1.0 INTRODUCTION

Nigeria is home to the largest population of sickle cell anemia patients, estimated to be in excess of 4 million patients with more than 150,000 children born annually with the disease (World Health Organisation, 2006). The condition is also incident in the African diaspora population in North America and Europe. The SCA patient’s quality of life and productivity are significantly reduced; the condition causes episodes of pain that can last for hours or days at a time (Steinberg, 2003; Todd et al., 2006; WHO, 2006; Hankins, 2009). There are few effective therapies for SCA, a chronic blood disorder which interferes with the body’s oxygen transfer process and can lead to the formation of small blood clots, which over time deprive organs and tissues of oxygen.

Despite decades of research, only one drug, hydroxyurea, approved by the American Food and Drug Agency, is available for the treatment of sickle cell anaemia (Hankins and Aygun, 2009; Brandow et al., 2010). This chemotherapeutic agent stimulates healthy production of fetal haemoglobin to counter the sickling process (Steinberg, 2003). Although long-term clinical studies have shown the drug to improve survival, while concerns of genotoxic and carcinogenic effects are yet to be ascertained (Lal and Ames, 2011). Developing novel drugs from traditional medicinal plants can serve as a means to improve the treatment of this disease.

Research on phytomedicine for the management of SCA has led to the development of a herbal based drug called Niprisan which has been patented by the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria. This development indicates that more of such herbal based drugs could be produced consequent upon scientific investigations on medicinal plants that are used in traditional African medicine.
1-1 BACKGROUND OF STUDY

Sickle cell anemia is inherited in an autosomal recessive manner. A person heterozygous for the disease is only a carrier with sickle cell trait (AS or Hb\textsuperscript{A}Hb\textsuperscript{S}). In some cases there are certain, mild symptoms but nothing close to life threatening. A person homozygous for the disease - referred to as SS or Hb\textsuperscript{S}Hb\textsuperscript{S} - has full fledged sickle cell anemia disease and exhibits a myriad of symptoms. An affected individual will begin to show symptoms after six months of age, this is when the fetal haemoglobin (HbF) subsides and the mutant HbS dominates.

Patients with sickle cell anemia suffer from episodic and painful vascular occlusions known as crises that can result in organ damage and, ultimately, premature death. Currently, in clinical practice, clotrimazole, hydroxyurea and erythropoietin are used in SCA management, but the side effects of these drugs limit their clinical use (Rifai et al.; 1995; Mehanna, 2001; Elliott et al., 2006). In Nigeria, herbal medications are used as alternatives to orthodox western medicine due to the perceived minimal side-effects, affordability and easy of acquisition (Ogunkunle and Ladejobi, 2006; Okigbo et al., 2009).

Potent bioactive compounds formed during normal metabolic processes are found in specific part(s) of medicinal plant(s), thus making such plant(s) components ideal in preparing pharmaceutical products for medical treatments. Scientific investigation on such medicinal plants could be of tremendous help in developing efficacious and safer drugs for SCA treatment.
1.2 STATEMENT OF PROBLEM

An affected (Hb$^S$Hb$^S$) individual becomes symptomatic, after six months of age, when the synthesis of fetal hemoglobin (HbF) subsides and the mutant HbS dominates. Under reduced oxygen tension, HbS polymerises and deforms the RBC. The multifaceted clinical features of this disease are the consequences of malformed properties of sickle cells due to a point mutation in the gene coding for the beta globin moiety of hemoglobin. The deformed structure of the red blood cells results in two problems that lead to a multitude of clinical manifestations. These two problems are haemolysis, which is the premature destruction of the red blood cells, and vaso-occlusion, which is the blockage of blood flow. Symptoms of sickle cell anemia can be categorized as either acute, episodic such as the trademark crises or they may be chronic. Many of the symptoms linked to haemolysis are due to the body trying to compensate by increasing erythrocyte production, resulting in abnormality of bone shape or size and heart dilation. The main clinical consequences of haemolysis include megaloblastic erythropoiesis, aplastic crisis, clinical jaundice and gallstones.

Even though scientists have carried out researches on sickle cell disease (SCD) for over fifty years they are yet to find a reliable therapy. Based on current knowledge of the disease, three different approaches to therapy have been clinically and laboratory tested (Hankins and Aygun, 2009). One approach is the chemical inhibition of HbS polymerization. This approach has not yet been successful enough to warrant implementation, and to date, none of the tested antisickling drug has an acceptable efficacy to toxicity ratio. The second approach is through the reduction of the intracellular hemoglobin concentration. Progress has since been made in the development of drugs that inhibit potassium and water loss from SS red cells. The most successful approach is the induction of fetal hemoglobin (HbF). The objective is to raise the levels of HbF which inhibits sickling. The antitumor drug hydroxyurea, which
is also a diuretic, succeeded in increasing HbF in primates as well as patients with SCA (Bunn, 1993), however not all patients respond to this drug and there are concerns over its toxicity and carcinogenic effects (Weinfeld et al., 1994; IARC, 2000; Lal and Ames, 2011; Latagliata et al., 2012).

1.3 AIMS AND OBJECTIVES

The aim of this study is to examine the efficacy of Senna alata and Senna podocarpa as herbal remedies for SCA.

The specific objectives are as follows:

(i) To evaluate the human HbSS erythrocyte membrane stabilising effect of the medicinal plant(s) extract and the influence of the medicinal plant(s) extract on polymerisation/depolymerisation of mutant haemoglobin

(ii) To determine the antioxidant activity of the medicinal plant(s) extract

(iii) To evaluate the long term toxicological effect(s) of the medicinal plant(s) extract using albino rats

(iv) To determine the effect of the medicinal plant(s) extract on rat RBC membrane protein profile

(v) To evaluate the cytotoxic effect of the medicinal plant(s) extract on K562 Cells

1.4 SIGNIFICANCE OF STUDY

The study will help identify a herbal preparation that is safe and efficacious in protecting the sickle cell membrane. This will help to reduce or eliminate RBC hemolysis, thereby inhibiting haemoglobin polymerisation thus decreasing frequency of vaso-occlusive events. Plant(s) / formulation(s) that can prevent dehydration, maintain the HbSS erythrocyte
membrane integrity and have minimal or no toxicity would be of potential benefit to HbSS patients.

1.5 HYPOTHESES

The working hypotheses are:

1. Increased RBC fragility, an hallmark of SCA may be reduced by protecting the membrane from oxidative stress thus improving the clinical outcome / outlook of SCA patients.

2. *Senna alata* and *Senna podocarpa* have been implicated in water homeostasis, and since the pathophysiological root of SCA is RBC dehydration, they may mediate the underlying process of dehydration.
1.6 OPERATIONAL DEFINITION OF TERMS

**Electrophoresis**: A technique for separating the components of a mixture of molecules by size or shape as a result of an electric field within a support gel.

**Aqueous-methanolic extract**: Freeze dried product of plant material(s) soluble in 80% methanol.

**Fetal haemoglobin (HbF)**: A type of haemoglobin made up of two alpha-globin chains and two gamma-globin chains found majorly in the erythrocytes of neonates.

**HbSS**: The specific mutated allele composition of an individual with respect to the beta globin gene located on chromosome 11, which codes for sickle haemoglobin.

**K562 Cell line**: Cell line originally established from a pleural effusion of a patient with chronic myelogenous leukemia in terminal blast crisis.

**Red Blood Cell (RBC)**: A type of cell in the blood that contains haemoglobin, which is the oxygen carrying pigment that gives blood its characteristic red colour.

**SDS-PAGE**: Sodium Deodocyl Sulphate PolyAcrylamide Gel Electrophoresis - A type of vertical gel electrophoresis used for separating mixture of molecules according to size.

**Sickle Cell Anemia (SCA)**: A type of genetic disorder resulting from a point mutation (A-T) on the sixth codon of the beta-globin gene located on chromosome 11, and often leading to anemia.

**Sickle haemoglobin (HbS)**: A type of haemoglobin made up of two alpha-globin chains and two attenuated beta-globin chains found in the erythrocyte.
2.0 LITERATURE REVIEW

Nigeria has the largest population of SCA patients, estimated to be in excess of 4 million patients with more than 150,000 children born annually with the disease (WHO, 2006). The homozygous state of SCA is a major health challenge in Nigeria and other developing countries, because the condition is associated with complications and reduced life expectancy. Carrier frequency of HbS varies significantly around the world, due to high malaria occurrence, since carriers are somewhat protected against malaria.

The genetic basis of the disease has long been established, but it is the molecular remedy that scientists are continually investigating. With advances such as the transgenic mice, hydroxyurea and new herbal therapies being discovered, complications and premature death of SCA patients may in the very near future, be a thing of the past. Hydroxyurea which is currently the drug of choice is potentially mutagenic and carcinogenic (Latagliata, 2012). Cancer and leukemia have been reported in hydroxyurea-treated sickle cell disease patients, but whether the incidence is higher than in the general population is not known (Steinberg et al., 2003). The relative risk of leukemia in hydroxyurea-treated sickle cell anemia is much less than the observed risk in myeloproliferative disorders. To put this into perspective, the risk of death from the complications of adult sickle cell disease appears to be at least 10-times greater than the possible incidence of leukemia in hydroxyurea-treated sickle cell anemia patients (Buchanan et al., 2004).

2.1 Historical Perspective

The acutely painful episodes that characterize sickle-cell anemia disease were described in 1872 by Africanus Horton, though the mechanism remained uncertain until, nearly thirty years later, when James Herrick (1910) coined the term ‘sickle cell’ to describe the peculiar
morphology of the red blood cell of his patient, a dental student who presented with pulmonary symptoms. He was not sure then, whether the blood condition was a new disease or a manifestation of another disease, but over the next 15 years, several similar cases were described, supporting the idea that this was a new disease and provided the platform for a preliminary clinical and pathological description. Hahn and Gillespie (1927) thereafter, suggested that anoxia caused RBC sickling by demonstrating that shape changes could be induced by saturating a cell suspension with carbon dioxide. This concept was proven by Scrver and Waugh (1930), in vivo by inducing venous stasis in a finger using rubber band in experiments that would undoubtedly not receive institutional review board approval today. They showed that stasis-induced hypoxia dramatically increased the proportion of sickle-shaped cells from approximately 15% to more than 95%. These seminal studies were noted by Linus Pauling, who was the first to hypothesize in 1945 that the disease might originate from an abnormality in the hemoglobin molecule. His hypothesis was validated in 1949 by the demonstration of the differential migration of sickle versus normal hemoglobin as assessed by starch gel electrophoresis. Both hemoglobins migrated towards the positive end but HbS migrated lesser, thus revealing that HbA had a greater net negative charge (Klug et al., 1974). That same year, the autosomal recessive inheritance of the disease was elucidated by James Neel and E.A. Beet through pedigree analysis, which revealed three phenotypes and genotypes controlled by a single pair of alleles (Hb\textsuperscript{A}, Hb\textsuperscript{S}). Around the same time, Watson et al. (1948) predicted the importance of fetal hemoglobin (HbF) by suggesting that its presence could explain the longer period necessary for sickling of newborn RBC compared with those from mothers who had “sicklemia”. Ingram and colleagues demonstrated shortly (1954) thereafter that the mutant sickle hemoglobin (HbS) differed from normal hemoglobin A (HbA) by a single amino acid in the primary structure of the globin portion. This finger printing technique involved the enzymatic digestion of protein portion of hemoglobin into
fragments which are then subjected to an electric field for initial migration and then placed in a solvent where chromatographic action causes a second migration. These migrations result in a two dimensional pattern. (Klug et al., 2010) This was followed by studies that analyzed the structure and physical properties of HbS, which formed intracellular polymers upon deoxygenation. These studies placed SCD at the leading edge of investigations to elucidate the molecular basis of human diseases.

Sickle cell anemia (SCA), one of the most prevalent hereditary disorders with prominent morbidity and mortality is an autosomal recessive disease caused by a missense point mutation in the haemoglobin beta gene found on chromosome 11p15.4 (Harrison’s, 1998). This mutation results in the substitution of valine (a hydrophobic amino acid) for glutamic acid (a hydrophilic amino acid) as the sixth amino acid residue on the beta globin chain of the structurally abnormal haemoglobin (Hb), called sickle haemoglobin (HbS). The sickle cell anemia pathology results from the acquisition of a novel property due to the change in amino acid sequence, rather than from the loss of normal function (Presely et al., 2010). Haemoglobin is an oxygen carrying protein that gives red blood cells their characteristic red colour. Patients who are homozygous for the mutation in the gene encoding the chain of hemoglobin (globin) that results in the substitution of valine for glutamic acid at position 6 (beta^{Glu6Val}) have hemoglobin S (HbS) and sickle cell anemia. Hemoglobin from these patients polymerizes in the deoxy conformation into long fibers composed of strands of HbS tetramers. The beta^{Glu6Val} mutation in deoxy-HbS favors a hydrophobic interaction between each strand and its neighbour. Other residues on the chain participate in the binding of adjacent tetramers within each strand and between strands. The interaction between the valine at position 6 with the phenylalanine at position 85 and the leucine at position 88 on the
partner strand is stereochemically unavailable in oxyhemoglobin. The fibers of polymerized deoxy-HbS are responsible for dehydration, rigidity, and lysis of red cells (Geva et al, 2004).

In persons who are heterozygous for the beta^{Glu6Val} mutation, hemoglobin can polymerize if the non-HbS allele encodes permissive mutant hemoglobin (such as HbC, HbD, or HbO Arab). In other words, patients with sickling disorders due to two heterozygous mutations in globin tend to have compound heterozygosity, such as HbS/C or HbS/D.

2.2 Red Blood Cells (RBC): Erythrocytes

During erythrocyte maturation, the blast forming unit-erythrocyte (BFU-E) matures into the colony forming unit-erythrocyte (CFU-E), which finally evolves into a mature erythrocyte after extrusion of the nucleus, acquisition of haemoglobin and a mature cytoskeletal support system. Mature RBCs are discoid-shape and devoid of a nucleus and mitochondria. They are rich in the heme-containing protein, haemoglobin.

Accelerated erythrocyte formation due to anemia may result in the release of RBCs prior to the loss of the nucleus. These immature erythrocytes known as reticulocytes can be found circulating in the blood in increased numbers (greater than 5% of the total RBCs). Once the red blood cell is released into circulation, it has a normal life span of 120 days. Damaged or senescent RBCs are sequestered in the spleen and destroyed by the splenic macrophages.

Pluripotent cells of the bone marrow differentiate by feedback inhibition mechanism into various blood components. Body cells deprived of oxygen due to reduced level of circulating oxygen transporting red blood cells triggers differentiation of bone marrow stem cells into erythroblast which are eventually released into the blood stream.
2.3 Hemoglobin

The biosynthesis of hemoglobin begins in the mitochondrion, continues in the cytoplasm and is completed in the mitochondrion. A functional hemoglobin molecule consists of four globin chains i.e two alpha and two non-alpha globin chains referred to as a tetramer, which are the expression products of genes on the alpha globin and beta globin loci located on chromosome 16 and 11 respectively. Transcriptional control elements positioned within (Persons and Nienhuis, 2000) and upstream (Li et al., 2004) of this region coordinate the sequential activation and silencing of these genes during three defined periods of human Hb-Grower1 ($\zeta_2 \varepsilon_2$), Hb-Grower2 ($\alpha_2 \varepsilon_2$) and Hb-Portland ($\zeta_2 \gamma_2$) (gestational weeks 4-8), fetal globin – Hb-F ($\alpha_2 \gamma_2$) (gestational week 8 through parturition) and adult globins – Hb-A($\alpha_2 \beta_2$) and Hb-A$_2$( $\alpha_2 \delta_2$) (from birth onward) (He and Russels, 2002). The expression of embryonic globin, like fetal hemoglobin (HbF), is normally silenced in adults despite the fact that its encoding gene remains structurally intact (Bank, 2005). The red blood cells are initially made in the liver and spleen, then in the long bones after which production in the liver and spleen ceases.

Polymerisation of deoxy-HbS is greatly reduced in the presence of substantial fractions of HbA or HbF within the RBC’s (Steinberg et al., 2003). Erythrocytes from heterozygous AS subjects, containing about 40% HbS do not sickle in vivo under normal oxygen tension, except under conditions where their internal Hb concentrations is markedly increased by osmotic shrinkage due to the hypertonic environment as of the renal medulla (Ataga and Orringer, 2000). Individuals with the sickle cell trait are asymptomatic and have normal haematological profiles, except under high stress of maximum exercise or low oxygen tension (high altitude or sudden decompression in aircraft) (Bunn and Forget, 1986; Gendreau and DeJohn, 2002; Lee et al., 2002), but individuals doubly heterozygous
(HbS HbF) for sickle Hb and for hereditary persistence fetal Hb, show no clinical evidence of sickling and have normal haematological profiles. In sickle cell (SS) patients Hb combinations that prevent sickling invivo also prevent the development of the haematological and clinical manifestations associated with dehydrated RBC’s, despite substantial proportions of HbS within the cells.

Human RBC’s are water permeable sacs with the highest soluble protein concentration of any cell approximately 7.2 mmol Hb/cell water. The RBC’s characteristic biconcave shape and flexibility is made possible due to its two dimensional spectrin-actin based meshlike cytoskeleton, with a highly structured link to integral membrane proteins embedded in the lipid bilayer. (Chasis et al., 1989; Palek and Lambert, 1990). Continued osmotic equilibrium in the plasma is achieved by the RBCs’ high water permeability so that they can swell or shrink only by the loss or gain of a fluid isoosmotic with surrounding plasma. Since the plasma protein concentration is less than 1mM, there is powerful pressure driving water into the cells. Osmotic stability over the approximate 120 day circulatory life span of the mature RBCs is actualised due to an evolved strategy of maintaining a nearly constant volume with minimal energy consumption. Because of their low Na+ and K+ permeabilities which require relatively few metabolically fuelled Na+ pumps per cell to balance their cation gradient driven leaks, RBCs have evolved an extremely efficient CO2 ferry between tissues and lungs, based on the high Cl− and HCO3− exchange (Lew and Bookchin,1986) and electrodiffusional fluxes (Knauf et. al., 1983). This homeostatic balance is markedly altered in sickle Hb containing RBCs whose ion content regulation, ion fluxes and hydration state become highly disrupted in the circulation. Enucleated erythroid cells termed reticulocytes released from the bone marrow into the circulation differentiate into mature RBCs within 2-3 days. The maturation process involves loss of residual RNA as well as protein synthesis with
concomitant reduction or outright loss of many metabolic functions including a gradual decline in monovalent cation transport activity and the reduction or inactivation of selected transporters such as $K^+\text{-}Cl^-$ cotransporter and $Na^+$ pumps, via the formation of exosomes (Johnstone, 1992).

### 2.4 Transport Proteins Involved In RBC Dehydration

Human RBC dehydration in vivo may arise from the single or synergistic activation of the $K^+\text{-}Cl^-$ cotransporter (KCC), the $Na^+$ pump or the Gardos channel. These three transporters expressed in the RBC plasma membrane differ considerably in their dehydrating potencies, modalities and distribution.

#### 2.4.1 $K^+\text{-}Cl^-$ Cotransporter (KCC)

The KCC, regulated by internal pH and cell volume, is functionally active in reticulocytes and much less so in mature AA and SS RBCs. Most of the $K^+$ traffic is mediated by this transporter, which may be regulated by cell swelling, intracellular acidification, and oxygenation state of Hb (Joiner and Franco, 2001) and to a lesser extent low intracellular Mg$^{2+}$ concentrations. Its functional state appears to be regulated by classical serine/threonine and tyrosine residue (Merciris et al., 2003) phosphorylation and dephosphorylation (Lauf and Adragna, 2000). Acidification in RBCs with active KCC may induce dehydration (Lauf et al., 1992; Gillen et al., 1996), the extent of which may be limited by the inhibitory effect of cell shrinkage, while in SS reticulocytes acid actification is abnormally exaggerated and may therefore overcome the intensity of the volume-regulatory “brake”. Inhibitory agents may act directly on the KCC or on its regulatory intermediates (Jennings and Schulz, 1991).
2.4.2 The Na\textsuperscript{+} Pump

The Na\textsuperscript{+}-K\textsuperscript{+} flux ratio through the Na\textsuperscript{+} pump is 3:2 (Lew et al., 1991). The high anion permeability of reticulocytes and RBCs, require that electroneutrality be maintained, majorly by anion efflux balancing the extra Na\textsuperscript{+} efflux. Elevated internal Na\textsuperscript{+}/K\textsuperscript{+} concentration ratios stimulate the Na\textsuperscript{+} pump, resulting in an increased NaCl flux, which if not compensated by passive fluxes would induce dehydration (Joiner et al., 1986). Model simulations (Glynn and Karlish, 1975; Brugnara et al., 1989; Skou and Esmann, 1992) confirms this effect to be rather slow, moreover, in SS cells Na\textsuperscript{+} pumps have been found to be substantially inhibited (Clark and Rossi, 1990; Ortiz et al., 1990), despite elevated internal Na\textsuperscript{+}/K\textsuperscript{+}. This inhibition was due to elevated Mg/ATP ratio, because Mg\textsuperscript{2+} released from 2, 3-diphosphoglycerate (2,3-DPG) during deoxygenation, pushed the ratio further away from the optimal (Ortiz et al., 1990). Thus Na\textsuperscript{+} pump-mediated fluxes may contribute only marginally to SS cell dehydration.

