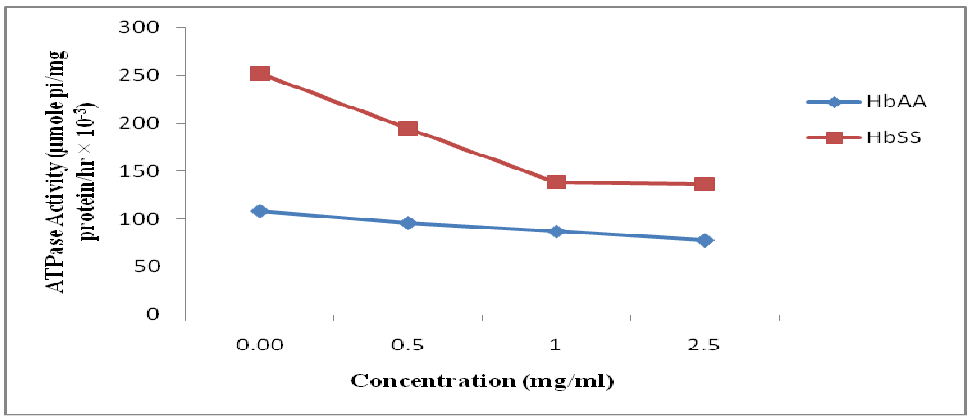

Fig.-1a: Effect of phenylalanine on erythrocyte membrane magnesium ATPase activity

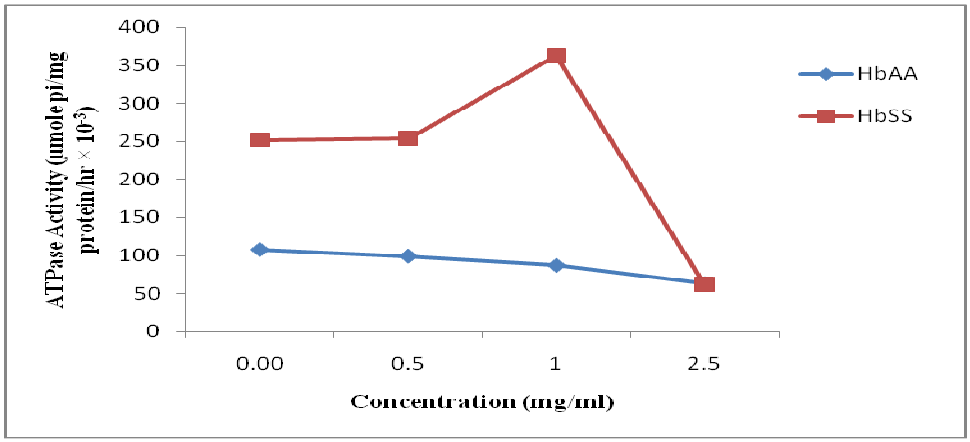

Fig.-1b: Effect of *C. albidium* on erythrocyte membrane magnesium ATPase activity

