USMANU DANFODIYO UNIVERSITY, SOKOTO

(POSTGRADUATE SCHOOL)

EFFECT OF SICKLO-N200 IN THE MANAGEMENT OF SICKLE CELL ANAEMIA

A Dissertation

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Dedication

This work is dedicated to my beloved parents; Late Sheikh Ayuba Ahmed and Hajia Karima for their guidance, financial and moral support.

Certification

This Dissertation by AYUBA, Sumayya Ahmed (15210304007) has met the requirements for the award of the degree of Master of Science (Biochemistry) of the Usmanu Danfodiyo University Sokoto, and is approved for its contribution to knowledge.

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CHAPTER FIVE

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List of Abbreviations

ACS	Acute Chest Syndrome
ANOVA	Analysis of variance
CAT	Catalase
CVD	Cardiovascular disease
DAF	Decay-accelerating factor
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
FAO	Food and Agriculture Organization
GPx	Glutathione peroxidase
GRA	Granulocytes
GSH	Glutathione
Hb	Haemoglobin
HbA	Haemoglobin A
HbAS	Sickle cell trait
HbS	Haemoglobin S
HbS/β^+	Sickle beta plus thalassaemia
HbS/β^0	Sickle beta zero thalassaemia
HbSC	Haemoglobin SC
HbSD	Haemoglobin SD
HbSE	Haemoglobin SE
HbSO	Haemoglobin SO
HbSS	Haemoglobin SS
HbF	Foetal Haemoglobin
HPLC	High Performance Liquid Chromatography
HU	Hydroxyurea
IL-1	Interleukin- 1
ITCs	Isothiocyanates
I3C	Indole-3-carbinol
LYM	Lymphocytes
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin
MCV	Mean Corpuscular Volume

MGO	Methylglyoxal
mHb	Methaemoglobin
MID	Monocytes
MIRL	Membrane inhibitor of reactive lysis
MPV	Mean Platelet Volume
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
PCT	Plateletcrit
PCV	Packed Cell Volume
PDW	Platelet Distribution Width
PLA	Placebo
RBCs	Red blood cells
RDW	Red blood cell Distribution Width
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SCA	Sickle cell anaemia
SCD	Sickle cell disease
SEM	Standard error of mean
SOD	Superoxide dismutase
TNF	Tumor necrosis factor
USDA	United States Department of Agriculture
USDHHS	United States Department of Health and Human Services
UV	Ultraviolet
WBCs	White Blood Cells
WHO	World Health Organization

Abstract

This study investigated the effect of Sicklo-N200 in the management of sickle cell anaemia (SCA). Thirty nine patients were recruited, randomly divided into 3 groups and administered placebo, 2.5g and 5g of Sicklo-N200. Haematological parameters, foetal haemoglobin (HbF), haemoglobin polymerisation rate, Fe^{2+}/Fe^{3+} ratio and antioxidant vitamins were analyzed before treatment and monitored every 4 weeks for 12 weeks using standard analytical procedures. HbF levels of patients treated with both doses of Sicklo-N200 were found to increase significantly ($P<0.05$) when treated for 8 and 12 weeks. A significant ($P<0.05$) decrease in haemoglobin polymerisation rate of patients administered 2.5g and 5g of Sicklo-N200 was observed at week 12 and 8 of treatment respectively. Both treatment groups showed significant increase in Fe^{2+}/Fe^{3+} ratio, serum vitamins C and E starting from the 8^{th} week. Placebo had no significant effect on HbF, haemoglobin polymerisation rate, Fe^{2+}/Fe^{3+} ratio. Patients administered 5g of Sicklo-N200 showed a significant increase in the value of the red blood cells count from the 8^{th} week of treatment, but the increase was not significant in the other groups. Haemoglobin and packed cell volume were not significantly affected by the treatment. White blood cells parameters and platelets decreased non significantly ($P>0.05$) in the treatment groups. This study showed that Sicklo-N200 is very beneficial in the management of SCA.

CHAPTER ONE

1.0 Introduction

Sickle cell disease (SCD) is an inherited blood disorder in which the body produces abnormally shaped red blood cells (Abboud, 2009) as a result of genetic changes that cause abnormalities in haemoglobin molecules (Cynthia *et al*., 2016). The abnormality is as a result of a mutation that occurs in the gene coding for haemoglobin in which valine is substituted for glutamate residue at position 6 from the N- terminus of the β globin chain (Bunn *et al*., 2010). This mutation leads to the production of abnormal haemoglobin (HbS). Sickle cell anaemia (SCA), the most common form of SCD, is as a result of homozygosity for the mutation that causes HbS (Howard *et al*., 2013). People who do not have SCD generally have haemoglobin A (HbA) in their blood cells. Haemoglobin S (HbS) is the abnormal variant of HbA, which occurs in sickled red blood cells (Leslie and Nancy, 2015). People who have SCA are born with two sickle genes, one from each of the parent. If one normal haemoglobin gene and one sickle cell gene are inherited, a person will have sickle cell trait (HbAS) and will not develop SCD, but will carry the trait that can be passed on to their children (Winfred, 2015).