2.4.3 Gardos Channel

The Gardos channel is a Ca\textsuperscript{2+}-sensitive, small-conductance, K\textsuperscript{+}-selective channel which is activated when the physiological intracellular RBC Ca\textsuperscript{2+} concentration levels of about 20-50 nM is increased above its activation threshold of 150 nM (Tiffert and Lew, 2001). A maximally activated Gardos channel would ensure fast dehydration of the RBC. Unlike erythroid cells from other mammalian species, whose Gardos channel activity is lost during maturation (Brown et al., 1978), mature human RBCs show persistent Gardos channel activity. The capability of Gardos channel, when fully activated, to mediate rapid RBC dehydration far exceeds that of the other transporters. Dehydration via Gardos channels may be reduced by direct inhibition of the channels with charybdoctoxin (IC\textsubscript{50} ≈1.2 nM) or clotrimazole (IC\textsubscript{50} ≈51 nM) (Brugnara et al., 1993), or indirectly, by inhibition of the anion
permeability (Bennekou et al., 2001). Mature RBCs, when compared with other cell types lack specialised Ca\(^{2+}\) accumulating organelles, Ca\(^{2+}\) signalling functions as well as minimal cytoplasmic buffering capacity (Tiffert and Lew, 1997).

### 2.5 Dehydration of RBC

The relationship between SS cell transport and sickling was investigated in 1955 by Tosteeson and co-workers, who showed that upon deoxygenation of fresh, heparinised whole blood obtained from HbSS patients their RBCs had a much larger gain of Na\(^{+}\) and loss of K\(^{+}\) than RBCs from HbAA (normal) controls. But on reoxygenation, HbSS cells gained K\(^{+}\) and lost Na\(^{+}\), affirming that the effects of deoxygenation were reversible. Further transport experiments with tracers of Na\(^{+}\), K\(^{+}\), and Cs\(^{+}\) indicated that sickling induced a reversible and poorly selective increase in the electrodiffusional cation permeability of the RBC membrane. A sickling induced permeability pathway was proposed by Lew et al. (1997) When HbSS RBCs was ultracentrifuged, a cell subpopulation accumulated at the bottom of the column, these cells had normal Hb content, and their high density reflected a dehydrated state. The distinct morphological features of this subpopulation earned the name irreversibly sickled cells (ISCs), due to the fact that when fully oxygenated, they still retained the sickled shape. Isotopic labelling techniques revealed that ISCs were a relatively young HbSS cell subpopulation which, after release from the bone marrow as reticulocytes, was rapidly transformed into ISCs within 4–7 days, and then disappeared from the circulation faster than other mature, non-ISC RBCs. ISCs were therefore a subpopulation of SS RBCs in rapid circulatory turnover. The exclusion of most high Hb F-containing HbSS cells (whose polymerization was reduced or inhibited) from the hyperdense, ISC-rich cell fraction suggested that sickling was necessary for ISC formation. Most research in the field was dominated by the notion of gradual dehydration, with attention focused on the three transport
systems potentially involved in HbSS RBC dehydration, notably, the Gardos channel, KCC and Na$^+$ pump.

2.6 Pathophysiology

The sickle phenotype results from the intracellular polymerization of deoxygenated $\alpha_2\beta^\prime_2$ heterotetramers (HbSS), into extended 14-strand fibres that disrupt both the shape and the function of the mature erythrocyte (Bunn, 1997). The deformation of RBC causes changes in their membrane structure (Ohnishi and Ohnishi, 2001), thereby enhancing the adhesion of the cells to vascular endothelial cells (Hoover, 1979; Brittain et al., 2004). This, together with elongated cell shapes, induces the obstruction of blood flow and causes the painful sickle cell crisis. The red blood cell membranes of SCA patients are osmotically and mechanically more fragile than those of normal subjects. For these reasons, sickle RBCs are easily destroyed and removed from the circulation in the spleen, thus causing anemia. The average life span of sickle erythrocytes is 10 - 20 days (Franco et al., 1998), as opposed the 120 days for the normal RBC. Patients suffer from chronic anemia, frequent painful episodes (crisis) and resultant malfunction of organs (especially the spleen) and degeneration of bone joints (Serjeant, 1997).

The total cellular calcium content of sickle RBC is markedly abnormal (50µmol/L as against 5µmol/L for normal RBC), but oxygenated sickle RBC have moderately enhanced permeability to external calcium and deoxygenation boosts calcium influx by about five fold (Etzion et al., 1993). Though excess calcium is stored internally in vesicles without increase in detectable ionized calcium, this influx activates a calcium-dependent potassium channel called Gardos channel, through which potassium is extruded from the sickle RBC, thus resulting in dehydration. Stimulation of sickle RBC membrane Ca$^{2+}$ATPase activity under optimized conditions among patients have been found to be increased, deficient and normal,
suggesting an insignificant effect on the calcium pumping capacity under physiological conditions. Stimulation of the \( \text{Na}^+\text{K}^+\text{ATPase} \) by deoxygenation of sickle RBC somewhat increases passive K efflux and Na influx three to five fold, this leak sites (Lew et al., 1991), areas of the membrane affected by speculation – a sickling effect – requires cell deformation for induction of monovalent cation leak, though oxidative perturbation of the sickle membrane may however not be ruled out. Some studies on RBC suggest that full homeostatic capacity of the cell (- Quabain) leads to cellular dehydration while the passive component of sickling-induced leak (+ Quabain) causes balanced leak. Cell swelling or low pH (<7.4) activates in sickle reticulocytes, a potassium-chloride cotransport (KCC) pathway. Irreversibly sickled cell (ISC) generated by repeated deoxygenation / oxygenation of sickle RBC, regarded as the end-stage defects of highly damaged cells, can constitute anywhere from a few percent to about 50% of RBC population. Formation of ISC \textit{in vitro} is generally prevented by an absence of external calcium, inhibition of Gardos channel, calcium channel blockers and calmodulin-mediated processes. The calcium antagonist, zinc, appeared to reduce ISC counts in patients consuming it, but formation of ISC formation \textit{in vitro} was not inhibited by in the presence of zinc. The implied role of calmodulin is interesting given its interaction with the RBC cytoskeleton and the effects of calcium on certain protein / protein interactions (Blackman et al., 2001; Chang and Low, 2001).

\section*{2.7 Cytoskeletal Dysfunction}

The flexibility and strength of the normal RBC is derived from the protein component of its membrane, the essential features of which is an underlying cytoskeleton (spectrin, actin, band 4.1 and other minor components) connected by linking units (ankyrin and band 4.1) to proteins embedded in the lipid bilayer (band 3 and glycophorin). In sickle cell anemia, this actin / spectrin lattice ‘locks’, making red blood cells much less deformable, and causing
them to obstruct the microcirculation. Sickle inside-out vesicles (IOV) have a diminished capacity for ankyrin-dependent binding of spectrin (Platt et al., 1985) and aminophospholipid, sickle IOV were found to have a slightly diminished quantity of band 4.1 (Schwartz et al., 1987) although artifacts due to protease action was not excluded. Sickle cell patients administered vitamin E tend to have normalised band 3 anion transport activity and high affinity ankyrin binding sites (Joiner et al., 1989). Careful analysis of sickle RBC membranes showed a small amount of abnormal spectrin / globin adduct (Presley et al., 2010), favoured by dehydration, reflecting an oxidative process possibly involving spectrin (not Hb) thiols in the presence of peroxide (Rank et al., 1985).

Sickle RBC fragility has been observed to increase in patients undergoing vigorous exercise. *Invitro* studies have documented notable reduction in the shear sensitivity of intact sickle cells on rehydration and ghosts made from most dense sickle RBC are mechanically fragile. This membrane instability reflects some protein/protein junctional association failure and destabilization of the cytoskeleton maybe due to free heme found to be in excess in both sickle membrane and sickle cytosol (Shalev and Hebbel, 1996).

Decreasing deformability of deoxygenating sickle RBC is highly dominated by development of Hb polymer and cytoplasmic viscosity. It may be anticipated that abnormal microrheology of sickle cell membrane would participate in vasoocclusion as increased static rigidity would enhance mechanical trapping of RBC during their attempted passage through the microvasculature, or capillaries of limiting diameter and flow velocity of cells therein (Ferrone, 2004).
2.8 Cell / Cell Interactions

Abnormal attachment of sickle RBC to mononuclear phagocytes or vascular endothelial cells indicates relevance to haemolytic anemia and vasocclusive events respectively. Sickle RBC have been found to be abnormally adherent to normal marrow, splenic or alveolar macrophages and normal peripheral blood monocytes, after which they are phagocytosed more readily than normal RBC. The ability of externalised phosphatidylserine (Yamaja Setty and Betal, 2008) in promoting adherence to macrophages in vitro is enhanced by deoxygenation, but exposure of normal RBC to peroxide or malondialdehyde (peroxidation by product) facilitates their phagocytosis. Abnormal adherence of sickle RBC to vascular endothelial cells derived from bovine aorta, rat microcirculation and human umbilical vein is enhanced by dehydration or calcium loading (Hoover et al., 1989; Kaul et al., 1993).

2.9 Pathophysiologic Role of Membrane Abnormalities

Emphases on HbS polymerization have dominated considerations of sickle cell disease pathophysiology generally but it is now recognised widely that this disease is exceedingly complex. Attempts to explain it based on a single feature would be artificial and the intriguing heterogeneity amongst patients would be grossly underestimated. Severity of anemia for sickle cell patients correlate directly with the magnitude of the very-dense cell population.

2.10 Oxidant Levels

Increased oxidant susceptibility of sickle red blood cells (RBC) has been demonstrated to play a major role in pathophysiology of the disease, in effect, reactive oxygen species (ROS) produced during redox cycles appear to cause premature ageing and altered morphology of RBCs, leading to their early removal from circulation. Another name for redox cycle
formation of reactive oxygen species and damage to the RBC is ‘oxidative stress’. In other cases toxicants can cause a disruption in oxidative metabolism within the cell, leading to generation of reactive oxygen species. An important potential consequence of free-radical formation is the occurrence of lipid peroxidation in the membranes within the cell. Lipid peroxidation occurs when free radicals attack the unsaturated bonds of fatty acids, particularly those in phospholipids. The free radical reacts with the fatty acid carbon chain, abstracting one hydrogen atom. This causes a fatty acid carbon to become a radical, with rearrangement of double bonds in the fatty acid carbon chain. This carbon radical in the fatty acid reacts with oxygen in a series of steps to produce a lipid hydroperoxide and a lipid radical that can then react with another fatty acid carbon. The peroxidation of the lipid becomes a chain reaction, resulting in fragmentation and destruction of the lipid. Because of the importance of lipids in membrane structure, the principal consequence of lipid peroxidation for the cell is loss of membrane function. The reactive products generated by lipid peroxidation can interact with other components of the cell as well, and this also could contribute to cellular perturbation. Protecting the RBC membrane from oxidative stress may thus improve the clinical manifestations of the patients (Ohnishi and Ohnishi, 2001).

2.11 Factors Contributing to Vasocclusion

HbS polymerization in vasocclusive events for most sickle RBC is never in equilibrium and sickling is dominated by kinetics, so that clinical effects depend critically on the relationship between microcirculatory transit time (short) and the unavoidable and relatively longer delay time (about 15 seconds) for the onset of Hb polymerization (Bunn, 1997). It is controversial whether or not the RBC membrane directly influences polymerization the occurrence of painful crises actually correlates inversely with percentage dense cells and percentage ISC, but correlates positively with adequate RBC deformability.
2.11.1 Environmental factors

The role of blood factors including plasma proteins (as modulators of RBC adhesivity), blood pH (as stimulant for cation depletion, as well as Hb polymerization), osmolarity (as determinant for dehydration) and zinc levels (as calcium antagonist) in vasocclusive crisis cannot be underemphasized. Since cellular pathobiology is influenced by oxidative phenomena, there is the possibility of accessibility to dietary factors like selenium, riboflavin, vitamins E and C, exerting some beneficial influence. Presence of other globins like HbA or HbF (levels varying enormously amongst patients), inhibit polymer formation while others e.g. HbD and HbO\textsuperscript{Arab} promote polymer formation.

2.11.2 Internal factors

Proper functioning of various RBC enzymes [(Gluthathione-S-transferase (GST), superoxidedismutase (SOD), Catalase (CAT), Methemoglobin reductase (NADHmr), Diaphorase (NADPHd) and Glucose-6-phosphate dehydrogenase (G-6PD)) systems play crucial roles in sickling. The SODs are a group of metalloenzymes that catalyze the conversion of reactive superoxide anions (O\textsubscript{2}\textsuperscript{-}) to yield H\textsubscript{2}O\textsubscript{2}, which is in itself, is an important ROS as well. Hydrogen peroxide is subsequently detoxified by two types of enzymes; catalases and glutathione-dependent peroxidases (GPOXs). Superoxide dismutases are considered to play a pivotal antioxidant role. Their importance is indicated by their presence in all aerobic organisims examined (Stegeman \textit{et.al.}, 1992). The SOD-CAT system provides the first defence against oxygen toxicity. Superoxide dismutase catalyses the dismutation of the O\textsubscript{2}\textsuperscript{-} to molecular oxygen and hydrogen peroxide which is detoxified by CAT activity to water and oxygen. (see equation below)

Equation. \[ 2\text{O}_2^- + \text{SOD} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 + \text{CAT} \rightarrow \text{H}_2\text{O} + \text{O}_2 \]
Fig. 1 A schematic diagram showing the interrelationship between the possible role of Hb polymerization, membrane defects and abnormal behavior of HbS in sickle cell anemia disease pathophysiology.
2.12 Histopathology

Indicators of stress at several levels of biological organisation have been used to evaluate effects of contaminants / toxicants at organizational level. These approaches vary from measures of genetic integrity to growth and reproductive competence of the individual (Teh et. al., 1996). As far as the individual organism is concerned, manifestations of stress at the tissue level represent an intermediate effect between the biochemical and reproductive levels (Hinton, 1990). Histopathological characteristics of specific organs express condition and represent time-intergrated endogenous and exogenous impact on the organism stemming from alterations at lower levels of biological organisation (Chavin, 1973). According to Segner and Braunbeck (1998), histological changes occur earlier than reproductive changes and are more sensitive than growth or reproductive parameters and, as an integrative parameter, provide a better evaluation of organism health than a single biochemical parameter. Many toxicants can damage specific cell types or organ systems.

2.13 Orthodox Treatment

The main drug therapy for sickle cell anemia, hydroxyurea, is thought to reduce the formation of sickle haemoglobin and hence ameliorate the structural consequences, while also decreasing neutrophil numbers which promote adhesion of sickled cells to blood vessel walls. However, a recent study suggests that hydroxyurea acts directly on the plasma membrane. The drug is known to decrease expression of adhesion molecules on the red blood cells, one of which is phosphatidylserine, usually expressed on the outer surface of some RBCs in sickle cell anemia. In a study by Covas et al., 2004, sickle cell patients receiving 12 months of hydroxyurea treatment had significantly reduced phosphatidylserine levels in their blood samples.
The multifaceted clinical features of this disease are the consequences of malformed properties of sickle cells. The deformed structure of the red blood cells results in two problems that lead to a multitude of clinical manifestations. These two problem mechanisms are haemolysis, which is the premature destruction of the red blood cells, and vaso-occlusion, which is the blockage of blood flow. Symptoms of sickle cell anemia can be categorized as either acute, episodic such as the trademark crises or they may be chronic. Many of the symptoms linked to haemolysis are the results of the body trying to compensate. The body compensates by increasing erythrocyte production, resulting in abnormality of bone shape or size and heart dilation due to increased heart action. The main clinical consequences of haemolysis include megaloblastic erythropoiesis, aplastic crisis, clinical jaundice and gallstones (Issa and Al-Salem, 2010).

The life span of erythrocyte in people with sickle cell disease is ten to twenty days as opposed to a normal 120 day span (Franco, 1998). This increased rate of destruction demands a significant increase in bone marrow activity. Because of the raised level of bone marrow activity a greater amount of folic acid is required. The shortage of this acid results in megaloblastic erythropoiesis with low reticulocyte counts, increasing mean cell volume, and reduced haemoglobin. The increased metabolic demand of the bone marrow competes with the demands of growth plates, usually resulting in impaired growth. Aplastic crisis, from bone marrow depression may occur and its characteristic symptoms include lethargy, dyspnea and possibly coma. The rapid haemolysis means increased bilirubin excretion which is associated with clinical jaundice and the formation of gallstones (Alexander-Reindorf et al., 1990; Issa and Al-Salem, 2010).

The second problematic mechanism, of the deformed red blood cells is the blockage of venous microcapillary, resulting in impeded blood flow. Tangled, sickled cells obstruct
vessels, causing tissue anoxia and possible necrosis. Manifestation of symptoms depends on which vessels that are being blocked. Damage to the splenic vasculature via obstruction, is an early pathological signature of sickle cell anemia. The spleen normally acts like a filter, removing damaged red blood cells and bacteria from the blood stream; however, in sickle cell patients the damaged red blood cells obstruct this filter, predisposing the individual to infection. The most common crisis, vaso-occlusive crisis, is caused by this blockage mechanism. Vaso-occlusive crisis (painful episode) is the term used for the excruciatingly painful period while the vessels are occluded by cells and tissue is being deprived of oxygen. It is characterized by severe thoracic, abdominal, muscular or bone pain. This form of crisis may last from a few hours to several weeks (Okpala and Tawil, 2002; Wright and Ahmedzai, 2010). Vessel blockage leads to extra complications in pregnant women, where mother and foetus face increased risk due to oxygen variability and children also face the devastating risk of stroke due to the occlusion of major cerebral vessels.

Sickle cell patients may develop chronic complications such as leg ulcers, below average growth rate and complications from organ infarctions such as retinopathy, nephropathy and possibly auto spleenectomy (Hassell, 1994).

Diminished solubility of HbS in its deoxygenated state results in the formation of a network of fibrous polymers, which stiffen and distorts the red blood cell’s shape. This polymerization produces the classic sickle shape. The kinetic feature of this polymerization is critical. When rapid deoxygenation occurs, polymerization doesn’t alter the cell’s disk-like shape. However, when HbS red cells are slowly deoxygenated a nucleus of HbS molecules combine, grow the fibers and transform into the sickle shape (Bunn, 1997).

The rate and extent of polymer formation in the SS red cells depends on three variables that dictate the extent of polymerization and thus, to an uncertain degree, the severity of sickle
cell disease. Those variables are the degree of deoxygenation, intracellular hemoglobin concentration, and presence of fetal hemoglobin. Deoxygenation causes polymerization induced damage which leads to dehydration resulting in formation of dense cells which cause sickling. This appears to be the basic domino effect. The polymerization rate of deoxygenated HbS, is dependent on the hemoglobin concentration. This translates into the fact that dense HbS cells are more likely to sickle. Dense cells are partly created by dehydration. There are two main contributors to dehydration in these mutant red blood cells. First is the greatly increased rate of potassium chloride co-transport in the erythrocytes. The second contributing factor results from the increased calcium triggering a potassium channel, thus providing another pathway for the loss of water (Bunn, 1997). The third variable in the severity of the expression of the disease is the presence of fetal hemoglobin, which until approximately the sixth month of life is the predominant type of haemoglobin (Stamatoyannopoulos, 2005; Oneal et al., 2006). Thus affected infants are more or less symptom free. After this time period the HbS is at normal levels of production and symptoms are revealed. In adults, the level of HbF correlates with the parental HbF levels in a genetically dominant pattern (Milner et al., 1984). HbF inhibits polymerization which halts the domino effect that generally results in sickled cells.

Research on sickle cell disease has been on for over fifty years and the current knowledge of the disease dictates three different approaches to therapy which have been clinically and laboratory tested. One approach is the chemical inhibition of HbS polymerization. The challenge faced is a formidable one, since the drug must be absorbed, circulate in the plasma without binding strongly to plasma proteins, penetrate the erythrocyte membrane and bind specifically to the HbS. To date, no antisickling drug has been tested with an acceptable efficacy to toxicity ratio (Bunn, 1993).
A second plan of attack is the reduction of the intracellular hemoglobin concentration. This approach relies on the dependency of the rate of polymerization of HbS on the HbS concentration. Progress has seen made in the development of drugs that inhibit potassium and water loss from SS red cells. This in turn reduces the intracellular hemoglobin concentration. The drug Clotrimazole inhibits potassium loss at a specific channel and has proven effective in transgenic mice and patients with sickle cell anemia (Bunn, 1993).

The other mode of cell dehydration, potassium-chloride co-transport, has not yet fully conformed to pharmacological manipulation, despite it’s inhibition by high intracellular mg$^{2+}$ (Buchanan et al., 2004)

The most successful of the three approaches is the induction of fetal hemoglobin (HbF). The objective is to raise the HbF levels because it inhibits sickling. The antitumor drug hydroxyurea succeeded in increasing HbF in primates as well as patients with SS disease (Bunn, 1993).