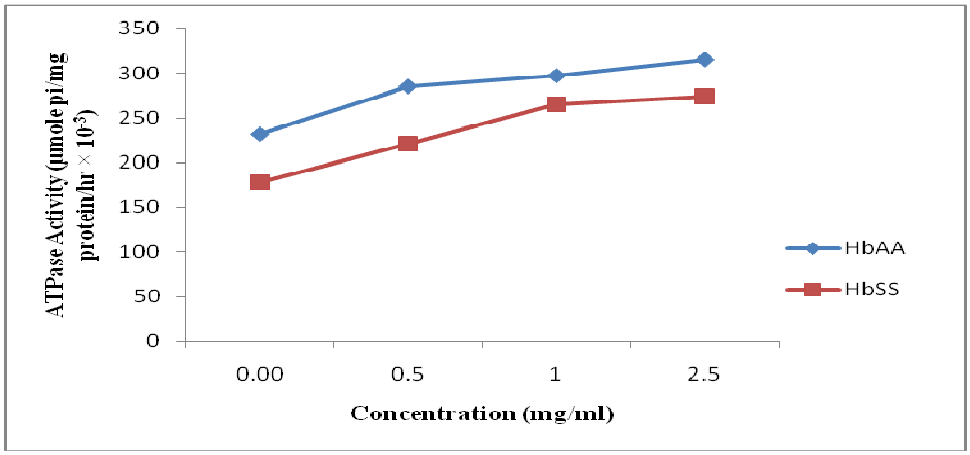

Fig.-2a: Effect of phenylalanine on erythrocyte membrane calcium ATPase activity

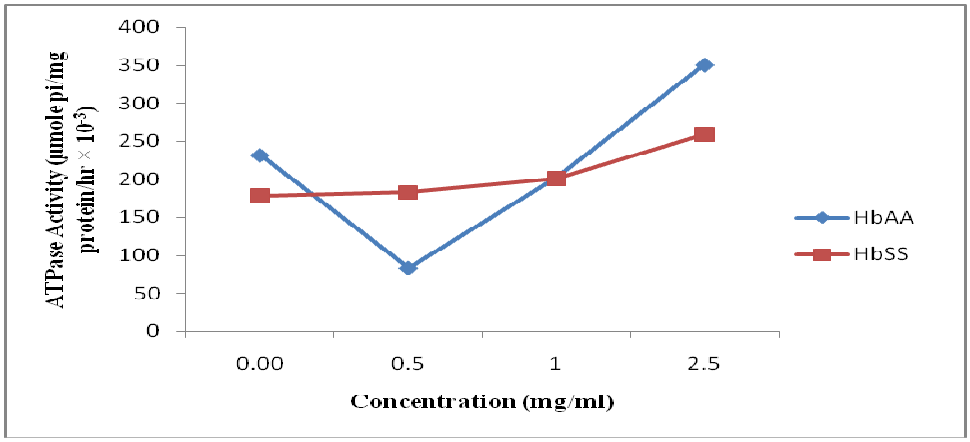

Fig.-2b: Effect of *C. albidium* on erythrocyte membrane calcium ATPase activity

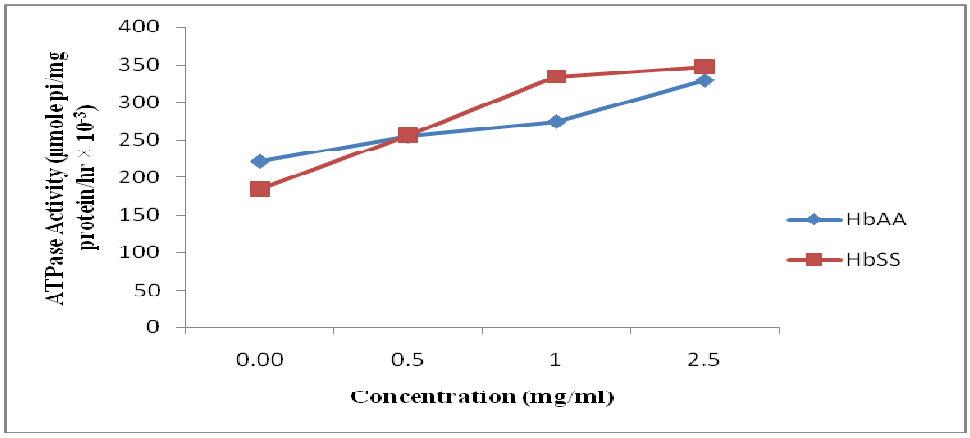

Fig.-3a: Effect of phenylalanine on erythrocyte membrane sodium-potassium ATPase activity

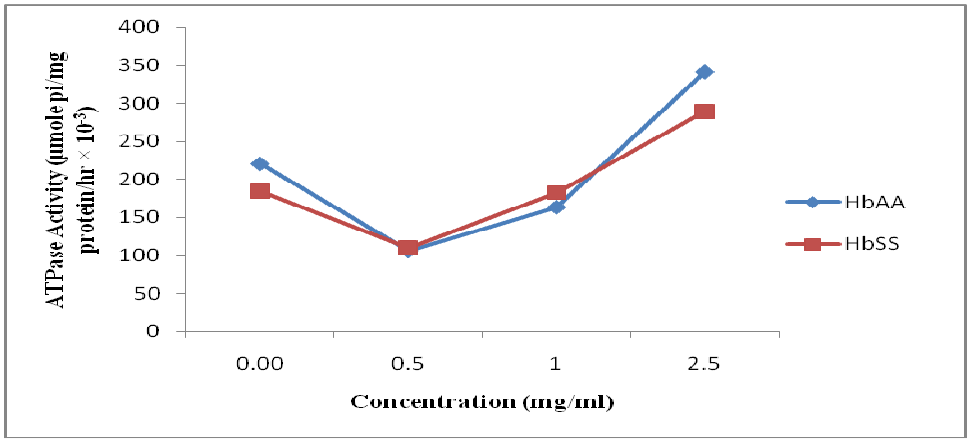

Fig.-3b: Effect of *C. albidium* on erythrocyte membrane sodium-potassium ATPase activity