The presence of the hydrophobic valine residue allows HbS to polymerize in the deoxygenated state causing the red blood cells (RBCs) to assume a sickle or crescent shape and making the cells both rigid and fragile (Serjeant, 2013). These cells stick to the walls of capillaries (blood vessels) and cannot squeeze through the blood vessels leading to reduced or blockage of blood flow in many parts of the body (Ohnishi *et al*., 2000). Consequently, tissues and organs are deprived of oxygen resulting in organ damage (Steinberg, 2011).

Sickle cells have a shorter life span (10 – 20 days) than normal red blood cells (90 – 120 days) (Gibson and Ellory, 2002). The body produces new RBCs to replace old ones daily, but sickle cells become destroyed so fast that the body cannot keep up with the demand for replacement resulting in the red blood cell count dropping, which results in anaemia (Makani *et al*., 2013).

The presence of sickle shaped RBCs in human blood was first reported by Herrick (1910) and it became the first disease where the exact genetic and molecular defect was elucidated (Neel, 1949). It has no definitive cure (Umar, 2017). About 80% of SCA cases are believed to occur in sub-Saharan Africa (Rees *et al*., 2010). It also occurs relatively frequently in parts of India, the Arabian peninsula, and among people of African origin living in other parts of the world (Elzouki, 2012), including Nigeria (Umar, 2017).

The pathophysiology of the disease is complex, but is ultimately due to the β-globin point mutation that allows HbS polymerization to occur under conditions of hypoxia, acidemia, fever, and erythrocyte dehydration (Catherine and Stephen, 2010). Sickle cell anaemia is medically complex, affecting virtually any organ in the body (kumar *et al*., 2011). The signs and symptoms of SCA usually begin in early childhood (5 to 6 months of age) (Emechebe *et al*., 2017). This is because the dominant haemoglobin (Hb) during the second and third trimester of gestation until shortly after birth is foetal haemoglobin (HbF) (Emechebe *et al*., 2017) and it decreases the polymerization of abnormal Hb (Yogen *et al*., 2004). Dietary iron can also increase the percentage of HbF in red blood cells which relieves the joint and bone pain of SCA (Jeanne, 2000). Anaemia, repeated infections, and periodic episodes of pain are among the characteristics features of this disorder (Gladwin and Sachdev, 2012). Additional

medical complications include: Pulmonary hypertension, bone and joint problems, acute chest syndrome, cardiac abnormalities, renal and liver disease, priapism, and splenic sequestration (Rogers, 2005; Khatib, *et al*., 2009; Paul *et al*., 2011). The severity of symptoms varies from person to person (Brousseau *et al*., 2010).

Treatment goals for SCA aim to relieve pain, prevent infections, and manage complications with vaccinations and antibiotics, high fluid intake, and folic acid supplementation (Mariane, 2008). Other measures may include the use of medication such as hydroxyurea, and blood transfusions to prevent worsening anaemia and stroke (Mirre *et al*., 2010). A small proportion of people can be cured by a transplant of bone marrow cells because the major barriers to its use is lack of a suitable donor and the risk of mortality and morbidity associated with the procedure itself (Panepinto *et al*., 2007). Additional emphasis is placed on disease prevention through genetic counseling of gene carriers for the abnormal haemoglobin and promotion of good nutrition (Wang, 2007).

Good nutrition is critical for people with sickle cell anaemia because it helps promote health and prevent complications in them (Hyacinth *et al*., 2010). Children with SCA need diets that provide plenty of calories, protein, vitamins and minerals (Barden *et al*., 2002; Mitchell *et al*., 2009). The subjects use more energy because of the increase in the rate of red blood cells break down (Al-saqladi *et al*., 2008). The sickle erythrocytes are fragile and dehydrated; they require a delicate balance of minerals and antioxidants to maintain hydration and cellular integrity (Malik, 1999; Okochi and Okpuzor, 2005). Fruits, vegetables and tea are good sources of bioactive phytochemicals and antioxidant nutrients (Mandal *et al*., 2009).