Jayabose and associates (1996) ran a study on treatment with hydroxyurea of children with severe sickle cell anemia. Their aim was to determine the effect on the hemoglobin levels, to evaluate the toxicity of the drug and to detect any change in the frequency of the vaso-occlusive crises. Group one was made up of children with sickle cell anemia who suffered frequent vaso-occlusive crises. The second group consisted of children with sickle cell and severe anemia. Each group was given 20-35mg/kg/day. The frequency of vaso-occlusive crises before and after treatment and the peak hemoglobin levels before and during treatment were recorded. The trial resulted in a statistically significant drop in the number of crises, hospital days and transfusions per year. The hemoglobin levels increased from 8-50% over baseline values. Multiple factors such as increasing mean corpuscular volume and decreasing
adhesion of red blood cells to the endothelium, are probably responsible for the clinical benefits of hydroxyurea.

One of the most important breakthroughs dealing with this disease did not even involve a human patient, but rather mice were in the limelight. Two separate research teams developed mutant mice that mimic human sickle cell anemia. The mice globins were completely eliminated and complement human genes, including the sickling mutant form of beta globin, were introduced. These mice make only the disease causing, mutant form of hemoglobin that clumps when deoxygenated and sickles. The mice suffer the same array of symptoms that a human with sickle cell disease endures. The creation of this genetically unique mouse is so valuable because now drugs and therapies can be tested just as they would in a person with the disease. The mice have exactly the same mutant beta globin that the majority of affected humans possess. Until the development of these mice, experiments were conducted in test tubes or on patients where a full range of possibilities couldn’t be tested (Barinaga, 1997). The effectiveness of the drug clotrimazole in reducing the intracellular hemoglobin concentration was also tested on these transgenic mice.

Prevention is the best form of treatment to prevent the intravascular sickling. The trademark crises of sickle cell are precipitated by fever, dehydration and cold exposure. To prevent these is an effort to prevent the extremely dangerous crises. Sudden transition to high altitudes and exposure to freezing temperatures should be avoided. Hydration is essential, as are immunizations.

The most effective of the non-controversial therapies available is the blood transfusion. This therapy includes packed red cell transfusions at three week intervals. This is generally enough of an effort to maintain the donor cell (HbA) circulation above fifty percent. The transfusions reduce the tendency for sickling by diluting the mutant host cells, temporarily suppressing the
production of erythrocytes containing HbS, thus improving blood and tissue oxygenation. However this therapy does have its downsides. It requires a tremendous blood resource and the risks of hepatitis and alloimmunization are potentially life threatening (Sandler, et al., 1997).

Bone marrow transplants have the potential to regulate the hemoglobin synthesis. This procedure causes life threatening complications in itself. Once these complications are improved, it will most likely become an option for severe sickle cell anemia patients (Ferster et al., 1992).

Since it is apparent that prevention of complications is the best form of treatment for this disease, it is helpful to detect the mutation early on. When it comes to genetic screening, sickle cell disease has had a rocky past. In the early 1970’s efforts were made to identify carriers of the recessive allele for the disease. The goal of this search was to advise the carrier which would help in family planning which would hopefully lower the disease incidence. However this plan backfired for several reasons. The counselling was not adequate and resulted in people confusing the harmless trait for the deadly disease. People were discriminated against at jobs and in society in general. It was even beleived that this was the white man’s way of medically reducing the reproduction of African Americans. Needless to say the screenings were stopped. Several key ideas were learned from this fiasco which is now been implemented in screenings for diseases from HIV to breast cancer. The test must have a direct benefit to the person tested. The results must be kept in the strictest confidence and the screening must be followed by counseling (Klug et al, 2010).

Prenatal diagnosis allows for preventative measures to begin while the child is still in the mother’s womb. Fetal cells may be acquired by amniocentesis. The DNA is extracted from the cells and digested with restriction enzymes. The enzyme would cut twice within the
normal beta globin gene, once at each site. Thus a normal gene produces two small DNA fragments. However, in the mutant alleles the second site has been destroyed by the mutation resulting in the production of one long restriction fragment. When run on a gel, one long band with two short fragments identifies a heterozygous state. This person will be a carrier. A gel showing one long band identifies a person homozygous for the sickle cell mutation (Klug et al, 2010).

With the aforementioned advances such as the transgenic mice and discovery of hydroxyurea, that positive headline may be in the very near future. Due to the genotoxicity, carcinogenicity of hydroxyurea as well as the non-responsiveness of some patients to hydroxyurea therapy, other therapeutic options are being considered.

2.13.1 Therapeutic options based on disease pathophysiology

2.13.1.1 HbS Polymerization

Deoxygenation induced polymerisation of HbS (α₂β²S) is the proximate cause of sickle cell anemia and a necessary but insufficient precursor of the disease phenotype. HbS and its polymer induce a variety of cellular and tissue injuries, but neither the fetal hemoglobin (HbF) tetramer (α₂γ₂) nor the α₂β²γ hybrid tetramer is incorporated into the HbS polymer, thus providing the rationale for treatments aimed at increasing HbF concentration.

2.13.1.2 Sickle Erythrocyte Damage and Dehydration

The presence of dense, dehydrated erythrocytes and abnormal reticulocytes is a distinguishing feature of sickle cell anemia. Since polymerization of HbS is uniquely dependent on the cellular concentration of HbS, the tendency for HbS polymerization and cell sickling is markedly enhanced by the increased cellular hemoglobin concentration of dehydrated sickle
erythrocytes. Of the four pathways that have been implicated in the dehydration of sickle erythrocytes, and modulation of these pathways, especially the Gardos channel and $\text{K}^+\text{-Cl}^-$ co-transport, is a potential means of treatment.

2.13.1.3 Endothelial damage

Cellular damage enables adhesive interactions between sickle cells, endothelial cells and leukocytes. Vasoconstriction may be favoured as nitric oxide (NO) production is impaired in a perturbed endothelium. Several agents directed at the endothelial receptors for sickle erythrocytes or leukocytes may interrupt cell-cell interactions.

2.13.1.4 Inflammation, reperfusion injury, oxidant radical production

Neutrophils mediate inflammation and tissue damage. Neutrophil numbers are increased and they may be abnormally activated and adherent. An "oxidant" environment may also be present in the sickle erythrocyte and endothelium. Novel ways of countering oxidant-induced injury have been proposed, chiefly, via exogenous antioxidant intake

2.13.2 HbS Polymerization

Several classes of drug, when titrated optimally, can increase levels of HbF in most patients with sickle cell anemia. Only one is approved by the American Food and Drug Agency for treating sickle cell anemia.

2.13.2.1 Hydroxyurea

Hydroxyurea, the sole US Food and Drug Administration (FDA)–approved drug for treating sickle cell anemia, could be used in all adults where indications for this treatment are present (Steinberg, 1999). Unfortunately, for complex reasons, only a fraction of patients who might benefit from treatment receive it. Hydroxyurea increases HbF in sickle cell anemia because its
cytotoxicity causes erythroid regeneration and perhaps because its metabolism leads to NO–related increases in soluble guanylate cyclase (sGC) with an increase of cGMP that augments gamma-globin gene expression (Cokic et al., 2003). A multicenter trial of hydroxyurea in adults with sickle cell anemia, where the drug was given at sub-toxic doses, showed that hydroxyurea reduced the incidence of pain and acute chest syndrome by nearly half, with little risk seen during more than 9 years of observation. Cumulative mortality was reduced nearly 40%, and a favourable result was related to the ability of the drug to increase HbF and reduce painful episodes and acute chest syndrome (Steinberg et al., 2003) No relationship between decrements in neutrophil counts and mortality was found. In infants, children and adolescents with sickle cell anemia, their HbF response to hydroxyurea is more robust than in adults. In a study of more than 100 children who received maximal drug doses, HbF increased to almost 20% and the treatment effects were sustained for 7 years without clinically important toxicity (Zimmerman et al., 2004). HbF levels achieved during treatment were associated with baseline HbF level, hemoglobin level, reticulocyte count, and leukocyte count and with compliance to treatment. According to some experts, pushing the drug dose to near toxic levels is not necessary for a clinically beneficial result. Some have proposed using hydroxyurea for secondary prevention of stroke in children (Ware et al., 1999). Hydroxyurea may also conserve resting energy expenditure by curbing the hypermetabolic state observed in children with sickle cell disease (Fung et al., 2001)

Hydroxyurea is potentially mutagenic and carcinogenic (Latagliata, 2010). Cancer and leukemia have been reported in hydroxyurea-treated sickle cell disease patients, but whether the incidence is higher than in the general population is not known (Steinberg et al., 2003). The relative risk of leukemia in hydroxyurea-treated sickle cell anemia is much less than the observed risk in myeloproliferative disorders. To put this into perspective, the risk of death
from the complications of adult sickle cell disease appears to be at least 10-times greater than the possible incidence of leukemia in hydroxyurea-treated sickle cell anemia patients (Buchanan et al., 2004).

2.13.2.2 Decitibine

A less-toxic analog of 5-azacytidine, 5-aza-2'-deoxycytidine (decitibine), may affect HbF levels by causing hypomethylation of the gamma-globin genes. In 8 symptomatic sickle cell anemia patients who failed to respond to hydroxyurea, decitibine treatment (0.2 mg/kg subcutaneously 1–3 times per week for 2 6-week cycles) led to an increase in HbF from 6.5% to 20.4%, with an increase in hemoglobin concentration from 7.6 to 9.6 g/dL and a fall in reticulocytes from 231 to 163 x 10⁹/L (Saunthararajah et al., 2003).

2.13.2.3 Short chain fatty acids

By acting as inhibitors of histone deacetylase (HDAC) and causing histone hyperacetylation and changes in chromatin structure, short-chain fatty acids, their derivatives, and other compounds with HDAC activity can enhance gamma-globin gene expression in erythroid cells of patients with sickle cell anemia and ß thalassemia. Very low concentrations of one HDAC inhibitor, an analog of trichostatin A, induced gamma-globin gene expression in an erythroleukemia cell line transfected with a reporter construct and in erythroid colonies of normal adults (Cao, 2004)

In the most advanced clinical trials of HDAC inhibitors, still only Phase II studies, arginine butyrate given by infusion once or twice a month was associated with a mean increase in HbF from 7% to 21% in 11 of 15 patients with sickle cell anemia. In some individuals, this level was maintained for 1–2 years (Atweh et al., 1999). No HDAC inhibitor of any class other than butyrate and phenylbutyrate has yet been used clinically.
2.13.3 Sickle Erythrocyte Dehydration

2.13.3.1 Inhibition of K\(^+\)-Cl\(^-\) co-transport

Oral magnesium supplementation inhibits erythrocyte K\(^+\)-Cl\(^-\) co-transport in vivo. Following studies that showed a beneficial effect on the erythrocyte membrane of transgenic sickle mice, a 6-month clinical trial of oral Mg pidolate improved erythrocyte hydration and was associated with a reduction in the number of painful days (De Franceschi \textit{et al.}, 2000). Studies to evaluate the effects of long-term magnesium supplementation in adult and pediatric patients with sickle cell anemia are ongoing, and additional studies are planned in HbSC disease patients where activated K\(^+\)-Cl\(^-\) co-transport and cell dehydration are likely to play a major pathophysiological role (Buchanan \textit{et al.}, 2004)

2.13.3.2 Inhibition of Gardos channel

Clotrimazole is an inhibitor of the human red cell Gardos channel but its use was associated with dysuria and reversible hepatocellular toxicity. ICA 17043, a clotrimazole derivative lacking the toxic imidazole residue, was a 10-fold more potent blocker of the Gardos channel than the native drug. In a recently completed Phase II trial of this agent in patients with sickle cell disease, cell density and hemolysis were decreased while hemoglobin concentration was increased. To see whether clinical benefit accrues from these erythrocyte and hematological changes will require a Phase III clinical trial (Ataga \textit{et al.}, 2008).

2.13.3.3 Inhibition of other channels

Movement of K\(^+\) via the Gardos channel requires the parallel movement of Cl\(^-\) anions to maintain electroneutrality. High-affinity blockers of Cl\(^-\) conductance can reversibly block human erythrocyte chloride conductance \textit{in vitro} without directly affecting the Gardos channel or the K\(^+\)-Cl\(^-\) co-transport. In sickle mice treated with a Cl\(^-\) conductance inhibitor,
packed cell volume (PCV) increased and mean corpuscular hemoglobin concentration (MCHC) decreased with an increase in cell $K^+$. A selective loss of the densest erythrocytes, with a shift from sickled to well-hydrated discoid erythrocytes, was seen. Clinical trials of this class of agent have not been done (Buchanan et al., 2004).

2.13.4 Anti-Adherence Therapy

Anti-adherence therapy for sickle cell disease targets the abnormal interactions among erythrocytes, endothelial cells, leukocytes and platelets that are part of the pathophysiology of the disease process. Potential anti-adherence agents have been studied in acute painful events, where, through poorly understood mechanisms, they restore microvascular circulation and improve tissue ischemia. In Phase II studies of a non-ionic surfactant copolymer, poloxamer 188, this agent reduced the duration and increased the resolution of acute painful episodes, an effect especially notable in children less than 15 years old and in patients receiving hydroxyurea (Orringer et al., 2001). Whether poloxamer 188 exerts its effects by modifying interactions of sickle cells or other blood cells to endothelium is not known. Its clinical effectiveness as a single agent to treat or prevent vasoocclusive complications is, at best, modest, and currently no additional trials of this agent are on-going.

Endothelium-dependent vasodilation is disturbed in sickle cell disease (Eberhardt et al., 2003) and superoxide generation induces chronic inflammation that may be inhibited by apolipoprotein (apo) A-1 mimetics. L-4F, an apoA-1 mimetic, inhibited superoxide production and improved vasodilation in sickle mice (Ou et al., 2003) While a mechanism of action is not yet totally clear, this agent may preserve endothelial function and endothelial nitric oxide synthase (eNOS) activity. This type of drug may have widespread application in vascular diseases including sickle cell anemia (Ansell et al., 2003). Most agents that might
disrupt the adhesive interactions and inflammation hypothesized to presage sickle vasoocclusion have not been studied clinically.

2.13.5 Nitric Oxide

A potent vasodilator, NO is an important regulator of vascular tone and, because of its interaction with hemoglobin, blood vessels and blood cells, has been hypothesized to have several advantageous effects in sickle cell disease. Generated from L-arginine by NO synthases, NO activates soluble guanylate cyclise (sGC), to produce the second messenger, cGMP (Buchanan et al., 2004). Nitric oxide inhibits the adhesive interactions among platelets, leukocytes and sickle erythrocytes (Hankins and Aygun, 2009). It also decreases vascular cell adhesion molecule-1 (VCAM-1) expression in endothelial cells. While a biologically sound rationale exists for using NO in the acute chest syndrome and pulmonary hypertension of sickle cell disease, a controlled trial of this treatment has not been reported. It should be remembered that this approach could be deleterious as NO can be metabolized to damaging oxidants like nitrite (NO$_2^-$) and peroxynitrite (ONOO$^-$). In a clinical trial, inhaled NO was associated with a small reduction in pain score and opioid use in children with acute painful episodes (Weiner et al., 2003).

2.14 Unorthodox Treatment

In Nigeria, herbal medications are used as alternatives to orthodox western medicine due to the perceived minimal side-effects, affordability and easy of acquisition (Ogunkunle and Ladejobi, 2006; Okigbo et al., 2009). Medicinal plants can be described as plants whose part / organ contains a substance that can be used for therapeutic purposes or as a precursor for the synthesis of antimicrobials or other useful drugs (Sofowora, 1982). World Health Organization (W.H.O.) as cited by Okigbo et al. (2009) defined medicinal plants as herbal
preparations produced from chemical, physical, or biological processes which may be used for immediate consumption or as a basis of herbal products. Medicinal and aromatic plants used in such herbal medications contain biologically active substances (secondary metabolites) such as saponins, tannins, flavonoids, alkaloids and other chemical substances which have curative properties (Okigbo et al., 2009). Most of these phytochemical constituents formed during normal metabolic processes are potent bioactive compounds found in specific plant parts, thus many plant components are used in preparing pharmaceutical products for medical treatments. It has also been noted that most treatments for diseases and infections in developing regions such as Africa largely depend on herbal preparations (Okigbo et al., 2009). WHO estimates that 80% of the world population, approximately 4 billion people, uses herbal medicine for primary health care (Macedo et al., 2008). Plant materials thus play a major role in primary health care as therapeutics in many developing countries. Herbal medicine has a long and respected history as the oldest form of healthcare to mankind being used worldwide in various forms.

The use of phytomaterials such as Piper guinensis, Pterocarpa osun, Eugenia caryophylla and Sorghum bicolor extracts for the treatment of SCA has been reported by Wabembe et al. (2001). The extracts of Pterocarpus santolinoides and Aloe vera were reported to increase the polymerisation time of Hbf and inhibit sickling in vitro (Ugbor, 2006). Sofowora and Isaac-Sodeye (1971) reported the reversal of sickling by root extracts of Fagara zanthoxyloides. Terminalia catappa could be effective antisickling agents that inhibit osmotically induced haemolysis of human erythrocytes (Mgbemene and Ohiri, 1999). The aqueous extract of Gracinia kola has been described by Elekwa et al.(2003) as having a higher membrane stabilising effect than phenylalanine, while 2 – hydroxybenzoic acid has been identified as the antisickling agent in aqeous extract of Zanthoxylum macrophylla roots (Elekwa et al.,
The in vitro HbS gelling time was reportedly increased by the extract of *Pterocarpus santolinoides* and *Aloe vera* (Mohanty *et al*., 2012). Antisickling activity of twelve Congolese plants namely *Alchornea cordifolia*, *Afromomum albo violaceum*, *Annona senegalensis*, *Cymbopogon densiflorus*, *Bridelia ferruginea*, *Ceiba pentandra*, *Morinda lucida*, *Hymenocardia acida*, *Coleus kilimandcharis*, *Dacryodes edulis*, *Caloncoba welwithsii*, and *Vigna unguiculata* have been reported (Mpiana *et al*., 2007). It has been suggested that *Cajanus cajan* seeds (Onah *et al*., 2002), aged garlic (Ohnishi and Ohnishi, 2001; Moriguchi *et al*., 2001), unripe *Carica papaya* fruit (Oduola *et al*., 2006; Thomas and Ajani, 1987), leaves (Imaga *et al*., 2009), the roots of *Cssus populnea L. CPK* (Moody *et al*., 2003), *Khaya senegalensis* (Fall *et al*., 1999), *Scoparia dulcis* (Ahmed and Jakupovic, 1990) as well as *Parquetina nigrescens* (Wambabe *et al*., 2005) have various degree of antisickling activities.

The Nigerian *Zanthoxylum* has been extensively studied (Sofowora and Isaacs-Sodeye, 1971; Sofowora, 1974; Sofowora, 1975a; 1975b; Adesanya and Sofowora, 1983). The bioactive compounds responsible for anti-sickling properties were identified as vanillic acid, p-hydroxy benzoic acid and p-fluoro benzoic acid. There are some other drugs prepared from medicinal plants in the market today under various names. The most prominent and widely used of them all is Ciklavit developed by Prof G Ekeke after eighteen years of intensive research in collaboration with Neimeth Pharmaceuticals, Lagos, Nigeria. (Okpuzor, *et al*., 2008)

Research on phytomedicine for the treatment of SCD has led to the development of Nicosan formerly called Niprisan (herbal based drug) which has been patented by the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria and produced to meet increasing global demand by sufferers of SCA. This development indicates that more of such herbal based drugs could be consequent upon scientific investigations on plants that are used in folklore medicine.
On the understanding that herbal remedies and medicinal plants products from indigenous flora have long been used in folk medicine in the management of SCA, it appears that proper and in-depth scientific investigation on such medicinal plants could be of tremendous help in developing efficacious and safer drugs for SCA treatment.

The species *Senna alata* (Fabaceae) and *Senna podocarpa* (Fabaceae) are examples of plants commonly used in popular Nigerian folk medicine. Both *S. alata* and *S. podocarpa* bear a common Yoruba name ‘asunwon’ while *S. alata* is referred to as ‘asunwon oyinbo’, the suffix meaning exotic or introduced species and *S. podocarpa* is known as ‘asunwon gidi’ the suffix meaning indigenous or native species (Odugbemi, 2006; Ogunkunle and Ladejobi, 2006).