Enzyme Activity Assay

The assay for the enzyme activities in SS and AA erythrocytes membranes followed the procedure outlined by [17]. Inorganic phosphate released from ATP was monitored using themodified methods of [14]. The effects of ethanolic

leaves extractof C. *albidium* on the transport ATPases (Na^+-, K^+- ATPase, Mg^{2+}-ATPase, Ca^+-ATPase) of HbSS and HbAA erythrocyte membranes were determined. The extract concentrations used in mg ml^{-1} were 2.5, 1.0 and 0.5 respectively. The tubes of each reaction mixture for Mg^{2+} ATPase contained 0.5ml of the extract, 0.5ml each of 21 mM $MgCl_2$, 10 mMTris-HCl (pH 7.4) and 8.0 mM ATP-Na_2. While that of Ca^{2+}-ATPase, contained 0.5ml of the extract, 21 mM $MgCl_2$, 17.5 mM $CaCl_2$, 10 mMTrisHCl (pH7.4) and 8.0mM ATP-Na. The reaction mixture for Na^+, K^+-ATPase contained 0.5ml of each extract, 0.35M NaCl, 17.5 mMKCl, 21.0 mM $MgCl_2$, 10 mMTris-HCl (pH7.4), and 8.0mM ATP-Na_2. The reaction was initiated by adding 0.2ml erythrocyte membrane preparation. The reaction mixture was incubated at 37^{o}C for one hour and the reaction terminated by the addition of 0.8ml ice-cold 10% (w /v) TCA. The resultant solution was left to stand for 20 min at 4^{o}C and then centrifuged at 4000g for 5 min. The concentration of phosphate produced was measured by the method of [14]. Different concentrations of the extract were treated separately and in duplicate. Data were reported as mean $\pm$ SEM, where appropriate. One way analysis of variance (ANOVA) was used to analyze the experimental data and Duncan multiple test range was used to compare the group means obtained after each treatment with control measurement. Differences were considered significant where $P \leq 0.05$. The statistical package used was SPSS, version.

RESULTS AND DISCUSSION

Erythrocyte membrane transport ATPase activity

The erythrocyte membrane transport ATPase activity was studied using the standard Phenylalanine model and the crude extract of *C. albidium*. The results of the effect of phenylalnine on erythrocyte membrane Magnesium ATP is presented in Fig. 1a while that of C. albidium is shown in Fig. 1b. At zero concentration Mg^{2+} ATPase was significantly elevated ($p<0.05$) in HbSS than in HbAA erythrocyte membrane preparation in both cases. Phenylalanine decreases Mg^{2+}ATPases activities in HbSS and HbAA erythrocytes membrane preparations while the effect of the extract on erythrocyte membrane Mg^{2+}ATpase activity (Fig. 1b) showed inconsistency. However this observation agrees with the results of other workers [4, 24]. The inconsistency could be attributed to unique pathophysiology of the genotypes of the erythrocytes or difference in physico-chemical properties of the membrane phospholipids or effect of interaction of the membranes with phytochemicals contained in the extract or even the difference in the conformational change of membrane phospholipids.The inconsistency could as well be introduced by the effect of Ca^{2+} inhibition of Mg^{2+} via Ca^{2+}/Mg^{2+} interactions on the enzymes or calmodulin binding site [24].

The results from the investigation of erythrocyte membrane transport on Ca^{2+} ATPase activity is shown in Fig. 2a for phenylalnine (which shows a dose dependent increase in activity) and Fig. 2b for *C. albidium* extract. At zero concentration Ca^{2+}ATPases were significantly decreased ($p<0.05$) in SS than AA erythrocyte. Fig. 2b shows a concentration dependent significant increase ($p< 0.05$) in activity for SS erythrocytes and there was a significant sharp decrease ($p<0.05$) at 0.5mg/ml concentration of the extract and afterwards the activity increased with increase in concentration of extract. This probably could be as a result of the synergistic effect of the high concentration of glycosides on the Ca^{2+}ATpase and combined effect of phytochemicals present in the extract on the ion, ion pump, membrane phospholipids and Mg^{2+} [28].

Fig. 3a and 3b shows the results of the effect of phenylalanine and *C. albidium* on Na^+, K^+ ATPase. The activities of Na^+, K^+- ATPases were significantly decreased ($p<0.05$) in SS than AA erythrocyte in the absence of extracts. The initial significant decrease in Na^+, K^+-ATPase activity at low concentration of *C. albidium* (Fig. 3b) could be attributed to the inhibitory effect of glycosides on the enzyme activity. The enhanced or increased Na^+, K^+-ATPase activity with increased concentration of extract could be attributed to the presence of a higher concentration of alkaloids present at a high concentration in the extract (Table 1). The presence of high concentration of alkaloids had been reported to cause such a change [6, 13].