Sickle cell anaemia patients have enhanced production of reactive oxygen species (ROS) and a decreased antioxidant status (Kiefmann, 2008; Nwaoguikpe and Braide, 2012). They also have a series of micronutrient deficiencies: vitamins A, B2, B6, B12, C, D and E, Folic Acid, Iron, Calcium, Magnesium and Zinc (Hasanato, 2006; Vanderjagt *et al*., 2008). The deficiencies of these antioxidants may account for some of the observed manifestations of sickle cell anaemia, such as increased susceptibility to infection and haemolysis (Fasola *et al*., 2007).

Nutraceutical can be defined as a food or part of a food that provides medical or health benefits, including the prevention and/or treatment of a disease (Elizabeth, 2002). Fruits and vegetables are valuable sources of health promoting substances active in neutralization of reactive oxygen species (Augustynowicz *et al*., 2014). They provide a range of micronutrients, including minerals, vitamins and antioxidant compounds, such as carotenoids, and polyphenols (Augspole *et al*., 2014). Increased consumption of fruits and vegetables containing high levels of phytochemicals has been recommended to prevent chronic diseases related to oxidative stress (Liu, 2003; Rao and Rao, 2007; Pandey and Rizvi, 2009). Carrot and cabbage are rich in bioactive compounds like carotenoids and thiocyanates with appreciable levels of several other functional components with significant health-promoting properties (Krishan *et al*., 2012; Leja *et al*., 2013; Joao, 2014). Honey also exhibits antioxidant property and contains trace amount of minerals (Beretta *et al*., 2007; Khalil *et al*., 2011). Nutrient antioxidants may act together to reduce reactive oxygen species level more effectively than single dietary antioxidants, because they can function as synergists (Eberhardt *et al*., 2000; Rossetto *et al*., 2002; Trombino *et al*., 2004).

1.1 Statement of the Problem

Sickle cell disease is a major public health disease worldwide (Piel *et al*., 2013). The morbidity and mortality in SCA is high (Weatherall, 2010). The progress in understanding the molecular basis of SCA and the pathophysiological mechanisms underlying the various complications observed in SCA has not been matched by the development of effective drugs for its treatment (Ademola, 2015). Current evidence suggests that available care is suboptimal (Galadanci *et al*., 2014). Prevention, control, and treatment of SCA in Nigeria are still in infancy. Sickle cell anaemia patients are under chronic oxidative stress induced by excessive production of free radicals from the abnormal haemoglobin (Adams *et al*., 2001). Current treatments are associated with some side effects (Nwaoguikpe *et al.,* 2013). Appropriate exploitation of dietary sources in the management of SCA has not been attained.

1.2 Justification

Sickle cell anaemia is a major public health concern and its management has remained inadequate (Keith, 2017). Globally, it is estimated that SCA occurs in approximately 300,000 births annually of which 80% occur in sub-Saharan Africa (Rees *et al*., 2010). In Nigeria, 25% of the population carries the sickle cell gene, resulting in estimated SCA prevalence of 2% (WHO, 2013). With our current population of 190 million, Nigeria thus has the largest population of SCA in the world (Galadanci *et al*., 2014). The health-care cost of the management of SCA patients is high and there is no effective and safe drug for the management of the disease as such research has to be carried out in order to determine the efficacy of certain natural agents having antisickling effect. This study is intended to find a nutritive therapy with little or no side effects for the management of SCA using carrot (*Daucus*

Carota), cabbage (*Brassica Oleracea*) and honey which are cheap, readily available and with high antioxidants and micronutrients content.

1.3 Aim and Objectives

The aim of this research was to formulate a carrot, cabbage and honey based nutraceutical (Sicklo-N200) that could be used in the management of sickle cell anaemia.

The objectives were to:

1. Formulate Sicklo-N200 in form of dried powder for reconstitution containing carrot, cabbage and honey.
2. Determine the effect of administration of Sicklo-N200 in sickle cell anaemia patients by monitoring the following parameters before and after supplementation:

    a. Full blood count {WBC differential, RBC, Hb, PCV, mean platelet volume (MPV), plateletcrit (PCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), RBC distribution width (RDW), platelets).

    b. Foetal haemoglobin level.

    c. Haemoglobin polymerisation rate and blood Fe^{2+}/ Fe^{3+} ratio.

    d. Serum antioxidant vitamins (A, C, and E) levels.