### 2.14.1 Physical Description of *Senna alata*

*Senna alata* (Fabaceae) is a plant growing 6-12 feet as a shrub with erect waxy yellow spikes resembling fat candles before individual blossoms open. The leaves are lanceolate in shape, bilaterally symmetrical opposed with smooth margins, folding together at night. The leaflets are 8-20 in number arranged in four pairs. The buds of the plant are rounded with five overlapping sepals, five free or less equal petals narrowed at the base. Fruits are winged pods with seeds small and square. It is found in the tropics (West Africa, Islands of Central and South Pacific, South America and Australia) in secondary vegetations or along riverbanks. It is commonly referred to as Ringworm Cassia, Candle Bush, Candelabra Bush, Empress Candle Plant, Ringworm Tree or “Candletree”. Its conservation status is secure. In Southwest Nigeria it is referred to as “Asunwon oyingbo” in the Yoruba language for its exotic origin; Crawcraw or Ringworm plant for its use in skin diseases (Odugbemi, 2006; Ogunkunle and Ladejobi, 2006).
2.14.1.1 Medicinal Attributes of **Senna alata**

*Senna alata* is a widely recognized medicinal plant amongst herbal traditional practitioners (Ogunkunle and Ladejobi, 2006). It is used in form of poultice, decoctions with plant parts such as leaves, stem, bark and pods used in preparations and are widely believed to be applicable in skin diseases, dysentery, abortifacient, antihelminthic, itch, ringworm, measles, eczema, bronchitis, black menstruation, venereal disease and stomach ache (Odugbemi, 2006). Other members of the Caesalpinioideae subfamily are recognized as well for their medicinal properties—*Senna podocarpa, Cassia fistula, S. tora, C. obtustifolia, C. nigricans, C.acutifolia, C. sieberiana, C. occidentalis, C. angustifolia, C. acutifolia, C. spectabilis* (Ayo and Amupitan, 2007). For example, *S. tora* has been noted for its anti-microbial and anti-oxidant abilities (Uddin *et al*., 2008). Out of the thirty-three species found in Nigeria, *Senna alata* has been noted for its use in the treatment of skin ailments and its use as a laxative or purgative with anti-microbial action against dermatophyte bacterial and fungal infections (Owoyale *et al*., 2005; Ayo and Amupitan, 2007). It has been stated that all the plant parts can be used as an oral laxative due to its anthraquinone content (Owoyale *et al*., 2005).

The leaves of *Senna alata* are used as infusions to treat constipation or poultice to treat oedema and skin disease (Okigbo *et al*., 2009). The leaf sap has been used to treat ringworm, scabies, ulcers, swellings, inflammation and skin parasites (Owoyale *et al*., 2005; Pieme *et al*., 2006). The decoction of the leaf, flower bark and wood has been used in treating eczema and allergy and the decoction of the leaf flower only has been used as an expectorant in bronchitis, dyspnoea and as an astringent and mouthwash (Pieme *et al*., 2006; Idu *et al*., 2006). The methanolic extract has been shown to have a strong growth inhibition against bacteria and fungi—*Mucor sp.*, *Rhizopus sp.*, *Aspergillus niger, Escherichia coli, Bacillus*
subtilis, Salmonella typhii, Pseudomonas aeruginosa and Staphylococcus aureus (Owoyale et al., 2005).

Further research has shown that the methanolic extract of S. alata also inhibited the growth of Microsporum canis, Trichophyton simii, Fusarium solani, Staphylococcus pyogenes, Corynebacterium diptheriae, Dermatophilus congoensis, Actinomyces bovis, Blastomyces dermatitidis, Trichophyton mentagrophytes, Candida albicans, Aspergillus flavus (Makinde et al., 2007). Plants possess primary metabolites and secondary metabolites; some secondary metabolites formed during normal metabolic processes may be responsible for their therapeutic ability. Phytochemical analysis of S. alata leaves shows the presence of phenolic compounds, eugenol, glycosides, saponin, flavonoids, alkaloids, tannins and anthroquinone. Anti-microbial activity of the methanolic extract of the leaves on the isolates of Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Bacillus subtilis, Microsporum gypseum and Trichophyton rubrum has been established (Phongpaichit et al., 2004; Ogunkunle and Ladejobi, 2006; Idu et al., 2006). S. alata has been found to contain azulene, guiane, 1, 5, 7-trihydroxy-3-methylantraquinone, rhein (1, 8-dihydroxyantraquinone), chrysophanic acid, chrysophenol and aloe-emodin (Hauptmann and Nazario, 1950; Phongpaichit et al., 2004; Ayo and Amupitan, 2007). Kaempferol is a flavonoid compound isolated from the methanolic extract of leaves of S. alata plant found to be responsible for the antioxidant effect of the extract (Panichayupakaranat and Kaewsuwan, 2004). Ethanolic extract of Cassia alata was found to exhibit protective antioxidant activities in rats exposed to carbon tetrachloride (Wegwu, 2005). The methanolic extract of S. alata leaves was found to be more effective than the chloroform, petroleum ether or water/aqueous extracts in anti-microbial activity (Owoyale et al., 2005; Idu et al., 2006; Makinde et al.,
2.14.2 Physical Description of *Senna podocarpa*

*Senna podocarpa* (Fabaceae) formally known as *Cassia podocarpa* is an herbal shrub that grows up to 5m high with dense racemes of yellow flowers and thin flat pods. It has a perennial life span and is widely distributed in Africa, and in some part of Asia. *S. podocarpa* is found in the savanna forest area of West Africa (Akanmu *et al*., 2005). The plant is found locally, on old farmlands in both western and northern parts of Nigeria (Dalziel and Hutchinson, 1958).

2.14.2.1 Medicinal Attributes of *Senna podocarpa*

This plant has been used extensively in the folklore medicines for the treatment of a variety of skin diseases such as scabies, ringworm and eczema (Sofowora, 1986) especially amongst the Igbo and Yoruba speaking tribes of Nigeria where it is locally known as Ageloo-ogala and Asunwon gidi, respectively (Adefemi *et al*., 1998). The leaves have been extensively used based on their antigonorheoeal and purgative properties as well as sore-healing remedy (Elujoba *et al*., 1994; Akanmu, 1999; WHO, 1999). The virucidal property against Herpes simplex virus and African swine fever virus and their infections was established by Silva *et al*. (1997) Decoction of the leaves is given as a mild laxative and in large doses, it acts as a purgative (Trease and Evans, 1996). In Liberia folk medicine, amongst the Kpelle people, the effects of stem bark extracts in vitro and in vivo on microfilaria were examined and found to possess curative effect (Kilian *et al*., 1990). According to Gomes *et al*. (1997) *S. podocarpa* is widely used by Fulani traditional healers living in Guinea Bissau to treat veneral diseases.
Ehtnobotanical and phytochemical studies of the leaves and pods of *S. podocarpa* have identified some of the plant constituents, which include rhein, emodin, chrsophanol, free or combined anthraquinones (Rai and Obayemi, 1973; Rai and Abdulahi, 1978; Elujoba *et al.*, 1994). Anthraquinones derivatives (Akinyemi *et al.*, 2000) obtained from the pods and leaves have been held responsible for the plants’ laxative as well as purgative activities. Other phytochemical groups in the leaf extracts of the plants include flavonoids, tannins, phlobatannins and saponins (Ogunkunle and Ladejobi, 2006)

### 2.15 Cytotoxicity testing

Sickle cell anemia (SCA) can potentially be cured by gene therapy (Perumbeti and Malik, 2010) and hematopoietic stem cell transplantation (Bernaudin *et al.*, 2007) but neither is currently applicable to most patients with this disease due to technical reasons, cost and lack of highly sophisticated medical care necessary to provide these therapies in Nigeria. Over 50 agents have been described that increase γ-globin expression and/or HbF in primary human erythroid cell cultures or when administered to humans. But most inducing agents, including histone deacetylase inhibitors, DNA methyltransferase inhibitors and cancer chemotherapy drugs may be cytotoxic, and may alter epigenic marks on a genome-wide basis, damage DNA or suppress erythropoiesis (Mabaera *et al.*, 2008). Hydroxyurea is the only approved drug for use in humans, though it has provided a major advance in the care of people with SCA, it is only effective in one – half of SCA patients (Steinberg *et al*. 1997a) and it produces significant suppression of blood counts. These and other issues have led to the low rate of compliance in Africa (Aliyu and Babdok, 2007) and United States (Hampton, 2008).

In view of the shortcomings of current inducing agents, there are no drugs that are effective, safe and have the ease of use that would make them applicable to most patients with SCA. There is therefore an urgent need to screen for natural inducers of HbF and identify new types
of agents that can induce HbF production with greater efficiency and lower toxicity (Lal and Vichinsky, 2004). In developing countries, so many constituents of plants capable of diseases treatment have been investigated by scientists with a view to contributing to the search for substances that would be effective in the management of the diseases. Crude extracts from plants have been used in treating many diseases since ancient times, although, the biological concentration and toxicity of such plant extracts are seldom known. The safety of any therapeutic agent is of paramount importance and appropriate toxicity assays to evaluate biological safety of biomaterials are essential. In vitro cytotoxicity assays with cultured cells are very common because they are rapid, inexpensive and do not have ethical implications (Ishiyama et al., 1996; Harbell et al., 1997; Fernandes et al., 2005). Several methods for determining cellular viability and/or growth following in vitro exposure to compounds have been reported. These methods used well known dyes that develop a colour, allowing a colorimetric measurement of cell viability.

Cytotoxicity is a technique for studying the effectiveness of substances on the survival and proliferation of cells before testing them in patients (Carney and Winkler, 1985). The assay measures the toxicity of a treatment on cells at a wide range of doses and it is frequently used in cancer research laboratories to determine the effect of drugs on proliferating tumor cells (Hoffman and Robert, 1991). In vitro cytotoxicity tests are based on the idea of ‘basal’ cytotoxicity – that toxic substances affect basic functions of cells which are common to all cells, and that the toxicity can be measured by assessing cellular damage. The development of in vitro cytotoxicity assays has been driven by the need to rapidly evaluate the potential toxicity of large numbers of compounds, to limit animal experimentation whenever possible, and to carry out tests with small quantities of compound (Barile et al., 1994). Evidence for the use of in vitro cytotoxicity tests has led many pharmaceutical companies to screen
compound libraries to remove potentially toxic compounds early in the drug discovery process (Davila et al., 1990; Barile et al., 1994 and Todd et al., 1999). Early identification of toxic effects can help project between chemical series and identify toxicity relationships that may exist between the chemicals and the cells (Todd et al., 1999).

A reduction in cellular ATP levels or mitochondrial activity is an early indication of cellular damage. It has been shown that changes in metabolic activity are better indicators of early cell injury, and that effects on membrane integrity are indicative of more serious injury, leading to cell death (Davila et al., 1990). The key features of cytotoxicity assay include low compound consumption and multiple assays. Mitochondrial enzyme, succinate-tetrazolium reductase, present in viable cells reduces tetrazolium salts to soluble or insoluble coloured formazan compounds that can be quantified spectrophotometrically. The most commonly used tetrazolim salt is MTT [3-(4, 5-Dimethylazol-2-yl)-2,5-diphenyltetrazolium bromide], however, the disadvantage is that the formazan dye produced from MTT is extremely water insoluble, so an additional extraction step is needed for spectrophotometric quantification, which can be avoided with the use of WST-1 [2,(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium]. Since the bio reduction of tetrazolium salt, is a sensitive chromogenic indicator for NADH produced by metabolically active cells in culture, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture (Riss and Moravec, 2004).

One parameter for cell death is the integrity of the cell membrane, which can be measured by the cytoplasmic enzyme activity released by damaged cells (Riss and Moravec, 2004). Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane (Korzeniewski and Callewaert, 1983). The LDH activity is determined in an enzymatic test.
The first step is the reduction of NAD+ to NADH/H+ by the LDH catalyzed conversion of lactate to pyruvate. In a second step, the catalyst (diaphorase) transfers H/H+ from NADH/H+ to the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is reduced to a red formazan (Nachlas et al., 1960; Decker and Lohmann-Matthes, 1988; Lappalainen et al., 1994). Neutral red (3-amino-ｍ-dimethylamino-2-methylphenazine hydrochloride) has been used previously for the identification of vital cells in cultures (DeRenzis and Schechtman, 1973). This assay quantifies the number of viable, uninjured cells after their exposure to toxicants; it is based on the uptake and subsequent lysosomal accumulation of the supravital dye, neutral red. Quantification of the dye extracted from the cells has been shown to be linear with cell numbers, both by direct cell counts and by protein determinations of cell populations (Borenfreund and Puerner, 1985, 1986).

Cell lines are often used for in-vitro cytotoxicity testing, and an experimental model system employing the human erythroid-like K562 cell line, isolated and characterized by Lozzio and Lozzio (1975) from a patient with chronic myelogenous leukemia in blast crisis, has been extensively employed as a very useful in vitro model to study the molecular mechanism(s) regulating the expression of embryonic and fetal human globin genes (Bianchi et al., 2000), as well as to determine the therapeutic potential of new differentiation-inducing compounds (Osit et al., 1997; Bianchi et al., 2001). K562 cells grow in culture as single, undifferentiated, cells in suspension, with low production of hemoglobins (Bianchi et al., 2007) but when stimulated by various agents, they respond within few days with a significant increase in the production of hemoglobins and γ-globin mRNA.
3.0 MATERIALS AND METHODS

3.1.1 Plant Materials

The *Cajanus cajan* (beans and leaves), *Smilax kraussiana* (roots), *Alchornea cordifolia* (leaves), *Senna alata* (leaves), *Senna podocarpa* (leaves) used in this study were sourced, during the rainy season (July – September) from 2006 - 2007, and authenticated by herbarium curators at the Forestry Research Institute of Nigeria (FRIN), Ibadan. The voucher specimens of *Senna alata* leaves (No.FHI.106929) and *Senna podocarpa* leaves (No.FHI.106995) were deposited at FRIN herbarium. “Jana”, an herbal formulation, was graciously provided by Prof. (Mrs.) Okpuzor of the Department of Cell Biology and Genetics, University of Lagos

3.1.2 Participants

The informed consent of the volunteers (SCA patients) was obtained after counseling in accordance with the approved University protocols (Appendix 1). Blood was collected from HbSS patients in steady states who had not been on any herbal therapy for at least six months, but were on sustainance therapy (folic acid and paludrin). Blood (3ml) from donors was taken into heparin or EDTA tubes and used within two hours post collection.

3.1.3 Experimental Animals

Male albino rats (94 -110 g) were purchased from the University of Ibadan animal house. The rats were allowed to acclimatize for 2 weeks at the University of Lagos, Department of Cell Biology and Genetics animal house experimental facility, provided standard feed for laboratory rodent, regular tap water *ad libitum*. All animals had free access to water and standard rat pellets (Pfizer). Rats were fasted (12-15hrs) prior to the administration of extract.
3.2 Preparation of Extracts

The plant materials (200g) were extracted with either 80% ethanol or 80% methanol using a Soxhlet apparatus, concentrated under reduced pressure to a viscous liquid, freeze dried, weighed and stored at 4 °C until use. For water extract, 10 g of powdered leaf material was mixed with boiling water (500 ml) for 15 min. The extract was filtered and evaporated under vacuum below 70 °C on a rotary evaporator according to Duh and Yen, (1997).

3.3 Preliminary Screening

The hydroethanolic extract of *Cajanus cajan* (beans and leaves), *Smilax kraussiana* (roots), *Alchornea cordifolia* (leaves), *Senna alata* (leaves), *Senna podocarpa* (leaves) as well as Jana were evaluated for their HBSS erythrocyte membrane stabilizing activity using the osmotic fragility test (Dacie and Lewis, 1991)

3.4 Phytochemical Screening

The phytochemical compositions of the extracts were evaluated according to the methods of Trease and Evans (2002) and Harborne (1991).

3.5 Proximate Analysis

3.5.1 Preparation of the plant materials for chemical analyses:

*Senna alata* leaves and *Senna* podocarpa leaves were washed with distilled water dried at room temperature to remove residual moisture, and oven-dried at 55 °C for 24 hours (Aletor and Adeogun, 1995; Abuye *et al*., 2003). The dried leaves were ground into powder using a blender (MARLEX; CM/L 7371373), and sieved through 20-mesh sieve. The leaf powder was used for the chemical composition analyses.
The methods recommended by the Association of Official Analytical Chemists were used to determine ash (#942.05), crude lipid (#920.39), crude fibre (#962.09) and nitrogen content (#984.13). All the proximate values were reported in % (AOAC, 1990).

3.5.2 Determination of Moisture Content

Moisture content of the leaf of the two Senna species were determined by drying leaf samples at 105°C in a hot air oven to constant weight (3 hrs) (AOAC, 1990).

3.5.3 Determination of Ash Content

Two gram aliquot of the sample was placed in a pre-weighed crucible; this was heated on a hot plate until no smoke was visible. The residue containing crucible was transferred into a muffle furnace set at 550°C for 3 hours after which the ash containing crucible was cooled in a desiccator and weighed. The weight of the ash containing crucible was subtracted from that of the pre-weighed crucible to obtain the ash content. (AOAC, 1990)

3.5.4 Determination of crude lipid and crude fibre content:

Two grams of dried leaves were weighed in a porous thimble of a Soxhlet apparatus. The thimble was placed in an extraction chamber, which was suspended above a pre-weighed receiving flask containing petroleum ether (b.p.40-60°C). The flask was heated on a heating mantle for eight hours to extract the crude lipid. After the extraction, the thimble was removed from the Soxhlet apparatus and the solvent distilled off. The flask containing the crude lipid was heated in the oven at 100°C for 30 min to evaporate the solvent, then cooled in a desiccator, and reweighed. The difference in weight was expressed as percentage crude lipid content. Crude fibre was estimated by acid-base digestion with 1.25 % H₂SO₄ (prepared by diluting 7.2 ml of 94 % conc. acid of specific gravity 1.835 g ml⁻¹ per 1000 ml distilled water) and 1.25 % NaOH (12.5 g per 1000 ml distilled water) solutions. The residue after
crude lipid extraction was placed in a 600 ml beaker and 200 ml of boiling 1.25 % H₂SO₄ added. The contents were boiled for 30 minutes, cooled, filtered through a filter paper and the residue washed three times with 50 ml aliquots of boiling water. The washed residue was returned to the original beaker and further digested by boiling in 200 ml of 1.25 % NaOH for 30 minutes. The digest was filtered to obtain the residue. This was washed three times with 50 ml aliquots of boiling water and finally with 25 ml ethanol. The washed residue was dried in an oven at 130ºC to constant weight and cooled in a dessicator. The residue was scraped into a pre–weighed porcelain crucible, weighed, ashed at 550ºC for two hours, cooled in a dessicator and reweighed. Crude fibre content was expressed as percentage loss in weight on ignition, (AOAC, 1990; Nesamvuni et al., 2001).

3.5.5 Determination of nitrogen content and estimation of crude protein:

Macro-Kjeldahl method was used to determine the nitrogen content, which is the major component of amino acid, a precursor for protein. Two grams of dried leaves were digested in a 100 ml Kjeldahl digestion flask by boiling with 10 ml of concentrated tetraoxosulphate (VI) acid (H₂SO₄) and a Kjeldahl digestion tablet (a catalyst) until the mixture was clear. The digest was filtered into a 100 ml volumetric flask and the solution made up to 100 ml with distilled water. Ammonia in the digest was steam distilled from 10ml of the digest to which had been added 20 ml of 45 % sodium hydroxide solution. The ammonia liberated was collected in 50 ml of 20 % boric acid solution containing a mixed indicator. Ammonia was estimated by titrating with standard 0.01 M HCl solution. Blank determination was carried out in a similar manner. Crude protein was estimated using the following formula:

\[
% \text{Crude protein} = % \text{Nitrogen content} \times 6.25 \quad (\text{AOAC, 1990}).
\]
3.5.6 Determination of carbohydrate and energy values:

Available carbohydrate was estimated, by subtracting the total sum of percent crude protein, crude lipid, crude fibre and ash from 100 % dry weight of the plant. The plant calorific value (kJ) was estimated by multiplying the percentages of crude protein, crude lipid and carbohydrate by the factors 16.7, 37.7 and 16.7 respectively and adding up the products. (AOAC, 1990).

3.6 Invitro Studies

3.6.1 Effect of Extract on HbSS Erythrocytes

3.6.1.1 Determination of Osmotic Fragility

Osmotic fragility of red cells was determined according to the modified method of Dacie and Lewis (1991). A 10% buffered saline solution was prepared by dissolving 45g NaCl, 6.828g Na$_2$HPO$_4$ and 0.935g NaH$_2$PO$_4$ in 500 ml of distilled water. A 1 % buffered saline solution was then prepared and its pH adjusted to 7.4 using a pH meter. Graded concentrations of buffered saline solution (0.00 - 0.85 %) were prepared and 5.0 ml of each concentration was dispensed into prelabelled tubes (control), while 4.0 ml of each concentration was dispensed into another set of eight tubes and 1.0 ml of buffered extract was added to each of the tubes (experimental). A drop (0.02 ml) of heparinised whole blood was then added to each tube and mixed thoroughly by five gently inversions, then incubated at room temperature for 30 mins after which the contents of the tubes was again mixed and centrifuged for five minutes at 2500 rpm. The supernatant was decanted into cuvettes and the optical density read at a wavelength of 540 nm using the supernatant from the 0.85 % tube as blank for each set of tubes. Lysis was recorded as a percentage (optical density of the solution of tube without NaCl was taken as 100 % hemolysis), and plotted against percentage sodium chloride to
obtain a fragility curve. The mean corpuscular fragility (MCF) index, which is the concentration of saline solution (NaCl g/L) that caused 50% lysis of erythrocytes, was interpolated from the plot and the % membrane stabilisation was calculated using the following formula: 

\[
\text{% Membrane Stability} = \left( \frac{\text{MCF}_{\text{control}} - \text{MCF}_{\text{test}}}{\text{MCF}_{\text{control}}} \right) \times 100
\]

(Okpuzor and Adebesin, 2006; Chikezie and Uwakwe, 2011)

3.6.1.2 Activity of the Extracts on Haemoglobin Polymerisation

The venous blood was washed with incubation media and collected by centrifugation at 2500 rpm for five minutes, until the supernatant was clear. A 10 % or 35% hematocrit was prepared for antisickling or desickling experiment respectively. All prepared blood samples were stored at 4°C until use and allowed to attain room temperature before use.