Results obtained from some studies [12, 13] indicated that an aqueous decoction of seed of *Cajanuscajan*was able to reverse and inhibit sickling of HbSS blood and this antisickling potency was attributed to phenylalanine. Antisickling activities of plant species on sickled erythrocyte may be due to inhibition of Ca^{2+} activated K^+ channels. Activation of this channel results in K^+ and water loss from sickled erythrocycles with subsequent dehydration which brings about increase in intracellular concentration of HbS leading to polymerization of deoxyHbS with its associated painful episodes [19]. Inhibition of this pathway increases K^+ cell content, rehydration of red blood cells. This approach results in cell swelling, decrease HbS concentration and decrease sickling [11].Thus increasing the solubility of deoxygenated haemoglobin S, by altering the RBC membrane permeability [8,22], decreasing the total concentration of haemoglobin in the erythrocyte, increasing the eryghtrocyte volume via manipulation of ion transport pumps or mechanisms and reduction of viscosity of the bloodand if excess Ca^{2+} in the red cells is pumped out [5], sickling of erythrocyte can be inhibited or reversed.

The result of this study, revealed that while phenylalanine decrease Mg^{2+}ATPases activities in HbSS and HbAA erythrocytes membrane preparations, it increases the Na^+, K^+ and Ca^{2+}ATPases activities in a dose dependent manner.

Phytochemical Screening

The preliminary phytochemical screening of the leaf extract carried out to evaluate the bioactive constituents of the plant is presented in Table 1. The result shows that *C. albidum* contained alkaloids, glycosides, tannins and resins in high proportion; flavonoids, saponins and carbohydrates in moderate proportion; reducing sugar and acidic compounds in small proportion while terpenoids and steroids were however absent.These phytochemicals are implicated as major bioactive constituents of medicinal plants used in sickle cell disease management [1, 11, 25].Glycosides are potent and specific inhibitors of Na^+ K^+ - ATPase by binding to or acting on the extracellular surface of the membrane [23]. Since *C. albidum* contains high concentration of glycosides it can also act as an inhibitor of Na^+K^+ ATPase. *C. albidum* is rich in alkaloids, which interacts with membrane phospholipids and possibly inhibit their modification stabilizing the functioning of Ca^{2+} channels and Ca^{2+} pump. Furthermore,alkaloids are nerve stimulants, convulsants and muscle relaxants hence; their presence in the plants portrays their usefulness in alleviating some of the pain symptoms associated with sickle-cell disease[1].Flavonoids, proteins and alkaloids have an aromatic amino acid in their structure of which phenylalanine a confirmed antisickling agent is an example. *C. albidum* which is shown to contain moderate flavonoids, very high protein and high alkaloids is likely to contain significant quantities of phenylalanine. The presence of carbohydrate and reducing sugars by the extract signifies readily source of ATP and NADPH reducing equivalent via glycolytic and pentophosphate pathways (PPP) respectively and hence reduction of oxidized glutathione, a reaction catalyzed by glutathione reductase. The net effect is stability and maintenance of erythrocyte membrane integrity.

CONCLUSION

At low concentration of the extract, Na^+,K^+ and Ca^{2+}- ATPase activities are decreased, which could result to decreased utilization of energy, increased hydration hence decreased haemomglobin concentration and reduced tendency of HbSS to polymerize. It appears that the synergistic effect of the extract on Na^+K^+ATpase outweighs that of increased activity of Mg^{2+} ATPase which causes increase viscosity of blood usually associated with sickling at low concentration. However at a higher extract concentration, the resultant effect of decrease in Mg^{2+} ATPase activity outweighed the effect of increase in the activity of Na^+K^+ATPase and Ca^{2+} ATPase. This results in decreased blood viscosity and possibly enhanced extrusion of Ca^{2+}, decreased energy utilization, reduced tendency for deformation of cell membrane and favours enhanced antisickling of the erythrocytes morphology. It maybe that the activity of the extract is influenced by the phytochemicals: glycosides, flavonoids, tannins, saponins and alkaloids present in the plant, which very likelyact in synergyto correct the faulty SS erythrocyte membrane ion pump.

Table-1: Results of phytochemical screening on *C. albidum*

	Chrypsophyllumalbidum
Flavonoids	++
Steroids	-
Terpenoids	-
Alkaloids	+++
Glycosides	+++
Tannins	+++
Saponins	++
Carbohydrate	++
Reducing Sugar	+
Acidic Compounds	+
Resins	+++
Fats and oils	-
Proteins	++++

Key:
- = Not Present
+ = Present in small concentration
++ = Present in moderate concentration
+++ = Present in high concentration
++++= Present in very high concentration

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