CHAPTER TWO

2.0 Literature Review

2.1 Sickle Cell Disease

Sickle cell disease (SCD) is the name for a group of genetic blood disorders caused by sickle haemoglobin (HbS) (Charles and Quinn, 2013). Sickle haemoglobin (HbS) is a structural variant of normal adult haemoglobin (HbA) that is inherited as an autosomal recessive Mandelian trait. Sickle cell disease occurs from genetic changes that cause abnormalities in haemoglobin molecules. The sickling mutation causes a single base change from adenine to thymine on the 17^{th} nucleotide of the beta globin chain gene (HBB). This invariably translates into substitution of valine for glutamate on the 6^{th} amino acid of the beta globin chain (Ademola, 2015).

The presence of the hydrophobic valine residue allows HbS to polymerise in the deoxygenated state. In addition to low oxygen tension, low pH and an increased concentration of HbS within the red blood cell (RBC) encourage polymer formation. HbS polymerisation ultimately causes RBCs to take on the characteristic sickle shape in a reversible fashion. Repeated episodes of polymerisation and sickling can cause red blood cells to be irreversibly sickled (Bunn *et al*., 2010).

The presence of sickle shaped RBCs in human blood was first reported by Herrick (1910). These sickle cells can block blood vessels leading to reduced or stopped blood flow in many parts of the body leading to vaso-occlusion, with resultant tissue ischemia. Tissues and organs are deprived of oxygen resulting to organ damage (Steinberg, 2011). When this blood flow slows or stops suddenly in a certain part of the body, the decrease in oxygen can cause severe pain (the sickle cell crisis). The

higher the concentration of sickle haemoglobin and the more acidic the environment, the faster the sickle cell process is. When blood cells dry out (dehydrate), the density of HbS within the cell increases, thereby speeding the sickling process. Many metabolic changes which occur in SCD that lead to anemia include sickling, generation of reactive oxygen species, and the general decrease in the rate of physical development (Okochi and Okpuzor, 2005).

2.1.1 Types of Sickle Cell Disease

Haemoglobin is the protein in red blood cells that carries oxygen. It normally has two alpha chains and two beta chains ($\alpha_2\beta_2$). There are many different kinds of haemoglobin genes. The normal haemoglobin gene is called A; however there are over 400 abnormal haemoglobin genes (Eric, 2009). The most common are: S, C, and beta thalassemia (also written as βThal).

It is possible to get many different combinations of genes because an individual inherits one gene from each parent. Each of these combinations will cause a different condition in the person who inherits it. The most common types of sickle cell disease, which are classified according to haemoglobin abnormality, are:

2.1.1.1 Sickle Cell Anaemia (HbSS)

Sickle cell anaemia (SCA) refers to the form of the disease when there is homozygosity for the gene mutation that is responsible for causing the production of sickle haemoglobin. Haemoglobin S in SCD contains an abnormal beta globin chain encoded by a substitution of valine for glutamic acid on chromosome 11 (Tae *et al*., 2014). This occurs when a child inherits the sickle gene from both parents and is the most serious form of the disease with the potential for serious complications. There

are various names that can be used to indicate sickle cell anaemia. These may include HbSS, SS disease, sickle cell disease-SS or hemoglobin S. Sickle cell anaemia is the most common form of sickle cell disease (CDC, 2013).

2.1.1.2 Sickle Cell Disease-SC (HbSC)

People who have this form of SCD inherit a sickle cell haemoglobin gene (S) from one parent and a gene for abnormal haemoglobin (C) from the other parent. Haemoglobin C is an abnormal haemoglobin in which substitution of a glutamic acid residue with a lysine residue at position 6 in the beta chain has occured (Monica, 2008). As a result of the two similar gene defects, it is associated with similar symptoms and effects. It can also be referred to as haemoglobin C with sickling disease or HbSC and is the second most common form of the disease (CDC, 2013).

2.1.1.3 Sickle Beta Thalassemia

Thalassaemia is another inherited health condition that affects the haemoglobin in the red blood cells. It occurs if a child inherits a sickle gene mutation from one parent and a thalassemia gene mutation from the other parent. The β-thalassemias represent an autosomal recessive disorder with reduced production or absence of β-globin chains resulting in anaemia (Tae and Bommy, 2014).

There are two kinds of sickle beta thalassaemia: sickle beta plus thalassaemia (HbS/β^{+}) and sickle beta zero thalassaemia (HbS/β^{0}). These are less common than the previous two types (CDC, 2013).

There are also some rare types of sickle cell disease, which include:

- Haemoglobin-SD disease (HbSD),

- Haemoglobin-SE disease (HbSE).
- Haemoglobin-SO Arab disease (HbSO).