3.6.1.2.1 Antisickling Activity of the Extracts

A 2000 µl aliquot of washed red blood cells (10 % hematocrit) was mixed with 2000 µl of buffered plant extract (5 mg ml\(^{-1}\)) or control. The mixture was gently shaken and at 30 mins interval, a 400 µl aliquot was withdrawn and mixed with 600 µl of freshly prepared 2 % sodium metabisulphite solution in a fresh tube. The resulting mixture was layered with 600µl of liquid paraffin and incubated at 37°C. A 100 µl aliquot was withdrawn every 35 mins into a fresh Eppendorf tube, mixed thoroughly with 200 µl of 5 % buffered formalin and then incubated at room temperature until ready to prepare slides. For the positive controls, namely NICOSAN (an herbal formulation currently used by SCA patients) (5 mg ml\(^{-1}\)), parahydroxybenzoic acid (synthetic drug) (5 mg ml\(^{-1}\)) and negative control (sodium chloride) a 1000 µl aliquot of the washed RBC and 1000 µl of control (NICOSAN / parahydroxybenzoic acid / sodium chloride) were thoroughly mixed at 37°C and immediately (zero time) a 200 µl aliquot was withdrawn and at intervals of 30mins thereafter, into fresh
Eppendorf tubes, mixed with 300 µl of freshly prepared 2 % sodium metabisulphite solution and the resulting mixture was further diluted with 500 µl of buffered formalin.

3.6.1.2.2 Desickling Activity of the Extracts

A 2000 µl aliquot of washed red blood cells (35 % hematocrit) was layered with liquid paraffin and 2000 µl of freshly prepared 2 % sodium metabisulphite solution was added with the aid of a needle and syringe. The mixture was gently rolled between the palms and incubated for 2 ½ hours at 37°C with occasional shaking. After incubation a 500 µl aliquot of the mixture was withdrawn and mixed with 500 µl of Senna alata (5 mg ml⁻¹), Senna podocarpa (5 mg ml⁻¹), parahydroxybenzoic acid (5 mg ml⁻¹) or physiological saline solution in a fresh tube layered with liquid paraffin and preincubated at 37°C. A 100 µl aliquot was withdrawn every 30 mins into a fresh Eppendorf tube, mixed thoroughly with 200 µl of 5 % buffered formalin and then incubated at room temperature until ready to prepare slides.

3.6.1.2.3 Preparation of Microscopic Slides

The mixture containing buffered formalin and control was centrifuged at 2000 rpm for 5 minutes and 250 µL of the supernatant was removed. With the aid of a capillary tube, a drop was taken and placed on a microscope slide. A smear was made and fixed with 95 % methanol. The slide was stained with Giemsa stain and excess stain was washed off. The slide was air dried and examined under light microscope. About 100 RBCs were counted from five different fields.
3.6.2 Antioxidant Potential of Extracts

3.6.2.1 Total Antioxidant Capacity

The total antioxidant capacity of the plant extract(s) was determined using the method of Prieto et al. (1999) with slight modifications. The assay is based on the formation of a green phosphate-molybdenum (V) complex by the reduction of molybdenum (VI) at acidic pH. Aliquots of 0.1 ml of each extract or the standard - ascorbic acid (100 µg ml\(^{-1}\)) was mixed with 3 ml of working reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95°C for 90 minutes, cooled to room temperature and the absorbance of each solution was measured at 695 nm against a blank (working reagent plus solvent-methanol/water) using a spectrophotometer. The experiment was performed in triplicates. The total antioxidant capacity was expressed as milligram equivalent of ascorbic acid.

3.6.2.2 Total Reducing Power

The reducing power of the plant extract(s), gallic acid and ascorbic acid was determined by assessing the ability to reduce ferric chloride solution (Athukorala et al., 2006). Aliquots of 1ml graded concentration (25-100 µg ml\(^{-1}\)) of the crude extract or standard (gallic acid or ascorbic acid) was mixed with equal volume of 1% ferricyanide and 200 mM phosphate buffer, pH 6.6. the mixture was incubated at 50°C for 20 minutes. An equal volume of 10 % trichloroacetic acid was then added and the resulting solution centrifuged at 6000 rpm for 10 minutes. The resulting supernatant, distilled water and 0.1 % ferric chloride were mixed in the ratio 1:1:2 and the absorbance measured at 700nm. A higher absorbance indicated higher reducing power.
3.6.2.3 Total Phenolic Content

The total phenolic content of the plant extract(s) was determined according to the method of Singleton and colleagues (1999), using the folin-ciocalteau reagent (FCR) which is reduced by phenols to a mixture of blue oxides with maximal absorbance at around 760 nm. Equal volumes (100 µl) of plant extract or gallic acid (0-100 µg) were mixed with 500 µl of FCR and 1.5 ml of 20 % sodium carbonate. The resulting mixture was thoroughly shaken, made up to 10 ml with distilled water and incubated at room temperature for 2 hours. The absorbance at 760 nm was determined against a reagent blank. The experiment was performed in triplicate and the total phenolic content expressed as gallic acid equivalents (GAE)

3.6.2.4 2, 2-Diphenyl-1-picryl-hydrazyl (DPPH) Radical Scavenging Activity

The DPPH radical scavenging activity (DRSA) of the plant extract(s) or standard (ascorbic acid) was assayed with slight modification of the method of Uddin et al. (2008) which depends on the production of yellow coloured diphenylhydrazine via reduction of purple DPPH (maximum absorbance at 517 nm). Graded concentrations (25-100 µl ml⁻¹) of the extract or vitamin C were prepared. A 2 ml aliquot of each test solution was then added to 0.5 ml of 1 mM DPPH solution in methanol and the mixture incubated in the dark for 15 minutes at 37°C, after which the absorbance was measured at 517 nm against a blank containing methanol and DPPH. This was performed in triplicate. The DPPH radical scavenging activity was calculated using the equation:

\[
\% \text{ DRSA} = 1 - \left( \frac{A_s}{A_b} \right) \times 100
\]

Where \( A_s \) and \( A_b \) are absorbance of the sample and blank respectively. A decrease in absorbance indicates an increase in DPPH radical scavenging activity.
3.6.2.5 DNA Protection Assay

Antioxidant / pro-oxidant activity of crude plant extracts assessed in aqueous system via the method of hydroxyl radical-induced DNA damage in plasmid pBR322 as described previously by Russo et al. (2001) with slight modification. To test for DNA damage induced by hydroxyl radicals, the reaction was conducted in an Eppendorf tube at a total volume of 12 µl containing 0.5 µg pBR322 DNA in 3 µl of 50 mM phosphate buffer (pH 7.4), 3 µl of 2 mM FeSO$_4$ and 2 µl of the hydromethanolic leaf extract of *S. alata* or *S. podocarpa* at concentrations of 10, 100 and 1000 µg ml$^{-1}$ followed by the addition 4 µl of 30% H$_2$O$_2$. The resulting mixture was incubated for 60 min at 37 °C. The DNA (super-coiled, linear and open circular) was analyzed on 1% agarose gels and visualized using ethidium bromide staining.

3.7 *In Vivo* Studies

3.7.1 Toxicological Studies

3.7.1.1 Organ Toxicity

3.7.1.1.1 Treatment of Animals

One hundred and twelve young albino rats (6 – 8 weeks old) were randomly distributed into seven groups of 16 rats each. Group A which served as control did not receive any dose of the extract but was administered 1 ml of distilled water daily. Groups B, C, D, received 1 ml daily dose of 200, 600 and 1000 mg kg$^{-1}$ body weight extract of *Senna alata* while groups E, F, G, received 1 ml daily dose of 200, 600 and 1000 mg kg$^{-1}$ body weight extract of *Senna podocarpa* respectively, for 15, 35 or 63 days. The animals were deprived of food but not water (8-14 hours) prior to administration of extract. During the period of administration, the animals were weighed, food and water intake were monitored daily. After 15, 35 or 63 days, all surviving animals were fasted overnight. Animals were sacrificed after anesthesia
(chloroform) by decapitation and blood from each animal was collected separately in labelled heparinised / EDTA tubes. Selected organs namely the brain, heart, kidney, liver, lungs, spleen, testes and cauda epididymes were harvested and washed instantly in normal saline. The washed organs were blotted dry with filter paper, weighed and labelled separately. About 0.2 g of the organs were cut with surgical scissors, kept separately in labelled containers and frozen (-20°C) for further analysis of biochemical parameters. The left over organs were immediately preserved in 1% formal saline for histopathological studies.

3.7.1.2 Haematological Parameters

Blood collected in anticoagulant (EDTA) tubes were used to determine populations of white blood cells (WBC) and red blood cells (RBC) according to Dacie and Lewis, 1991. Packed cell volume (PCV) was also evaluated.

3.7.1.2.1 Determination of packed cell volume (PCV)/ Haematocrit

PCV was determined by the microhaematocrit method. Haematocrit is the volume of red blood cells expressed as a percentage of the volume of whole blood in the sample. Lithium heparinised capillary tube coated with anticoagulant were filled with blood obtained from EDTA bottles. One end of the capillary tube was sealed with plastercine, and centrifuged for 5 mins at 13000 rpm in a high speed haematocrit centrifuge. The packed cell height of the sample expressed in percentage (%) was determined using a PCV chart.

3.7.1.2.2 Determination of white blood cell count (WBC)

A white blood cell count estimates the total number of white blood cells in 1 mm³ of blood. A 20 µl aliquot of blood sample was mixed with 380 µl of Turk’s fluid and the mixture was put in a Neubauer counting chamber which was subsequently viewed under low power objective
(X10) of a light microscope. The four larger corner squares containing uniformly distributed WBCs were observed and the cells therein counted.

### 3.7.1.2.3 Determination of differential white blood cell count

A differential white blood cell count provides information on the different types of white blood cells present in the circulating blood which include neutrophils, lymphocytes, monocytes, basophils (rarely seen) and eosinophils. Blood from EDTA tube was used to prepare a thin blood smear on a clean grease free labelled microscope glass slide. The smear was air dried, fixed with absolute methanol and stained with Leishman stain. The stained slide was allowed to dry and then examined under the light microscope using the oil immersion objective.

### 3.7.1.3 Biochemical Assay

Blood collected into anticoagulant (lithium heparin) tubes were centrifuged at 3000 rpm for 10 minutes at room temperature. The plasma samples were obtained and stored at -4°C in labeled sample bottles until required. The resulting plasma and 10 % liver homogenate was analyzed to determine the activity of some marker enzymes. Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were analyzed by the method of Obidike et al., 2011 and total protein using the Biuret method. The catalase and thiobarbituric acid reactives substances (TBARS) levels, (a measure of antioxidant status) of plasma as well as organ homogenates were also determined according to the method of Niehaus and Sammuelson, (1968). The 10 % homogenate of selected organs were also prepared by macerating 1 g of each organ with 9 ml of phosphate buffered saline. The mixture was centrifuged at 5000 rpm for 5 minutes and the supernatant transferred into prelabeled Eppendorf tubes.
3.7.1.3.1 Determination of biochemical Parameters

3.7.1.3.1.1 Determination of albumin

A 100 µl aliquot of water (blank), plasma or standard (albumin) (100 mg dl$^{-1}$) was mixed with 2500 µl of buffered bromocresol green and incubated for 10 mins at room temperature after which the absorbance was read against a blank.

Albumin concentration = (OD sample / OD standard) × 100 mg dl$^{-1}$

3.7.1.3.1.2 Determination of total protein (TP)

Proteins react with cupric ions in alkaline solution to form a purple complex. A 20µl aliquot of plasma sample, water (blank) or standard (80 g l$^{-1}$ protein and 0.095 % sodium azide) was mixed with 1000 µl aliquot of working solution [sodium hydroxide (200 µl), potassium sodium tartarate (32 mmol l$^{-1}$), copper sulphate and potassium iodide (30 mmol l$^{-1}$)], incubated for 10 mins at room temperature then transferred into cuvettes and the absorbance of the samples and standard read against the reagent blank within 30mins at 546 nm.

The total protein concentration (g l$^{-1}$) = 80 × (OD sample / OD standard)

3.7.1.3.2 Determination of enzyme activities

3.7.1.3.2.1 Determination of alanine aminotransferase (ALT)

Alanine aminotransferase catalyse the production of pyruvate from α-ketoglutarate and L-alanine. Pyruvate is a measure of ALT activity, in the presence of 2,4-dinitrophenylhydrazone forms pyruvate-hydrazone complex.

A 100 µl aliquot of water/plasma samples was mixed with 500 µl aliquot of working solution [100 mmol l$^{-1}$ phosphate buffer (pH 7.4), L-alanine (200 mmol l$^{-1}$ and α-ketoglutarate (2.0
mmol l⁻¹)] and incubated for exactly 30 mins at 37 °C, then 500 µl of 2,4-
dinitrophenylhydrazine (2 mmol l⁻¹) was added. The resulting solution was further incubated
for 20 mins at room temperature, after which 5000 µl of sodium hydroxide (0.4 mmol l⁻¹) was
added and the absorbance read against the blank at 546 nm in a spectrophotometer.

3.7.1.3.2 Determination of aspartate aminotransferase (AST)

Aspartate aminotransferase catalyse the production of oxaloacetate from α-oxoglutarate and
L-aspartate. oxaloacetate a measure of AST activity, in the presence of 2,4-
dinitrophenylhydrazone forms pyruvate-hydrazone complex.

A 100 µl aliquot of water (blank) or plasma sample was mixed with 500 µl aliquot of
working solution [100 mmol l⁻¹ phosphate buffer (pH 7.4), L-aspartate (200 mmol l⁻¹) and α-
oxoglutarate (2.0 mmol l⁻¹)] and incubated for exactly 30 mins at 37 °C, then 500 µl of 2,4-
dinitrophenylhydrazine (2 mmol l⁻¹) was added. The resulting solution was further incubated
for 20 mins at room temperature, after which 5000 µl of sodium hydroxide (0.4 mmol l⁻¹) was
added and the absorbance read against the blank at 546 nm in a spectrophotometer.

3.7.1.3.3 Determination of alkaline phosphatase (ALP)

A 500 µl aliquot of plasma sample was mixed rapidly with 3000 µl aliquot of working
reagent (p-nitrophenylphosphate (10 mmol l⁻¹), diethanolamine buffer (1 mol l⁻¹, pH. 9.8) and
0.5 mm l⁻¹ magnesium chloride) in a cuvette and the absorbance read at 1 min interval for 3
mins at 405 nm, in a spectrophotometer.
3.7.2 Antioxidant studies

3.7.2.1 Determination of Catalase (CAT) activity

Catalase activity was assayed colorimetrically at 620 nm and expressed a μmoles of hydrogen peroxide consumed per min or hydrogen peroxide consumed per min per mg of protein as described by Sinha, 1972. The reaction mixture (1.5 ml) contained 1ml 0.01 M phosphate buffer (pH 7.0), 100 μl of sample and 400 μl of H₂O₂.

Catalase enzyme activity = (ΔOD/min/E) x (V/v) x 10³
Where:
ΔOD/min = change in absorbance/min
E = Molar concentration co-efficient (40)
V = Total volume of reacting sample
v = Volume of sample

3.7.2.2 Determination of lipid peroxidation

Lipid peroxidation resulting in the formation of thiobarbituric acid reactive substances (TBARS) was measured by the method of Rael et al. (2004). A 100 μl aliquot of plasma was mixed with 2000 μl working reagent (equal volumes of thiobarbituric acid (0.37 %), trichloroacetic acid (15 %) and 0.25 M HCl). The mixture was boiled for 15 mins in a water bath, cooled, centrifuged at 1000 rpm for 10 mins at room temperature and the absorbance of the clear supernatant was measured against a blank at 535 nm in a spectrophotometer.

The TBARS level expressed as malonyldialdehyde (MDA) level (nmol ml⁻¹)

= (OD/E) x (V/v) x 10⁶
Where: OD = change in absorbance/min
E = Molar concentration co-efficient (40)
V = Total volume of reacting sample
v = Volume of sample

3.7.3 Electrophoresis of Red Blood Cell (RBC) Membrane Proteins

Albino rat erythrocyte plasma membranes (ghosts) were prepared using a modification of the procedure of Johanning and O'dell (1989). Blood obtained from rats dosed daily for 63 days with the plants extract was collected into EDTA bottles and centrifuged at 2500 rpm for 15 minutes at 4°C. The plasma and buffy coat were then carefully removed using a Pasteur pipette. Erythrocytes were then washed 3 times in cold 5 mM sodium phosphate / 0.15 M sodium chloride and centrifuged as before. Careful aspiration was performed after each wash, sacrificing packed cells at the interphase to ensure removal of the buffy coat. Packed cells (1 mL) were then lysed in 20 mL of cold 0.01% saponin in 5 mM sodium phosphate, pH 8.0. The mixture was incubated at 4°C for 10 minutes and centrifuged at 16,000 rpm for 20 minutes at 4°C. The resultant deep red supernatant lysates (enriched cytosolic content) were discarded, leaving a red pellet of packed ghost over a creamy white layer of leukocyte debris. The ghost was carefully separated from this protease rich layer, by tilting and rotating the tube to free the ghost and then aspirating it into a new tube containing ice cold 5 mM sodium phosphate, pH 8.0 and washed severally by centrifuge until an opaque pellet was obtained. The protein content of the resulting pellet was assayed using the Biurets method and final pellet was solubilised with 2 volumes of SDS-PAGE sample loading buffer and boiled for 5 minutes. The membrane protein profile was analysed by SDS-polyacrylamide (10 %) gel electrophoresis under denaturating conditions, using the discontinuous buffer system of Laemmli (1970). Equal concentrations of protein were loaded per track on each gel.
3.8.1 Genotoxicity Studies

3.8.1.1 Genotoxicological Investigation of Extract Using an Animal (Albino Rat) Model

3.8.1.1.1 Sperm-Head Morphology Assay

Male albino rats (35) were divided into seven experimental groups comprising of 5 animals per group and one group served as control. The control group received by gavage, 1 ml of water daily for 63 days, while the experimental groups received also by gavage, 1 ml of sub chronic doses of 200, 600 and 1000 mg kg⁻¹ body weight, corresponding to low, medium and high doses of each plant extract. After 63 days from the first exposure, the animals were sacrificed under anaesthesia, and the cauda epididymis removed and 1mg macerated in prelabelled tubes containing 5 mls of normal saline. A fraction of each suspension was mixed with 1% eosin Y solution and a smear made on a clean glass slide. The air dried smear slides were coded (to avoid bias) and scored for the different sperm head morphologies.

3.8.2 CELL LINE STUDIES

The cytotoxic effect of the extracts on the human erythroid-like K562 cell line was determined using WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] cell viability and proliferation assay kit (ScienCell), neutral red uptake assay (Creppy et al., 2004) and LDH leakage assay (Upur et al., 2008).

3.8.2.1 Cell Culture

Human chronic myelogenous leukemia K562 cells purchased from Cell Line Services (Germany), were cultured and maintained in a Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma Aldrich). Cells were grown at 37º C in a humidified atmosphere of 5% CO₂/air.
3.8.2.2 Cell viability Assay (WST-1)

The hydromethanolic leaf extract of *Senna alata* and *Senna podocarpa* were tested for *in vitro* cytotoxicity, using K562 cells. Cytotoxicity was measured by the reduction of tetrazolium salt - WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] - to colored formazan compounds by succinate-tetrazolim reductase which exists in viable cells. Briefly, 50 μl of media (RMPI 1640) was added to wells in the 96-well plates from row A to row F (triplicate). Then, 100 μl of diluted plant extract (1mg/ml) was added to the wells on the first column of rows A - F. Starting from the first column the 100 μl of solution (50 μl + 50 μl media) was mixed and 50 μl transferred to wells on the second column by using multichannel micro-pipettor and a serial dilution was done up to the tenth column. Finally, excessive 50 μl from the tenth column were discarded. The final volume for each well was 50 μl. The cultured K562 cells were resuspended in RPMI 1640 at 1.39 x 10⁴ cells / ml after verifying cell viability by a trypan blue dye exclusion assay. Then, 50μl aliquot of the suspension was pipetted into each well, except the 12th column wells, in the micro-titer plate containing medium or extract (final volume 100 μl). Finally, 50 μl of medium was added to the 12th column wells as medium control, while column eleven wells served as cell control. Each sample was replicated 3 times and the cells incubated at 37°C in a humidified 5% CO₂ incubator for 24 h. After the incubation period, WST-1 reagent (10 μl of 3.26 mg/ml) was added into each well, the plates carefully shaken and the cells incubated for 3h. The absorbance values of each well at OD₄₅₀ – OD₆₃₀ were read using an ELISA Reader (Molecular Device, Menlo Park, USA). The IC₅₀ values were determined by plotting the drug concentration versus the survival ratio of the treated cells. Assays were performed at least three times, and data shown are representative of those assays.

\[
\text{% Cytotoxicity} = 1 - \left[ \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100 \right]
\]
A dose-response curve was plotted to enable the calculation of the concentration that killed 50% (CC\textsubscript{50}) of the K562 cells.