2.1.1.4 Sickle Cell Trait (HbAS)

Sickle cell trait refers to individuals that have inherited only one abnormal sickle gene and also possess one normal adult haemoglobin gene. It is unusual for people with the trait to experience symptoms, but they are carriers who have the potential to pass the gene on to their children, who may suffer from sickle cell anaemia if the other parent is also a carrier of the gene mutation (David *et al*., 2010).

2.1.2 Signs and Symptoms of Sickle Cell Anaemia

Problems in SCA typically begin five to six months of age. This is because the dominant haemoglobin (Hb) during the second and trimester of gestation until shortly after birth is foetal haemoglobin (HbF) (Boyer *et al*., 1975) and it inhibits polymerisation of sickle cell Hb. The signs and symptoms of SCA are caused by the sickling of red blood cells. When red blood cells sickle, they breakdown prematurely, leading to anaemia. Painful episodes can occur when sickled red blood cells, which are stiff and inflexible, get stuck in small blood vessels. Anaemia, repeated infections, and periodic episodes of pain are among the characteristics features of this disorder. The severity of symptoms varies from person to person. Some have mild pains while some have severe pains that need to be hospitalized (Gladwin and Sachdev, 2012).

The hallmark of SCA is the sickle cell crisis which includes:

2.1.2.1 Vaso-occlusive Crisis; which is caused by sickle-shaped red blood cells that obstruct capillaries and restrict blood flow to an organ resulting in ischaemia, pain, necrosis, and often organ damage (Franco, 2009).

2.1.2.2 Splenic Sequestration Crisis; which are acute, painful enlargements of the spleen, caused by trapping of red cells within the spleen and resulting in a precipitous fall in haemoglobin levels with the potential for hypovolemic shock (McCavit *et al*., 2011).

2.1.2.3 Aplastic Crisis; which occurs as a result of an infection caused by parvovirus B19 leading to temporary cessation of red cell production, which in turn leads to low levels of red blood cells during the infection. Infections (especially by parvovirus) are common in SCA patients (Cauff and Quinn, 2008).

2.1.2.4 Haemolytic Crisis; which refers to rapid decrease in haemoglobin level because red blood cells break down at a faster rate leading to anaemia. Anaemia can cause shortness of breath, fatigue, and delayed growth and development in children. The rapid breakdown of RBCs may also cause yellowing of the eyes and skin which are signs of jaundice (Franco, 2009).

2.1.2.5 Acute Chest Syndrome (ACS); this is a common and potentially life threatening complication of painful crises, and is often precipitated by a chest infection, occurs when the lung tissues are deprived of oxygen during a crisis. It is caused by infection, blockage of blood vessels and asthma. Signs and symptoms include: chest pain, fever, pulmonary infiltrate, respiratory symptoms and hypoxemia (Glassberg, 2011).

2.1.2.6 Additional Medical Complications; this include pulmonary hypertension; which develops when pressure in the arteries of the lung increase, stroke; which is caused by blockages of vessels carrying oxygen to the brain, infection, bone and joint problems, renal and liver disease, priapism, eye problems and leg ulcers (Rogers, 2005; Halasa *et al*., 2007; Gladwin and Sachdev, 2012).

2.1.3 Sickle Cell Anaemia and Diet

Good nutrition while essential for everyone is critical for people with SCA. It helps to promote health and prevent complications. The beneficial effects of high intake of plant products to human health have been partly attributed to the compounds which possess antioxidant activities (Gundgaard *et al*., 2003; Gosslau and Chen, 2004). Some sources of phytochemicals include broccoli, lettuce, cabbage, spinach, tomatoes, soybean, green tea, ginger, chilli pepper, turmeric , grapes, garlic, aloe and carrot (Arts and Hollman, 2005; Aggarwal and Shishodia, 2006).

The major antioxidants of vegetables are vitamins C and E, carotenoids, and phenolic compounds, especially flavonoids. Nutrient antioxidants may act together to reduce reactive oxygen species level more effectively than single dietary antioxidants, because they can function synergistically (Rossetto *et al*., 2002 and Trombino *et al*., 2004). Examples of antisickling plants include: Fermented mixture of dried unripe fruit pulp of *C. papaya* (pawpaw) and dried *S. bicolor* (sorghum) leaves (Mojisola *et al*., 2009), extract of *Allium sativum* (garlic) (Takasu *et al*., 2002), boiled and crude ethanolic extracts of edible *Cajanus cajan* beans (pigeon pea) (Osuagwu, 2010), the use of phytomaterials of *Piper guineense* (African pepper), *Pterocarpa osun* (black camwood), *Eugenia caryophyllala* (clove) and S. *bicolor* extracts for the treatment of SCA has been reported (Mehanna, 2001). Vegetables and fruits play a significant role

in human nutrition, especially as sources of vitamins (A, E, B6, C, thiamin, riboflavin, niacin and folate), minerals, and dietary fiber (Wargovich, 2000; Kader, 2002).