### 3.8.2.3 Cell Viability (Neutral Red Uptake Assay)

Cells were seeded in 24-well microplates (x10\textsuperscript{4} cells ml\textsuperscript{-1}), routinely cultured in a humidified incubator for 24 h. Cells were maintained in culture and exposed to \textit{Senna alata} or \textit{Senna podocarpa} aqueous methanolic leaf extract as well as nicosan – a herbal drug, over a range of concentrations (0.0 – 100.0 µg ml\textsuperscript{-1}). After 24 h exposure to the test substance, neutral red (NR) uptake test was performed according to the procedure described by Upur et al. (2008). Briefly, at the end of the treatment, the medium with or without test substance was discarded and 200 µl of freshly prepared neutral red solution (50 µg ml\textsuperscript{-1}) was added to every well and re-incubated for an additional 4 h at 37°C. Thereafter, the cells were carefully washed twice with 200 µl of PBS to eliminate extracellular neutral red. The incorporated dye was eluted from the cells by adding 200 µl elution medium (50% ethanol supplemented with 1% acetic acid, v/v) into each well followed by gentle shaking of microplate for 15 min. The plates were then read at 540 nm using a microplate reader (Molecular Device, Menlo Park, USA). The percentage of viable cells was calculated and the IC\textsubscript{50} was determined and expressed as microgram per milliliter.

### 3.8.2.4 Cell Death (Lactate Dehydrogenase Leakage Assay)

Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme, present in most eukaryotic cells, which is released into the culture medium upon cell death due to plasma membrane damage. K562 cells (5 x 10\textsuperscript{4} cells ml\textsuperscript{-1} well\textsuperscript{-1}) were pre-incubated in 24-well plates for 24 hours at 5% \textit{CO}\textsubscript{2}–95% air 37°C. Cell viability was assessed by lactate dehydrogenase (LDH) leakage through the membrane into the medium. Aliquots (150µl) of the cell culture supernatants
from control and serial concentration of extract treated cultures were tested after 6 or 7 days incubation for the presence of LDH using a LDH assay kit (ScienCell). In this test, three wells were used for each concentration of extract, and three independent experiments were performed.

3.9 DATA ANALYSIS

Statistical Analysis: Data was analyzed using one-way analysis of variance (ANOVA) followed by Tukey Post Hoc Test, using SPSS 16.0 computer software package (SPSS Inc; Chicago, U.S.A). Differences at P<0.05 were considered significant.
4.0 RESULTS

4.1 PRELIMINARY SCREENING

Five plants namely *Cajanus cajan* (beans and leaves), *Smilax kraussiana* (leaves), *Alchornea cordifolia* (leaves), *Senna alata* (leaves), *Senna podocarpa* (leaves) and Jana, a herbal formulation were screened for their membrane stabilizing activity, using the osmotic fragility test.

4.1.1 MEMBRANE STABILISING EFFECT OF AQUEOUS ETHANOLIC EXTRACT

Membrane stabilization was conferred on HbSS RBC by the hydroethanolic extract of five medicinal plants namely *Cajanus cajan* seed (38.34 – 40.18%)(Fig.2) and leaf extract of (Fig.3) *Smilax kraussiana* (18.97 – 45.52%), (Fig.4) *Alchornea cordifolia* ( -67.63 – 62.85%), (Fig.5) *Senna alata* (34.63 – 44.17%), (Fig.6) *Senna podocarpa* (34.25 – 67.12%), (Fig.7) *Cajanus cajan* (31.85 - 57.53%), as well as (Fig.8) Jana-herbal formula ( 64.34 - 77.26%). Optimal membrane stabilizing concentration of *Senna podocarpa* and JANA was 1000 µg ml\(^{-1}\), while 600 µg ml\(^{-1}\) was recorded for the other extracts. Two plants *Senna alata* and *Senna podocarpa* were thus selected for further investigation, since there is no documented report on these plants in relation to SCA management.
Fig. 2 Osmotic fragilogram of HbSS erythrocytes incubated for 30mins with ethanolic leaf extract of Cajanus cajan.
Fig. 3 Osmotic fragilogram of HbSS erythrocytes incubated for 30 mins with ethanolic seed extract of Cajanus cajan.
Fig. 4 Osmotic fragilogram of HbSS erythrocytes incubated for 30 mins with ethanolic leaf extract of *Smilax kraussiana*. 
Fig. 5 Osmotic fragilogram of HbSS erythrocytes incubated for 30 mins with ethanolic leaf extract of *Alchornea cordifolia*
Fig. 6 Osmotic fragilogram of HbSS erythrocytes incubated for 30 mins with ethanolic leaf extract of Senna alata
Fig. 7 Osmotic fragilogram of HbSS erythrocytes incubated for 30 mins with ethanolic leaf extract of *Senna podocarpa*
Fig. 8 Osmotic fragilogram of HbSS erythrocytes incubated for 30 mins with "jana" an herbal formula.
4.1.2 EXTRACTION YIELD AND PHYTOCHEMICAL COMPOSITION

For *S. alata*, aqueous methanolic extract gave the highest yield, followed by water extract and lastly aqueous ethanolic extract (Table 1). On the other hand, *S. podocarpa* water extract gave the highest yield, followed by aqueous methanol and then aqueous ethanol.

*Senna alata* contained keto-sugar, while alkaloid was detected in *S. podocarpa* and both *S. alata* as well as *S. podocarpa* contained deoxy-sugar (Table 2a). Presence of tanins, saponins, flavonoids, steroids, anthraquinones and cardiac glycosides were confirmed (Table 2b) in *S. alata* and *S. podocarpa*, while phlobatanin was found only in *S. podocarpa*.
Table 1. Percentage yield of plant material(s) after extraction with different solvents

<table>
<thead>
<tr>
<th>Plant Material</th>
<th>Ethanol (80%)</th>
<th>Methanol (80%)</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alchornea cordifolia (Leaves)</td>
<td>26.51</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cajanus cajan (seeds)</td>
<td>20.32</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cajanus cajan (leaves)</td>
<td>22.01</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Senna alata (leaves)</td>
<td>23.17</td>
<td>27.67</td>
<td>24.03</td>
</tr>
<tr>
<td>Senna podocarpa (leaves)</td>
<td>5.50</td>
<td>9.81</td>
<td>13.95</td>
</tr>
<tr>
<td>Smilax kraussiana (leaves)</td>
<td>11.90</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
### Table 2a Phytochemical screening of ethanolic (80%) extracts

<table>
<thead>
<tr>
<th>TEST</th>
<th>AC</th>
<th>CCB</th>
<th>CCL</th>
<th>SA</th>
<th>SK</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fehlings soln</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Fehlings +H2SO4 + Heat</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Dragendoff</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Meyers</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Barfoed’s (Monosacchs)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Resorcinol (Keto-sugar)</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Phlorogucinol (Pentose)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Keller-Killanni (Deoxy-sugar)</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Key:** - Negative; + positive; ++ more positive; +++ most positive

AC – *Alchornea cordifolia* leaves; CCB – *Cajanus cajan* beans; CCL – *Cajanus cajan* leaves; SA – *Senna alata* leaves; SK – *Smilax kraussiana* leaves; SP – *Senna podocarpa* leaves

### Table 2b Phytochemical screening of methanolic (80%) extracts

<table>
<thead>
<tr>
<th>TEST</th>
<th><em>S. alata</em></th>
<th><em>S. podocarpa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>TANIN</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SAPONIN</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLAVONOID</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PHLOBATANIN</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>STEREOIDS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TERPENOID</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ANTHRAQUINONE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CARDIAC GLYCOSIDE</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Key:** - Negative; + positive
4.1.3 PROXIMATE COMPOSITION

The chemical compositions (Table 3) of the two plants showed no significant difference in their ash or moisture content. *S. alata* had significantly higher lipid, protein and crude fibre contents while the carbohydrate and energy contents of *S. podocarpa* were significantly higher.
Table 3 Proximate composition of aqueous-methanolic leaf extract of *Senna alata* and *Senna podocarpa*

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>S. alata</em></th>
<th><em>S. podocarpa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>%Moisture</td>
<td>5.67±0.14</td>
<td>6.20±0.15</td>
</tr>
<tr>
<td>%Lipids</td>
<td>11.2±0.18</td>
<td>3.35±0.10</td>
</tr>
<tr>
<td>%Protein</td>
<td>5.28±0.09</td>
<td>1.97±0.06</td>
</tr>
<tr>
<td>%Crude fibre</td>
<td>33.33±0.18</td>
<td>20.72±0.59</td>
</tr>
<tr>
<td>%Ash</td>
<td>6.61±0.20</td>
<td>6.78±0.16</td>
</tr>
<tr>
<td>%Carbohydrate</td>
<td>43.58±0.81</td>
<td>67.18±0.13</td>
</tr>
<tr>
<td>Energy (Kcal)</td>
<td>295.94±0.62</td>
<td>306.19±0.97</td>
</tr>
</tbody>
</table>

Values (g/100gwt) expressed as percentage mean ± SEM; n=3; superscript (a) shows significant difference (P<0.05) between means on the same row.
4.2 IN VITRO STUDIES

4.2.1 MEMBRANE STABILISING EFFECT OF AQUEOUS METHANOLIC EXTRACT

The mean corpuscular fragility values of *S.alata* and *S.podocarpa* hydromethanolic leaf extract treated HbSS RBC were significantly (P < 0.05) lower than the untreated HbSS RBC. The hydromethanolic leaf extract of *S.alata* (Fig.9) exhibited a membrane stabilizing effect (44.44 – 66.01%) with a 200 µg ml\(^{-1}\) optimal concentration, whereas hydromethanolic leaf extract of *S.podocarpa* (Fig.10) showed a much lower level of membrane stabilization (5.72 – 17.71%) with 800 µg ml\(^{-1}\) supplementation conferring optimal stabilizing effect.
Fig. 9 Osmotic fragilogram of HbSS erythrocytes incubated for 30 mins with methanolic leaf extract of *Senna alata* Values represent mean of triplicates ± SEM.
Fig. 10 Osmotic fragilogram of HbSS erythrocytes incubated for 30 mins with methanolic leaf extract of *Senna podocarpa*. Values represent mean of triplicates ± SEM.
4.2.2 EFFECT OF EXTRACT ON HEMOGLOBIN POLYMERIZATION

The test substances, *S.podocarpa*, *S.alata*, parahydroxybenzoic acid and Nicosan, had significantly higher antisickling activity when compared to the untreated control at all sampling times (Fig.11). After 120 minutes of incubation, Nicosan exhibited a significantly (P < 0.05) higher antisickling activity than *S.podocarpa* as well as parahydroxybenzoic acid, while after 150 minutes incubation time the antisickling activity of *S.podocarpa* was significantly lower than that of *S.alata*, parahydroxybenzoic acid and Nicosan in that order (Fig.12).

Pre incubation of HBSS RBCs with *S.alata* for up to 90 minutes did not significantly alter the antisickling profile after 175 minutes. Optimal pre incubation time of 30 minutes prior to deoxygenation conferred an antisickling effect that lasted for 210 minutes (Fig.13). Pre incubation of HBSS RBCs for 150 minutes with *S.podocarpa* prior to deoxygenation, conferred an antisickling effect that lasted for 210 minutes, while no significant (P > 0.05) changes in antisickling profile was observed for 30, 60, 120 and 180 minutes pre incubation time (Fig.14).
Fig. 11  Antisickling efficiency of *Senna alata*, *Senna podocarpa*, nicosan, parahydroxybenzoic acid and buffered saline. Suspension (10% hematocrit) of HbSS erythrocytes were incubated with test solution (2.5 mg ml$^{-1}$) for three hours at 37°C, aliquots were then withdrawn, deoxygenated with 2% sodium metabisulphite and the percentage of sickle cells determined. (PABA- Parahydroxybenzoic acid)
Fig. 12 Antisickling potencies of *Senna alata*, *Senna podocarpa*, nicosan, parahydroxybenzoic acid and buffered saline. Suspension (10% hematocrit) of HbSS erythrocytes were incubated with test solution (2.5 mg ml\(^{-1}\)) at 37°C, then aliquots were withdrawn at various times, deoxygenated with 2% sodium metabisulphite and the percentage of sickle cells determined. (PABA- Parahydroxybenzoic acid)
Fig. 13  Antisickling potency of *Senna alata*. Suspension (10% hematocrit) of HbSS erythrocytes was incubated with *S. alata* (2.5 mg ml\(^{-1}\)) at 37°C, then aliquots were withdrawn every thirty minutes, deoxygenated with 2% sodium metabisulphite at 37°C, and the percentage of sickle cells determined at thirty-five (35) minutes interval.
Fig. 14 Antisickling potency of *Senna podocarpa*. Suspension (10% hematocrit) of HbSS erythrocytes was incubated with *S. podocarpa* (2.5 mg ml$^{-1}$) at 37°C, then aliquots were withdrawn every thirty minutes, deoxygenated with 2% sodium metabisulphite at 37°C, and the percentage of sickle cells determined at thirty-five (35) minutes interval.
4.2.3  EFFECT OF EXTRACT ON HEMOGLOBIN DEPOLYMERIZATION

A contact time dependent significant (P < 0.01) increase in reversal activity by *Senna podocarpa* (2.5 mg ml\(^{-1}\)) was observed (Fig. 15) after 60 mins, though the level of reversal activity was extremely significantly (P < 0.001) lower when compared with the positive control para-hydroxybenzoic acid (PABA) (2.5 mg ml\(^{-1}\)) and *Senna alata* (2.5 mg ml\(^{-1}\)). *S. alata* showed a highly significantly (P < 0.001) increased contact time dependent reversal activity when compared to with normal saline, *S. podocarpa* and PABA.
Fig. 15 Effect of *Senna alata* (SA), *Senna podocarpa* (SP), parahydroxybenzoic acid (PABA) and buffered saline (NS) on presickled RBC. Suspension (35% hematocrit) of HbSS erythrocytes was layered with liquid paraffin and incubated with 2% sodium metabisulphite for 2½ hours at 37°C. After incubation, the test solution was carefully introduced into the solution under oil, aliquots were then withdrawn at 30 minute intervals, and the percentage of sickle cells determined. Data expressed as mean ± sem; ANOVA and Tukey Post Hoc test; *** significantly different from control (buffered saline) \( P \leq 0.001 \)
4.2.4 ANTIOXIDANT POTENTIAL OF EXTRACTS

Table 4 shows that the total phenolic content of the extracts expressed as mg gallic acid equivalent per gram dry weight of extract was highest in the aqueous-methanolic extract of *S. podocarpa* (154±0.34) followed by aqueous-methanolic extract of *S. alata* (102.21±0.11), then aqueous extract of *S. podocarpa* (79.28±0.06) and lastly aqueous extract of *S. alata* (68.81±0.03). There were significant differences (p<0.05) amongst the four extracts in total phenolic compounds content. Both *S. podocarpa* and *S. alata* aqueous-methanolic extract had higher total phenolic content than their corresponding aqueous extracts.

The aqueous extracts (Fig.16) showed poor reducing power, but gallic acid, ascorbic acid, aqueous-methanolic extracts of *S. podocarpa* and *S. alata*, exhibited EC50 (effective concentration) values of 28.13, 38.02, 49.47 and 61.45 µg ml⁻¹ respectively.

The DPPH radical scavenging activity of the extracts showed a dose-dependent response. The aqueous-methanolic extract (Fig.17) of *S. podocarpa* and *S. alata* had similar IC50 (inhibitory concentration) values of 64.56 µg ml⁻¹ and 65.16 µg ml⁻¹ respectively which were about five times less potent than ascorbic acid (13.59 µg ml⁻¹).
Table 4. Total amount of phenolic compounds, free amino acids and total antioxidant capacity of aqueous and methanolic (80%) plant extract

<table>
<thead>
<tr>
<th>Plant</th>
<th>Total phenolic (mgGAE/g plant extract)</th>
<th>Free amino acids (mg/100g)</th>
<th>Total antioxidant capacity (mgAAE/g plant extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methanol (80%)</td>
<td>Aqueous</td>
<td>Methanol (80%)</td>
</tr>
<tr>
<td>S.alata</td>
<td>102.21±0.11</td>
<td>68.81±0.03</td>
<td>192±0.12</td>
</tr>
<tr>
<td>S.podocarpa</td>
<td>154.85±0.34a</td>
<td>79.28±0.06a</td>
<td>418.31±0.99a</td>
</tr>
</tbody>
</table>

a- significant difference (p<0.05) exist within column
Fig. 16 Reducing power of the aqueous and aqueous-methanolic plants leaves extract with gallic acid and ascorbic acid as control. *S. alata*(Sa) / *S. podocarpa* (Sp) aqueous-methanolic (m) / aqueous (Aq) extract (values are mean±SEM of three replicates)
Fig. 17 DPPH radical scavenging activity of aqueous extract (Aq) / aqueous-methanolic extract (m) of *S. alata* (Sa) and *S. podocarpa* (Sp) leaves
4.2.5 DNA PROTECTION ASSAY

Aqueous-methanolic leaf extract of *S.podocarpa* exhibited a dose dependent DNA protection activity, while aqueous-methanolic leaf extract of *S.alata* at 10 µg ml\(^{-1}\) and 1000 µg ml\(^{-1}\) exhibited pro-oxidant activity, but 100 µg ml\(^{-1}\) showed DNA protection activity (Fig. 18).
Fig. 18 Effect of aqueous-methanolic leaf extract of *S. alata* / *S. podocarpa* on hydroxyl radical induced DNA damage.

Lanes 1-pBR322; 2-plasmid + FeSO₄; 3-plasmid + H₂O₂; 4-plasmid + FeSO₄ + H₂O₂; 5-7 plasmid + FeSO₄ + H₂O₂ + *S. alata* (1000 µg ml⁻¹, 100 µg ml⁻¹, 10 µg ml⁻¹ respectively); 8-10 plasmid + FeSO₄ + H₂O₂ + *S. podocarpa* (1000 µg ml⁻¹, 100 µg ml⁻¹, 10 µg ml⁻¹ respectively)
4.3 IN VIVO STUDIES

4.3.1 Sub acute toxicological effects of *S.alata* and *S.podocarpa* on biochemical parameters of albino rats

Following the sub acute administration of *S.alata* for 15 days (Fig.19), the AST activity was significantly (*p* < 0.05) reduced in the plasma at doses of 200- and 600 mg ml\(^{-1}\), but at 1000 mg ml\(^{-1}\) dose, it showed a very significant (*p* < 0.01) reduction in AST activity. A concomitant, though non significant (*p* > 0.05) reduction in ALT activity was also revealed. A significant (*p* < 0.05) and very significant (*p* < 0.01) reduction in plasma urea level by 200- and 600 mg ml\(^{-1}\) of *S.alata* respectively was observed with no significant (*p* > 0.05) changes in the albumin level (Fig.20). *S.podocarpa* (Fig.21) on the other hand, did not significantly (*p* > 0.05) alter the plasma AST and ALT activity at all doses tested, but reduced the urea level very significantly (*p* < 0.01) at 1000 mg ml\(^{-1}\) dose over the control while the albumin level was not significantly (*p* > 0.05) altered (Fig.22).
Fig. 19 Effect of hydromethanolic extract of *Senna alata* leaves on activity of selected enzymes in the plasma of albino rat administered 1ml daily gavage dose of water (CONTROL), 200mg/kg.bwt (SAL), 600mg/kg.bwt (SAM) or 1000mg/kg.bwt (SAH) for 15 days. Data are mean ± SEM; n=5; ANOVA (P < 0.05); significantly different from control at *(P < 0.05) **(P < 0.01)
Fig. 20 Effect of hydromethanolic extract of *Senna alata* leaves on the level of selected analytes in the plasma of albino rat administered 1ml daily gavage dose of water (CONTROL), 200mg/kg.bwt(SAL), 600mg/kg.bwt(SAM) or 1000mg/kg.bwt(SAH) for 15 days. Data are mean ± SEM; n=5; ANOVA (P < 0.05); significantly different from control at *(P < 0.05)* or ***(P < 0.01)***
Fig. 21 Effect of hydromethanolic extract of *Senna podocarpaa* leaves on activity of selected enzymes in the plasma of albino rat administered 1ml daily gavage dose of water (CONTROL), 200mg/kg.bwt(SAL), 600mg/kg.bwt(SAM) or 1000mg/kg.bwt(SAH) for 15 days. Data are mean ± SEM; n=5; ANOVA (P< 0.05).
Fig. 22 Effect of hydromethanolic extract of *Senna podocarpa* leaves on plasma analyte level of albino rat administered 1ml daily gavage dose of water (CONTROL), 200mg/kg.bwt(SAL), 600mg/kg.bwt(SAM) or 1000mg/kg.bwt(SAH) for 15 days. Data are mean ± SEM; n=5; ANOVA (P < 0.05); Significantly different from control at **(P < 0.01).**
4.3.2 Sub chronic toxicological effect *S. alata* and *S. podocarpa* on body and organ weights of albino rats

*S. alata* at 200 mg kg.bwt\(^{-1}\) significantly \((p < 0.05)\) increased weight of the animals (Fig.23). The weight of liver relative to the body weight (Fig.24) was significantly increased by 600 mg kg.bwt\(^{-1}\) of *S. alata*, while no significant changes in the relative weights of other organs was noticed when compared to the control. The high dose (1000 mg kg.bwt\(^{-1}\)) of *S. alata* induced a very significant \((p < 0.01)\) increase in the liver weight relative to brain weight (Fig.25) when compared to the control.

*Senna podocarpa* (Fig.26) at 200 mg kg.bwt\(^{-1}\) dose significantly \((p < 0.05)\) increased liver weight and concomitantly decreased the kidney weight, with a highly significant \((p < 0.01)\) increase in the weight of the lungs and testes when compared with the control. The 600 mg kg.bwt\(^{-1}\) dose however, induced a high significant \((p < 0.01)\) increase and decrease in lungs and testicular weights respectively. The liver and lungs weight was significantly \((p < 0.05)\) increased and decreased respectively by 1000 mg kg.bwt\(^{-1}\) dose of *S. podocarpa* when compared with the control. The liver weight expressed as fraction of the brain weight (Fig.27) was very highly significantly \((p < 0.01)\) increased over the control in the 1000 mg kg.bwt\(^{-1}\) dose group.
Fig. 23 Effect of *Senna alata* (SA)/*Senna podocarpa* (SP) hydro-methanolic leaf extract on live weight of Albino rats administered 1ml daily gavage dose of 200mg/kg.bwt (L), 600mg/kg.bwt (M) or 1000mg/kg.bwt (H) for 35 days.
Fig. 24  Effect of *Senna alata* (SA-) hydro-methanolic leaf extract on live weight of Albino rats administered 1 ml daily gavage dose of 200 mg/kg.bwt (L), 600 mg/kg.bwt (M) or 1000 mg/kg.bwt (H) for 35 days
Fig. 25 Effect of *Senna alata* (SA-) hydro-methanolic leaf extract on live weight of Albino rats administered 1ml daily gavage dose of 200mg/kg.bwt (L), 600mg/kg.bwt (M) or 1000mg/kg.bwt (H) for 35 days
Fig. 26 Effect of Senna podocarpa (SP-) hydro-methanolic leaf extract on live weight of Albino rats administered 1ml daily gavage dose of 200mg/kg.bwt (L), 600mg/kg.bwt (M) or 1000mg/kg.bwt (H) for 35 days.
Fig. 27 Effect of *Senna podocarpa* (SP) hydro-methanolic leaf extract on live weight of Albino rats administered 1ml daily gavage dose of 200mg/kg.bwt (L), 600mg/kg.bwt (M) or 1000mg/kg.bwt (H) for 35 days.
At nine weeks, daily dosage of *S.alata* or *S.podocarpa* (Fig.28) increased with very high significance (p < 0.001) the weight of the rats at all doses tested, except the 1000 mg kg.bwt\(^{-1}\) dose of *S.alata* when compared to the control. The liver weight compared to the control was significantly (p < 0.05) reduced by 600- and 1000 mg kg.bwt\(^{-1}\) dose of *S. alata*, (Fig.29) while the liver weight expressed as a fraction of the brain weight (Fig.30) was however increased very significantly (p < 0.01) and very highly significantly (p < 0.001) at 200- and 1000 mg kg.bwt\(^{-1}\) dose respectively. *S. podocarpa* (Fig.31) did not significantly (p < 0.05) induce weight change in the organs at all the tested doses when compared to the control. The liver weight as a fraction of brain weight (Fig.32) was very significantly (p < 0.01) elevated over the control by 200- and 600 mg kg.bwt\(^{-1}\) dose of *S.podocarpa*, while no significant weight change was noticed in other organs.
Fig. 28 Effect of 63 day daily administration 1ml gavage dose of water (Control), 200mg/kg.bwt (L), 600mg/kg.bwt (M) or 1000mg/kg.bwt (H) of *S. alata* (SA-) or *S. podocarpa* (SP-) hydromethanolic leaf extract on weight change. Values represent mean ± SEM; n=5; ANOVA. ***Significantly different from the control (p<0.001)
Fig. 29 Effect of 63 day daily administration 1ml gavage dose of water (Control), 200mg/kg, bwt (-L), 600mg/kg, bwt (M) or 1000mg/kg, bwt (H) of S. alata (SA-) hydromethanolic leaf extract on organ weight. Values represent mean ± sem; n=5; ANOVA; Significantly different from the control at *(p < 0.05) and **(p < 0.01)
Fig. 30 Effect of 63 day daily administration 1ml gavage dose of water (Control), 200mg/kg.bwt(L), 600mg/kg.bwt(M) or 1000mg/kg.bwt(H) of S.alata(SA-) hydromethanolic leaf extract on organ weight. Values represent mean± sem; n=5; ANOVA; Significantly different from the control at **(p < 0.01) and ***(p < 0.001)
Fig. 31  Effect of 63 day daily administration 1ml gavage dose of water (Control), 200mg/kg.bwt (L), 600mg/kg.bwt (M) or 1000mg/kg.bwt (H) of S. podocarpa (SP-) hydromethanolic leaf extract on organ weight. Values represent mean ± sem; n=5; ANOVA (p<0.05)
Fig. 32  Effect of 63 day daily administration 1ml gavage dose of water (Control), 200mg/kg.bwt (L), 600mg/kg.bwt (M) or 1000mg/kg.bwt (H) of *S. podocarpa* (SP-) hydromethanolic leaf extract on organ weight. Values represent mean± sem; n=5; ANOVA (p< 0.05)
4.3.3 Sub chronic toxicological effect \textit{S.alata} and \textit{S.podocarpa} on hematologic parameters of albino rats

There were no significant (p > 0.05) changes in WBC (total and differential) and platelets populations (Table 5) between the control and the experimental (\textit{Senna alata} or \textit{Senna podocarpa}) groups, and between the experimental groups. The PCV was however significantly (p < 0.05) increased in the experimental groups when compared with the control.
Table 5. Effect of nine weeks administration of hydromethanolic leaf extract of *Senna alata* and *Senna podocarpa* on hematologic parameters of albino rats.

<table>
<thead>
<tr>
<th>HEMATOLOGIC PARAMETER</th>
<th>CONTROL (Water)</th>
<th>200 mg kg.bwt-1</th>
<th>600 mg kg.bwt-1</th>
<th>1000 mg kg.bwt-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S.alata</em></td>
<td><em>S.podocarpa</em></td>
<td><em>S.alata</em></td>
<td><em>S.podocarpa</em></td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>31.6±0.68</td>
<td>41.00±1.03*</td>
<td>39.40±2.04*</td>
<td>42.17±0.95*</td>
</tr>
<tr>
<td></td>
<td>41.00±1.03*</td>
<td>39.40±2.04*</td>
<td>42.17±0.95*</td>
<td>41.00±1.03*</td>
</tr>
<tr>
<td>WBC (x10³) (x10³/mm³)</td>
<td>6.04±3.31</td>
<td>7.33±6.23</td>
<td>6.64±7.54</td>
<td>6.32±5.18</td>
</tr>
<tr>
<td></td>
<td>6.64±7.54</td>
<td>6.32±5.18</td>
<td>6.24±5.87</td>
<td>4.44±1.20</td>
</tr>
<tr>
<td></td>
<td>4.44±1.20</td>
<td>5.70±3.42</td>
<td>4.44±1.20</td>
<td>5.70±3.42</td>
</tr>
<tr>
<td>Platelets (x10³/mm³)</td>
<td>40.20±3.10</td>
<td>44.67±1.41</td>
<td>41.40±1.66</td>
<td>42.17±0.91</td>
</tr>
<tr>
<td></td>
<td>41.40±1.66</td>
<td>42.17±0.91</td>
<td>34.80±3.37</td>
<td>44.00±1.38</td>
</tr>
<tr>
<td></td>
<td>44.00±1.38</td>
<td>38.20±3.41</td>
<td>44.00±1.38</td>
<td>38.20±3.41</td>
</tr>
<tr>
<td>Leucocytes (x10³/mm³)</td>
<td>58.80±3.81</td>
<td>51.00±1.91</td>
<td>56.80±2.13</td>
<td>54.33±0.99</td>
</tr>
<tr>
<td></td>
<td>56.80±2.13</td>
<td>54.33±0.99</td>
<td>64.80±3.23</td>
<td>51.00±1.18</td>
</tr>
<tr>
<td></td>
<td>51.00±1.18</td>
<td>61.40±3.70</td>
<td>51.00±1.18</td>
<td>61.40±3.70</td>
</tr>
<tr>
<td>Monophils (x10³/mm³)</td>
<td>0.40±0.4</td>
<td>2.00±0.00*</td>
<td>0.80±0.37</td>
<td>2.00±0.00*</td>
</tr>
<tr>
<td></td>
<td>0.80±0.37</td>
<td>2.00±0.00*</td>
<td>0.00±0.00</td>
<td>1.80±0.37*</td>
</tr>
<tr>
<td></td>
<td>1.80±0.37*</td>
<td>0.40±0.40</td>
<td>1.80±0.37*</td>
<td>0.40±0.40</td>
</tr>
<tr>
<td>Eosophils (x10³/mm³)</td>
<td>0.60±0.6</td>
<td>2.67±0.61</td>
<td>1.00±0.45</td>
<td>1.50±0.22</td>
</tr>
<tr>
<td></td>
<td>1.00±0.45</td>
<td>1.50±0.22</td>
<td>0.00±0.00</td>
<td>3.20±0.97*</td>
</tr>
<tr>
<td></td>
<td>3.20±0.97*</td>
<td>0.00±0.00</td>
<td>3.20±0.97*</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

Values are expressed mean ± SEM (n=5); P<0.05 level of significant using ANOVA followed by tukey multiple comparism test. Asterisk (*) shows that the mean is significantly different (P<0.05) when compared with the control on the same column.
4.3.4 Biochemical effects of *S.alata* and *S.podocarpa* on ALT, AST and ALP activity in albino rats

Table 6 Shows that nine weeks orally administered daily gavage dose of aqueous methanolic leaf extract of *S.alata* or *S.podocarpa* had no significant (P > 0.05) effect on the levels of ALT and AST activities in the liver at all doses tested compared to the control group, but 200- and 600 mg kg.bwt\(^{-1}\) dose of *S.alata* caused a significant (P < 0.05) and very highly significant (P < 0.001) reduction in total protein level respectively. Neither extract significantly (P > 0.05) altered the activity of plasma ALT, but 1000 mg kg.bwt\(^{-1}\) dose of *S.alata* decreased significantly (P < 0.05) the AST activity while ALP activity was significantly (p < 0.05) increased when rats were treated with 600 mg kg.bwt\(^{-1}\) dose of *S.podocarpa* versus the control. Also albumin level was increased, very significantly (P < 0.05) when 1000 mg kg.bwt\(^{-1}\) dose of *S.podocarpa* was given, while all other treatments were not significantly (P > 0.05) different from the control.
Table 6. Effect of 63 days daily administration of hydromethanolic leaf extract of *Senna alata* and *Senna podocarpa* on activity of some plasma enzymes of albino rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dosage (mg kg⁻¹)</th>
<th>ALT (U L⁻¹)</th>
<th>AST (U L⁻¹)</th>
<th>ALP (U L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>24.18±1.05</td>
<td>19.16±0.79</td>
<td>63.29±10.42</td>
</tr>
<tr>
<td><em>Senna alata</em></td>
<td>200</td>
<td>27.73±1.67</td>
<td>13.61±2.64</td>
<td>73.29±10.00</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>32.24±7.20</td>
<td>16.41±1.56</td>
<td>78.20±16.78</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>22.86±0.80</td>
<td>10.05±1.79*</td>
<td>76.18±10.79</td>
</tr>
<tr>
<td><em>Senna podocarpa</em></td>
<td>200</td>
<td>21.88±1.23</td>
<td>15.39±1.20</td>
<td>76.36±12.87</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>40.16±4.23</td>
<td>17.38±0.10</td>
<td>172.00±26.40*</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>25.34±4.73</td>
<td>17.38±0.97</td>
<td>90.90±19.33</td>
</tr>
</tbody>
</table>

Values are expressed mean ± SEM (n=5); P<0.05 level of significant using ANOVA followed by tukey multiple comparism test. Asterisk (*) shows that the mean is significantly different (P<0.05) when compared to the control on the same column.
4.3.5 Antioxidant activity of *S.alata* and *S.podocarpa* in albino rats

*S.alata* and *S.podocarpa* (Table. 7) did not significantly alter plasma catalase activity at all doses tested when compared to the control, but 200 mg kg.bwt$^{-1}$ dose of *S.alata* reduced significantly (P<0.05) the liver catalase activity. The level of liver lipid peroxidation was reduced in a very highly significant (P<0.001) manner over the control, by all the tested doses of *S.alata*, while *S.podocarpa* on the other hand had no significant (P < 0.05) effect on the liver lipid peroxidation level. The level of lipid peroxidation in the plasma was significantly (P<0.05) reduced over the control by 200 and 1000mg/kg.bwt of dose of S.alata, but *S.podocarpa* at 200 and 600mg/kg.bwt caused a very high significant (P<0.001) reduction in the level of lipid peroxidation in the plasma.
Table 7. Antioxidant effect of 63 days daily administration of hydromethanolic leaf extract of *Senna alata* and *Senna podocarpa* on albino rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dosage (mg kg$^{-1}$)</th>
<th>CATALASE</th>
<th>TBARS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LIVER</td>
<td>PLASMA</td>
</tr>
<tr>
<td>CONTROL</td>
<td></td>
<td>0.34±0.04</td>
<td>0.084±0.05</td>
</tr>
<tr>
<td><em>Senna alata</em></td>
<td>200</td>
<td>0.07±0.10*</td>
<td>0.297±0.10</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>0.08±0.09</td>
<td>0.037±0.40</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>0.05±0.08</td>
<td>0.042±0.25</td>
</tr>
<tr>
<td><em>Senna podocarpa</em></td>
<td>200</td>
<td>0.33±0.05</td>
<td>0.024±0.02</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>0.40±0.04</td>
<td>0.012±0.00</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>0.17±0.04</td>
<td>0.098±0.04</td>
</tr>
</tbody>
</table>

Values are expressed mean ± SEM (n=5); P<0.05 level of significant using ANOVA followed by tukey multiple comparison test. Asterisk (*) shows that the mean is significantly different (P<0.05) when compared to the control on the same column.
4.3.6 Effect of plant extract on albino rat erythrocyte membrane protein profile

The protein profile (Fig. 33) of albino rat RBC of the control and *S. alata* experimental groups did not show distinct differences in their banding pattern. No dose related difference in the albino rat RBC protein profile (Fig. 34) existed between the control and *S. podocarpa* experimental groups.
Fig. 33 Erythrocyte membrane protein electrophoreogram of albino rats administered 1ml daily gavage doses of water or *Senna alata* aqueous-methanolic leave extract for nine weeks. Lane 1: Protein ladder (kDa); 2: Control; 3 & 4: 200 mg kgbw t\(^{-1}\); 5 & 6: 600 mg kgbw t\(^{-1}\); 7 & 8: 1000 mg kgbw t\(^{-1}\).

kDa - Kilodalton
Fig. 34  Erythrocyte membrane protein electrophoregram of albino rats administered 1ml daily gavage doses of water or *Senna podocarpaa* aqueous-methanolic leave extract for nine weeks. Lane 1: Protein ladder (kDa); 2: Control; 3 & 4: 200 mg kg\text{bwt}^{-1}; 5 & 6: 600 mg kg\text{bwt}^{-1}; 7 & 8: 1000 mg kg\text{bwt}^{-1}
kDa – Kilodalton.
4.3.7 Histopathology

Control liver (Plate 1) exhibited dilated central veins, moderate lymphocytes in portal tracts, while the *S. podocarpa* group showed (Plate 2) congestion of sinus and hepatic veins with moderate lymphocytic inflammation. Moderate lymphocytes in the portal tracts with destruction of limiting plate were noticed (Plate 3) in *S.alata* group.
Plate 1. Control liver with normal parenchyma and dilated central vein (arrowed) harbouring moderate lymphocytes. Section stained with haematoxylin - eosin (HE) solution for light microscopy
Plate 2. *Senna podocarpa* liver Sinusoidal congestion of parenchyma with dilated central vein (arrowed) and lymphocytic inflammation. Section stained with haematoxylin - eosin (HE) solution for light microscopy.
Plate 3. *senna alata* liver moderately congested parenchyma with dilated central vein (arrowed) harbouring lymphocytes. Section stained with haematoxylin - eosin (HE) solution for light microscopy.
4.3.8 Genotoxic potential of plant extract on albino rats

There was no significant (p > 0.05) increase in the average weight of the testis and cauda epididymis in the hydromethanolic extract fed albino rat groups (Table 8). In the 1000 mg kg\(^{-1}\) \textit{S.podocarpa} extract treated group, increase in testes weight was significant when expressed as relative weight (organ weight/brain weight).

Figure 35 shows the frequency of occurrence of the different types of spermheads observed in albino rats exposed to extracts of \textit{S.alata} and \textit{S.podocarpa}. The mean percentage of normal sperm ranged from 51\% to 97\% in all treatment groups, and not different significantly (P < 0.05) among \textit{S. alata} treated group or between \textit{S. alata} treated groups compared with control. However, treatment with \textit{S. podocarpa} significantly (P < 0.001) reduced the occurrence (50.8\% to 53.33\%) of normal sperms in rats, while various morphological (Plate 4) sperm abnormalities were recorded in the control and the \textit{S. alata} / \textit{S. podocarpa} treatment groups. Significant increase (P < 0.001) in frequency of spermhead abnormalities including pinhead, wide acrosome and amorphous head were observed in the \textit{S. podocarpa} treated group, while the short hook rat spermhead morphology has the highest occurrence (5.21\%) in the 1000 mg kg\(^{-1}\) \textit{S. podocarpa} treated group.
Table 8 Effects of methanolic extract of *Senna alata* and *Senna podocarpa* on testis and cauda epididymides of albino rats after 9 weeks exposure

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>TESTIS</th>
<th></th>
<th>CAUDA EPIDIDYMIS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute Weight</td>
<td>% Body weight</td>
<td>% Brain weight</td>
<td>Absolute Weight</td>
</tr>
<tr>
<td>CONTROL</td>
<td>0.99±0.07</td>
<td>0.73±0.04</td>
<td>66.78±4.37</td>
<td>0.16±0.02</td>
</tr>
<tr>
<td>200 mg kg.bwt⁻¹</td>
<td>1.10±0.04</td>
<td>0.73±0.04</td>
<td>75.91±3.10</td>
<td>0.21±0.02</td>
</tr>
<tr>
<td><em>Senna alata</em></td>
<td>600 mg kg.bwt⁻¹</td>
<td>1.02±0.02</td>
<td>0.65±0.04</td>
<td>68.89±3.40</td>
</tr>
<tr>
<td></td>
<td>1000 mg kg.bwt⁻¹</td>
<td>1.15±0.03</td>
<td>0.67±0.04</td>
<td>77.77±2.26</td>
</tr>
<tr>
<td>200 mg kg.bwt⁻¹</td>
<td>1.07±0.02</td>
<td>0.72±0.02</td>
<td>77.47±5.05</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td><em>Senna podocarpa</em></td>
<td>600 mg kg.bwt⁻¹</td>
<td>1.02±0.05</td>
<td>0.60±0.03</td>
<td>73.99±2.80</td>
</tr>
<tr>
<td></td>
<td>1000 mg kg.bwt⁻¹</td>
<td>1.29±0.07</td>
<td>0.67±0.01</td>
<td>79.76±2.24</td>
</tr>
</tbody>
</table>

*Significantly different from the control (P<0.05)*
Fig. 35 Frequency of occurrence of the different types of spermheads induced by 63 days exposure of albino rats to *Senna alata* -200/kg.bwt (SAL), -600mg/kg.bwt (SAM), -1000mg/kg.bwt (SAH), *Senna podocarpa* – 200mg/kg.bwt (SPL), 600mg/kg.bwt (SPM) or – 1000mg/kg.bwt (SPH). Values represent mean ± SEM; ANOVA (P < 0.05)
Plate 4 Observed shapes of normal and abnormal sperm heads: (a) short hook; (b) long hook; (c) tailless head; (d) long hook; (e) amorphous head; (f) normal; (g) pin shaped; (h) wide acrosome.
4.4 Cytotoxic potential of plant extract on K562 cell line

Figure 36 shows the viability of K562 cells based on WST-1 assay following exposure to varying concentrations of different extracts. At concentrations below 75 µg ml$^{-1}$ all the extracts sustained cell viability. *Senna podocarpa*, Nicosan and *Senna alata* had cell cytotoxicity (CC$_{50}$) values of 104.5 µg ml$^{-1}$, 115.3 µg ml$^{-1}$ and 131.8 µg ml$^{-1}$ respectively. The neutral red uptake assay revealed that the cytotoxic effect of *Senna podocarpa* on K562 cells (Fig. 37) was not significantly different (p > 0.05) from that of Nicosan at all concentrations tested, while the cytotoxicity of *Senna alata* was significantly (P < 0.05) lower than that of *Senna podocarpa* and Nicosan at concentrations greater than 64 µg ml$^{-1}$. While the lactate dehydrogenase leakage assay showed (Fig. 38) that, of all the tested concentrations of the different extracts, only 8.125 µg ml$^{-1}$ of *Senna alata* showed significant (P < 0.05) cytotoxicity.
FIG. 36 Cytotoxic effect of aqueous methanolic leaf extract of Senna alata, Senna podocarpa and an herbal drug, Nicosan, on K562 cells. K562 cells grown on 96-well microplates were incubated for 48 hours in RPMI 1640 medium supplemented with 1% fetal bovine serum to which was added varying concentrations of extract. Succinate dehydrogenase activity was determined using WST-1 assay kit. Data expressed as mean ± sem.
FIG. 37 Cytotoxic effect of aqueous methanolic leaf extract of Senna alata (SA), Senna podocarpa (SP) and an herbal drug, Nicosan (NIC), on K562 cells. K562 cells grown on 24-well microplates were incubated for 48 hours in RPMI 1640 medium supplemented with 1% fetal bovine serum to which was added varying concentrations of extract. The cells were washed and then exposed to neutral red for 4 hrs and the quantity of lysosmally accumulated dye was determined. Data expressed as mean ± sem; * significant difference exist between SA and SP (p < 0.05).
FIG. 38 Cytotoxic effect of aqueous methanolic leaf extract of Senna alata, Senna podocarpa and an herbal drug, Nicosan, on K562 cells. K562 cells grown on 24-well microplates were incubated for 48 hours in RPMI 1640 medium supplemented with 1% fetal bovine serum to which was added varying concentrations of extract. Lactate dehydrogenase levels in supernatants were then determined using an analysis kit. Data expressed as mean ± sem; * significant difference exist between SA and SP (p < 0.05).
5.0 DISCUSSION

The low value of the ash content could be indicative of low mineral value especially the macro minerals in the leaf of *S. alata* and *S. podocarpa*. The crude fat value for *S. alata* was moderate, while that for *S. podocarpa* was high as compared to those of *T. triangulare*, *Amaranthus hybridus*, *Calchorus africanum* (Akinhunsi and Salawu, 2005).