2.1.4 Therapy for SCA

Many researches (Brugnara and Steinberg, 2002; Bunn *et al*., 2010) stated that in treatment of SCA, it is required that one focuses on the ways of inhibiting sickle cell haemoglobin polymerisation, prevention or repair of red cell dehydration and interrupting the interaction of sickle cells with the endothelium. Current treatment focuses on prevention of complications through early diagnosis by newborn screening, prophylactic antibiotics usually penicillin, folic acid supplementation which helps to replace red blood cells, hydration in which the oral rehydration therapy has recorded great success in the management of sickle cell anaemia (Joy *et al*., 2008), pain relief medications ranging from nonprescription non-steroidal anti-inflammatory drugs (NSAIDs) to opiods are given to control pain, and transcranial Doppler screening (with subsequent transfusion therapy) if needed for stroke prevention. Additional emphasis is placed on disease prevention through genetic counseling of gene carriers for sickle haemoglobin. New therapies to cure or ameliorate symptoms include haematopoietic cell transplantation and hydroxyurea therapy which induces foetal haemoglobin production (Tae and Bommy, 2014). The induction of foetal haemoglobin has been the most promising of all the lines of orthodox treatment used in the management of SCA (Vadolas *et al.*, 2004; Bianchi *et al*., 2009). Foetal haemoglobin is believed to interfere with the polymerised globin chains whose interaction with each other results in rigidity of the cells. The anti sickling properties of certain amino acids such as phenylalanine, alanine, lysine, arginine etc have also been reported (Ekeke and Shode, 1990).

Gene therapy is being experimented and level of success as recorded in transgenic animal models and few human subjects is encouraging (Wu *et al.*, 2006). It has progressed to the point of human trial and was reported in 2017 in a patient having SCD (Ribeil *et al*., 2017). Gene therapy is an experimental technique that aims to treat genetic diseases by altering a disease-causing gene or introducing a healthy copy of a mutated gene to the body. Genetically altering patient's haematopoietic stem cells is the strategy employed by researchers in treating SCA with gene therapy. One strategy is to remove some of the pateints's haematopoietic stem cells, replace the mutated HBB gene in these cells with a healthy copy of the gene and then transplant those cells back into the patient using a modified, harmless virus (Hoban *et al*., 2016). Another strategy is to genetically alter another gene in the patient's haematopoietic stem cells so they boost the production of HbF by using a highly specific enzyme to cut the cell's DNA in the section containing one of the genes that suppresses production of HbF resulting in more production of HbF since the gene no longer works when the cell repairs its DNA. Gene therapy offers an advantage over bone marrow transplant, in that complication associated with a bone marrow donation (finding the right match) is not a concern (Hoban *et al*., 2016).

Many investigations have been carried out on the role of some dietary supplements, such as thiocyanate (Okochi and Okpuzor, 2005) in the management of sickle cell anaemia. The sickle cell erythrocytes are fragile and dehydrated and it is important that minerals and antioxidants are constantly supplied to maintain hydration and membrane integrity. Therefore many tropical plants have been investigated for their micronutrients and antioxidative properties. Some of the plants examined so far include *M. charantia* (Semiz and Sen, 2007), *Allium cepa* (Onion), *Allium sativum* (Garlic) and *Telferia occidentalis (*Ugu) (Nwaoguikpe, 2009), *Cymbropogon citrates*

(lemon grass) and *Camellia sinensis* (Ojo *et al*., 2006), *Scoparia dulcis* (Adaikpoh *et al*., 2007), and *Picrorhiza kurroa* (Rajaprabhu, *et al*., 2007). The use of Niprisan; a mixture of *Piper guineense, Pterocarpa osun, Eugenia caryophyllala* and *Sorghum bicolor* extracts as an antisickling phytomedicine has been reported (Mehanna, 2001). Patients with SCA have a series of micronutrient deficiencies: vitamins A, B2, B6, B12, C, D and E, folic acid, iron, calcium, magnesium and zinc (VanderJagt *et al*., 2008). It has been suggested that a better nutrition could improve body composition, especially lean mass, and have a positive impact on SCA morbidity and mortality (Al-saqladi *et al*., 2008).