The increased left shift in osmotic fragilogram of erythrocytes supplemented with the extracts, suggests conferment of membrane stabilization, with Jana-herbal formula having the highest, followed by the hydroethanolic leaf extract of *Senna podocarpa*, *Cajanus cajan*, *Senna alata*, *Smilax kraussiana*, *Alchornea cordifolia*, and *Cajanus cajan* seed in descending order respectively. Optimal membrane stabilization was achieved by *Senna podocarpa* and Jana at highest concentration tested. The fragilogram generated by *Alchornea cordifolia* was erratic and showed membrane destabilizing action, while that of *S. kraussiana* at the highest concentration tested crenated the RBCs. The hydromethanolic leaf extract of *S. alata* appeared to be a better membrane stabilizing agent than *S. podocarpa*, which may be indicative of higher activity of its extractable secondary compounds composition. Plants are known to contain compounds which facilitate the stabilization of red blood cell membranes (Smith *et al.*, 1983) hence the membrane stabilizing effects of these extracts may be due to the presence of one or more naturally occurring compounds. The attenuating and potentiating effects of the extracts on membrane stability needs further investigation with a view to understanding the mechanism involved.

Both plant extracts inhibited the red blood cell sickling process. *S. alata* appears to be an early inhibitor while *S. podocarpa* is a late inhibitor as indicated by the morphological normalization of HbSS RBCs following treatment with the hydromethanolic extract. This suggests some influence on the propensity of HbSS RBC to sickle. Although the mechanism
of action of has not been elucidated, HbSS erythrocyte membrane stabilization by the plants extract may play a role in enhancing RBC rehydration rate which would increase the solubility of deoxy HbS or the extracts may possibly possess protein binding affinity, which could disrupt formation of tactoid or fibrilary structures due to deoxy HbS polymerization inside the RBC, thus increasing the concentration of unpolymerised deoxy HbS in solution.

There were significant (P < 0.05) differences amongst the four extracts in total phenolic compounds content. Both S.podocarpa and S.alata hydromethanolic extract had higher total phenolic content than their corresponding aqueous extracts. Plant polyphenolic compounds play an important role in stabilizing lipid oxidation, including radical scavenging properties and may thus act as primary antioxidant free radical terminators in sickle cell anemia which can also be viewed as having oxidant etiology. Several studies have described the antioxidant properties of medicinal plants, foods and beverages which are rich in phenolic compounds (Krings and Berger, 2001; Wang et. al., 2008).

The aqueous extracts of S.podocarpa and S.alata had a significantly (P < 0.05) higher content of free amino acids than their hydromethanolic extracts. These plants may serve as a reservoir of available amino acid when consumed as teas.

The hydromethanolic extracts of S.podocarpa and S.alata showed significantly (P < 0.05) higher antioxidant capacities than their respective aqueous extracts. The hydromethanolic extract of S.podocarpa showed highest overall total antioxidant capacity, which may be attributed to its higher phenolic content. The DPPH radical scavenging activity showed a dose-dependent response. The hydromethanolic extract of S. podocarpa and S.alata had similar IC₅₀ values, which were about five times less potent than ascorbic acid. This may be attributed to its redox properties which allow them act as a reducing agent, hydrogen donors,
singlet oxygen quenchers, metal chelating properties. On the other hand aqueous extract of both plants showed very weak antioxidant activity.

The aqueous leaf extract of *S.podocarpa* and *S.alata* showed poor reducing power. Gallic acid exhibited an EC$_{50}$ (effective concentration) value $<$ ascorbic acid $<$ hydromethanolic leaf extract of *S.podocarpa*. While the hydromethanolic leaf extract of *S.alata* had the highest EC$_{50}$ value. This might be due to the reducing power since a positive correlation existed between total phenolic content (TPC) and total antioxidant capacity (TAC), which is in accordance with previous studies (Zheng and Wang, 2001; Unver *et al*., 2009).

The antioxidative activity of the phenolic compounds may be attributed to their redox properties which allow them act as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelating agents (Huang *et al*., 2004) or inhibit various oxidizing enzymes and regenerate endogenous alpha tocopherol in the lipid bilayer (Letelier *et al*., 2009). According to Osman *et al*., (2004) reducing power has a direct positive correlation with antioxidant properties. Little quantities of different active principles are present in these extracts which could provoke multiple and synergistic antioxidant effects (Letelier *et al*., 2009).

Different values of each antioxidant assay from the same sample are found in various publications (Surrasmo *et al*., 2005; Okoro *et al*., 2010) which could be caused by variation in geographical location, environmental conditions or the mechanism of action of the compounds in the natural sample.

The increased DRSA and TAC values of hydromethanolic over aqueous extract could be due to the solvent of extraction which may have selectively solubilised more phenolic compounds as shown by the increase in phenolic content. The hydromethanolic extracts contain more
apolar compounds than the water extract since increase in the polarity of a compound causes an increase of solubility of the compound in the apolar phases in which peroxidations occur. The free radical scavenging property may be one of the mechanisms by which these plants are effective in traditional medicine. Phenolic compounds may be responsible for antioxidant properties of many plants (Uddin et al., 2008). Therefore hydromethanolic extract of *S. podocarpa* and *S. alata* can be more effective antioxidants than their water extract.

The results of the DNA protection assay indicate that hydromethanolic leaf extract of *S. podocarpa* (at all doses tested) and *S. alata* (at 100 µg ml⁻¹) protects DNA against hydroxyl mediated damage generated by Fenton chemistry. The ability of the extract to protect DNA can be attributed to its ability to scavenge hydroxyl radicals before the radicals oxidize DNA, thus suppressing the formation of linear DNA and induced a partial recovery of super coiled DNA.

*Senna alata* and *Senna podocarpa* are medicinal plants widely used in south west Nigeria to treat various diseases. The application of these herbs in the management of sickle cell anemia is being sought; hence the toxicity profile of these herbals was investigated. A subchronic (35 day) study of the hydromethanolic leaf extract of these plants at the tested doses did not appear to retard growth or affect food consumption, indicating that feed intake and utilisation of protein and other nutrients were not affected by the intake of the herbals.

Analysis of organ weight in toxicology studies is paramount for identification of potentially harmful effects of chemicals (Sellers, 2007). Anthropometric results revealed a higher body weights in the group treated for 63 day treatment of rats with *S. alata* (26.28-68.15% above controls) and *S. podocarpa* (59.75-64.62% above controls). Liver weight relative to body weight was statistically decreased in rats dosed with 600- and 1000 mg kg.bwt⁻¹ of *S. alata*, whereas liver weight relative to brain weight was statistically increased in rats dosed with
200- and 1000 mg kg.bwt\(^{-1}\) of S.alata. The liver weight effects may be considered test substance related since according to Bailey et al., (2004) analysis of organ-to-body weight ratios is predictive for evaluating liver toxicity. Other organ weights relative to body/brain weight from the different treatment groups did not reveal any statistical differences except the increase in liver weight relative to brain weight of the 200- and 600 mg kg.bwt\(^{-1}\) S.podocarpa treated groups.

The rats treated with *S. alata* and *S. podocarpa* showed no changes in their daily physical and behavioural activities. The intake of the extract did not affect the functions of the bone marrow as reflected by the values of WBC (total and differential) and platelets. They were neither quantitatively nor qualitatively affected. However, the increase in the PCV values may be taken to be a reflection of an increased RBC volume, indicative of an impairment of the cellular dehydration pathway, mediated by K\(^{+}\)-Cl\(^{-}\) cotransporter (KCC) or Ca\(^{2+}\)- activated K\(^{+}\) channel (Gardos pathway) (Muzyamba and Gibson, 2003).

It is established that the liver plays a significant role in various metabolic processes, and its xenobiotic function is vital. Therefore, emphasis was laid on the effect these extracts might have on the function of the organ, 63 days consumption of either *S.alata* or *S.podocarpa* did not alter its total protein level.

*S.alata* at 200- and 1000 mg kg.bwt\(^{-1}\) significantly and very significantly reduced plasma AST activity respectively while 600 mg kg.bwt\(^{-1}\) of *S.podocarpa* increased plasma ALT activity. The changes in aspartate (AST) and alanine (ALT) aminotransferase enzyme levels found in the rats correlate with the relative organ weight changes, though the values were not double that of control, which is one indicator of biological significance (Rhomberg et al., 2007). Both enzymes are not liver specific but are also found in skeletal and cardiac muscle (Thrall, 2004). These findings are therefore not considered toxicologically significant. The
were no significant changes in the WBC (total and differential) and platelets counts between the control and the experimental groups. A significant increase in the RBC counts of the experimental groups over the control suggests that the hydromethanolic leaf extracts of *S.alata* and *S.podocarpa* may not be toxic as they do not affect leucopoiesis but affected the circulating erythrocytes and possibly hematopoiesis as reflected by changes in packed cell volumes (PCV) and eosinophils. The increased PCV may also be a reflection of the hydration state of the RBCs.

Assessment of TBARS was used to measure plasma and tissue concentrations of malondialdehyde (MDA), a decomposition product of oxidized lipids, thus serving as an index of plasma and tissue lipid peroxidation. The increased lipid peroxidation level of the brain of rats administered 35 day daily dosage of S.alata leaf extract, may be due to either artifactual *ex vivo* oxidation (Janero, 1990) or that the extract or its product of metabolism crossed the blood brain barrier, whereas the reduction in hepatic, renal as well as plasma TBARS supports the former

Thiobarbituric acid reactive substances (TBARS), as a marker of lipid peroxidation, and catalase a cellular antioxidant were also estimated in the plasma and liver, of rats after 63 day administration of plant extract. There was a significant reduction in hepatic (*S.alata* dose group) and plasma (both plant extracts) TBARS. Overall result of the TBARS indicated potential of crude extracts to inhibit oxidation in lipid system. The findings of this study revealed that the leaf extract of *S.alata* and *S.podocarpa* increased the antioxidant capacity of blood and had an inhibitory effect on the basal level of liver lipid peroxidation, a major consequence of free radical cell damage, which may alter intrinsic membrane properties. Natural antioxidants, e.g. plants extract, could also protect erythrocyte membrane from lipid peroxidation induced by oxo-heme oxidants, by terminating the chain reaction or reacting
with free radicals, particularly peroxy radicals, which are major propagators of the auto-
oxidation chain of fat. This lends scientific support to the therapeutic use of the plant leaves
in traditional medicine.

*Senna alata* and *Senna podocarpa* leaves are employed traditionally in the treatment of a
variety of disease (either singly or in conjunction with other plants) in southwestern Nigeria.
The sperm head morphological evaluation is considered to be essential for the assessment of
male germ cell toxicity by several regulatory bodies like the Organization for Economic
Cooperation and Development (Trivedi, 2010).

The mechanism by which *Senna podocarpa* leaf extract caused increased a significant
increase in sperm head abnormalities is not quite clear, but generally damage to the sperm
cell may have occurred via physiological, cytotoxic or genetic means.

Testicular germ cells carrying minor gene mutations are not eliminated but are manifested as
morphologically deformed sperm. It is also documented that certain substances are germ cell
mutagens, affecting gene loci in spermatogonial cells thereby increasing the percentage of
sperm abnormality (Letz, 1990). In mice, a rodent, sperm cell morphology is genetically
controlled by numerous autosomal and sex-linked genes (Krazanowska, 1986), thus, the
abnormal sperm population observed in the *Senna podocarpa* treated group in this study
maybe due to mutagenic effects of some constituents of these plants extract on specific gene
loci of germ cell chromosomes involved in the maintenance of normal sperm structure. And
since no significant testicular weight loss was recorded in this study, testicular germ cells
may not have been destroyed either due to membrane damage or macromolecular
degradation, although the increased incidence of abnormal sperms is of great concern.
Toxicological disturbance of male reproductive function can occur at many sites producing a range of effects, some primary, and some secondary to the initial insult. Target cells include testis, epididymis, mature sperm and hormonal regulatory system (Creasy, 2001). *Senna podocarpa* exposure could have produced pituitary-hypothalamic or sex hormonal effects which may have affected spermatogenesis (Bruce and Heddle, 1979; Letz, 1990). Spermatogonia located within the basal compartment of the testis, is outside the blood-tubule barrier, hence it is exposed to any toxicant entering the interstitial fluid. Genotoxicants that gain access to the lumenal compartment (containing spermatocytes and spermatids) may result in irreversible DNA damage which may manifest as head abnormalities.

The sensitivity of the assays used in determining cell cytotoxicity depends on their endpoints. The results from the WST-1 assay, indicate that both *Senna alata* and *Senna podocarpa* possessed some active principles which increased cell viability at concentrations lower than 100 µg ml⁻¹, these constituents might have increased the activity of mitochondrial dehydrogenase, induced the expression of growth stimulation or exerted mitogenic effect. But since some plant extracts have been found to reduce MTT in the absence of cells (Shoemaker *et al*., 2004), which could also have been the case with WST-1. The dose-dependent cytotoxicity observed with higher concentrations of both plant extracts maybe due to biologically active alkaloids (Ganapaty *et al*., 2010) present in *Senna podocarpa* or antioxidants which can be oxidised in culture medium with deleterious effect on cells *in vitro* (Long *et al*., 2000; Morita *et al*., 2003; Halliwell, 2008). The lysosome is regarded as the garbage disposal unit of the cell and its functionality connotes cell survival. Lysosomal activity was most severely affected by *Senna podocarpa* (8.13 µg ml⁻¹) followed by *Senna alata* (16.25 µg ml⁻¹) and nicosan (65 µg ml⁻¹). The results from the neutral red uptake assay revealed that *Senna alata* at concentrations greater than (16.25 µg ml⁻¹) increased lysosomal
activity, while the converse was the case for *Senna podocarpa* as concentrations greater than (8.13 µg ml⁻¹) impaired lysosomal activity. K562 cell membrane perturbation leads to increased extracellular lactate dehydrogenase activity which implies cell cytotoxicity. *Senna alata* appeared to cause greater membrane perturbation at lower concentrations than *senna podocarpa* probably due to the presence of higher saponin content. The observation in this study maybe of therapeutic significance, since these plants are used in complementary and alternative medicine (CAM).

**CONCLUSION**

The hydromethanolic leaf extracts of *Senna alata* and *Senna podocarpa* exhibited appreciable anti-sickling as well as de-sickling effect. Both plants provide exogenous sources of antioxidants (possibly in form of phenolic phytochemicals) with remarkable activity, which may be beneficial if incorporated into supplements formulated for SCA patients, to lower DNA, membrane and possibly protein oxidative damage. The use of these extracts may help reduce anemia, with indications that the prolonged use at high concentrations may also induce infertility due to reduced spermatozoa. Thus the extensive prolonged utilization of these plants orally in traditional medicine is cautioned when fertility is an issue. The results of the present research have paved the way to envisaging *in vivo* studies of these extracts as a therapeutic agent in patients with SCD and may provide a rational explanation for their use in managing SCD by Nigerian traditional practitioners.
### OBJECTIVES

<table>
<thead>
<tr>
<th>To evaluate the HbSS RBC membrane stabilising effect of <em>S. alata</em> and <em>S. podocarpa</em> and their influence on polymerisation/depolymerisation of mutant HbS</th>
<th>The result indicated a significant reduction in lysis of HbSS RBC under osmotic stress and inhibition/reversal of the sickling process by <em>S. alata</em> and <em>S. podocarpa</em> extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>To determine the antioxidant activity of <em>S. alata</em> and <em>S. podocarpa</em></td>
<td>Hydromethanolic leaf extract of <em>S. alata</em> and <em>S. podocarpa</em> significantly increased the blood antioxidant capacity and had an inhibitory effect on the basal liver lipid peroxidation level</td>
</tr>
<tr>
<td>To evaluate the long term toxicological effect(s) of <em>S. alata</em> and <em>S. podocarpa</em> on albino rats</td>
<td>Long term oral administration of hydromethanolic leaf extract of <em>S. alata</em> and <em>S. podocarpa</em> appeared toxic</td>
</tr>
<tr>
<td>To determine the effect of <em>S. alata</em> and <em>S. podocarpa</em> on RBC membrane protein Profile of albino rats</td>
<td>Both extract did not alter albino rat erythrocyte membrane profile</td>
</tr>
<tr>
<td>To evaluate the cytotoxic effect of <em>S. alata</em> and <em>S. Podocarpa</em> on K562 cells</td>
<td>Both extract reduced K562 cell viability</td>
</tr>
</tbody>
</table>
CONTRIBUTION(S) TO KNOWLEDGE

1. This study has established the membrane stabilizing potential of hydromethanolic leaf extract of *Senna alata* and *senna podocarpa*, via reduction in the median corpuscular fragility

2. Hydromethanolic leaf extract of *Senna alata* possess greater antisickling activity than that of *Senna podocarpa*

3. Hydromethanolic leaf extract of *S.alata* and *S.podocarpa* increases the antioxidant capacity of blood and also suppress the basal level of lipid peroxidation of liver.

4. Hydromethanolic leaf extract of *Senna alata* and *Senna podocarpa* functions as an antianemic agent by increasing the hematocrit level.
REFERENCES


Surassmo, S., Nukoolkarn, V., Gritsanapan, W and Chomnawang, M. T. (2005). Effects of Thai Medicinal Plants on Inflammation caused by Acne-Inducing Bacteria 31st Congress on Science and Technology of Thailand at Suranaree University of Technology


Ref. No. ADM/DST/229/3/318

Dr. E. O. Temile,
Consultant,
Department of Pediatrics,
LUTH, Surulere.

APPROVAL OF RESEARCH & ETHICS COMMITTEE

I wish to refer to your request in respect of the above stated subject matter.

Approval has been granted you to continue with the study titled "EVALUATION OF EKALVIT IN THE MANAGEMENT OF SICKLE CELL DISEASE: A MULTI CENTRE CLINICAL TRIAL.

Wishing you all the best in the study.

A. O. ENSIPRUP (MRS),
Chairman, Research & Ethics Committee.
Photograph of *Senna alata*
Photograph of *Senna podocarpa*