2.2 The Blood

Blood is a specialized bodily fluid that delivers necessary substances to the body's cells, such as nutrients and oxygen and transports waste products away from these same cells (Manmohan *et al*., 2013). It is the vehicle for long distance, mass transport of materials between the cells and external environment or between the cells themselves. Blood represents about 7-8% of total body weight (Dennis, 2013). Blood consists of a complex liquid plasma in which the cellular elements; erythrocytes, leukocytes, and platelets are suspended. The circulating blood carries out the critical functions of transporting oxygen and nutrients to body cells and getting rid of carbon dioxide, ammonia, and other waste products. It also plays a vital role in our immune system, coagulation, and in maintaining a relatively constant body temperature (Lockard and Robert, 2019)

2.2.1 Red Blood Cells

Erythrocytes are essentially plasma membrane enclosed bags of haemoglobin that are necessary for the delivery of oxygen to tissues. They are the most abundant cells of

the blood; red cells normally make up 40-50% of the total blood volume (Lauralee, 2014). Erythrocytes have a smooth surface and exist as biconcave disc which allows for flexibility and movement through vessels of different shapes and sizes, they lack a cell nucleus, and the cytoplasm contains mostly haemoglobin molecules. The red cells are produced continuously in the bone marrow from stem cells at a rate of about 2-3 million cells per second. Red cells remain viable for only about 4 months before they are removed from the blood and their components recycled in the spleen (Lauralee, 2014).

The association of two mutant β -globin subunits forms haemoglobin S (HbS). Under low - oxygen conditions, the absence of a polar amino acid at position six of the β - globin chain promotes the non - covalent polymerization of haemoglobin, which distorts red blood cells into a sickle shape and decreases their elasticity (Kotue 2016). The loss of red blood cell elasticity is central of the pathophysiology of sickle cell disease (Forget and Bunn, 2013). In people homozygous for HbS, the presence of long-chain polymers of HbS distort the shape of the red blood cell, from smooth dount-like shape to ragged and full of spikes, making it fragile and susceptible to breaking within capillaries (Serjeant, 2013). Normal red blood cells are quite elastic, which allows the cells to deform to pass through capillaries. In sickle cell disease, low oxygen tension promotes red blood cell sickling and repeated episodes of sickling damage the cell membrane and decreases the cell's elasticity. These cells fail to return to normal shape when normal oxygen tension is restored (Kitadi *et al.,* 2015). As a consequence, these rigid blood cells are unable to deform as they pass through narrow ccapillaries, leading to vessel occlusion and Ischaemia (Steinberg, 2011). The anaemia of the illness is caused by haemolysis in the spleen, because of their misshape. Although, the bone marrow attempts to compensate by creating new red

cells, it does not match the rate of destruction (Makani *et al*., 2013). In people heterozygous for HbS, the polymerization problems are minor, because the normal allele is able to produce over 50% of the haemoglobin. Carriers have symptoms only if they are deprived of oxygen or while severely dehydrated (David *et al*., 2010).

2.2.1.1 Red Blood Cells Destruction

Premature destruction of sickle erythrocytes occur both extravascularly and intravascularly. Extravascular haemolysis results from abnormalities of the sickle cell that permit its recognition and phagocytosis by macrophages and from impaired deformability of sickle red cells, enabling their physical entrapment (Nur *et al.*, 2011). Elevations of free plasma haemoglobin in patients suggest that one-third of the total haemolysis in sickle cell anaemia is intravascular (Rother *et al*., 2005). One mechanism of intravascular haemolysis is sickling - associated exovesiculation (Westerman *et al*., 2008) of vesicles rich in phosphatidylinositol anchored membrane proteins depleting the cell of the complement regulatory proteins Decay-accelerating factor (DAF) and Membrane inhibitor of reactive lysis (MIRL) and leaving the cells susceptible to complement - mediated intravascular lysis (Nur *et al.*, 2011). Another component of intravascular haemolysis is increased mechanical fragmentation of sickle cells which accounts for the accelerated haemolysis of sickle cell patients during exercise (Makani *et al*., 2013). Haemoglobinuria and oliguria may be present. Pallor develops rapidly and icterus is common. Frequently the pallor, listlessness and mild icterus develop more insidiously. The urine may be dark in colour due to the presence of excess urobilinogen. The anaemia is usually ortho chromic but it may be either normocytic or macrocytic. Reticulocytosis is often marked and there may be many erythroblasts in the peripheral blood (Franco, 2009).

2.2.2 White Blood Cells

White cells, or leukocytes, exist in variable numbers and types but make up a very small part of blood's volume; normally less than 1% in healthy people (Lockard and Robert, 2019). All leukocytes ultimately originate from common precursor undifferentiated pluripotent stem cells in bone marrow that also give rise to erythrocytes and platelets (Sembulingam and Prema, 2012). Leukocytes are found throughout the body including the spleen, liver, and lymph glands. They are the immune system's mobile defense units and are transported in the blood to sites of injury or of invasion by disease causing microorganisms. Individual white cells usually only last 18-36 hours before they are removed, though some types live as much as a year (Sembulingam and Prema, 2012).

There are five types of circulating WBCs; neutrophils, eosinophils, basophils, monocytes, and lymphocytes (Lynne, 2019). Each has a characteristic structure and function. All white blood cells are nucleated, which distinguishes them from the anucleated red blood cells and platelets. The types of leukocytes fall into two main categories; depending on the appearance of their nuclei and the presence or absence of granules in their cytoplasm when viewed microscopically. Neutrophils, eosinophils, and basophils are categorized as polymorphonuclear (many-shaped nucleus) granulocytes (granule containing cells). Monocytes and lymphocytes are mononuclear agranulocytes (Lynne, 2019).

Neutrophils are the most abundant white blood cell, constituting 60-70% of the circulating leukocytes (Bruce *et al*., 2002). They are phagocytic specialists; they engulf and destroy bacteria intracellularly. Eosinophils compose about 2-4% of the WBC total. An increase in circulating eosinophils is associated with allergic

conditions (such as asthma and hay fever) and with internal parasite infestations (for example, worms) (Saladin, 2012). Basophils are the rarest of the white blood cells (less than 0.5% of the total count) (Falcone *et al*., 2000). They excrete two chemicals that aid in the body's defenses: histamine and heparin. Histamine is responsible for dilating blood vessels and increasing the flow of blood to injured tissue. Heparin is an anticoagulant that inhibits blood clotting and promotes the movement of white blood cells into an area. Basophils can also release chemical signals that attract eosinophils and neutrophils to an infection site (Saladin, 2012). Monocytes, the largest type of WBCs, are like neutrophils, they become professional phagocytes after emerging from the bone marrow. Lymphocytes provide immune defense against targets for which they are specifically programmed. There are two types of lymphocytes, B and T lymphocytes (B and T cells), which look alike. B lymphocytes produce antibodies, which circulate in the blood and are responsible for antibody-mediated immunity, T lymphocytes do not produce antibodies; instead, they directly destroy their specific target cells by releasing chemicals, a process called cell-mediated immunity (Lauralee, 2014).

2.2.3 Haemoglobin (Hb)

Haemoglobin is an oxygen carrying pigment which is composed of a protein and prosthetic group (heme). The protein part of haemoglobin consists of four polypeptide chains, usually two pairs of identical chains, attached to a heme group which has an organic part (a protoporphyrin ring made up of four pyrrole rings) and a central iron ion in the ferrous state (Fe^{2+}). During development from the embryonic to the adult state, at least six different polypeptide chains; alpha (α), beta (β), gamma (γ), delta (δ), epsilon (ε) and zeta (ζ) are synthesized and incorporated in the various

haemoglobin molecules, several of which may be present at a particular gestational age (Brittain, 2004). Mutations in the genes for the haemoglobin protein in a specie result in haemoglobin variants. Many of these mutant forms of haemoglobin cause no disease but some of these mutant forms of haemoglobin cause a group of hereditary diseases termed the "haemoglobinopathies". The best known haemoglobinopathy is sickle cell disease (Hardison and Ross, 2012). Abnormality in its structure leads to various disorders and diseases. In humans, as in all vertebrate species studied, different α-like and β-like globin chains are synthesized at progressive stages of development to produce haemoglobins characteristic of primitive (embryonic) and definitive (fetal and adult) erythroid cells (Zhang and Gerstein, 2004).

In the fetus, the zeta (ζ) and epsilon (ε) genes are initially expressed resulting in the formation of the four common embryonic haemoglobins, Hb Portland-1 ($\zeta_2\gamma_2$), Hb Portland-II ($\zeta_2\beta_2$) Hb Gower-1 ($\zeta_2\varepsilon_2$), and Hb Gower-2 ($\alpha_2\varepsilon_2$) (Brittain, 2004). Their down regulation in early embryonic life is followed by the expression of the 2 α -genes and the 2 γ –genes; this causes the accumulation of haemoglobin F ($\alpha_2\gamma_2$), which predominates in the last 2 trimesters of gestation. The α genes remain fully active at birth, but the γ genes are effectively down regulated and the β-like (δ and β) genes are up regulated so that, normally, by the end of the first year of life, the "adult" haemoglobin (haemoglobins A) is predominant (more than 95%) (Alan, 2017).

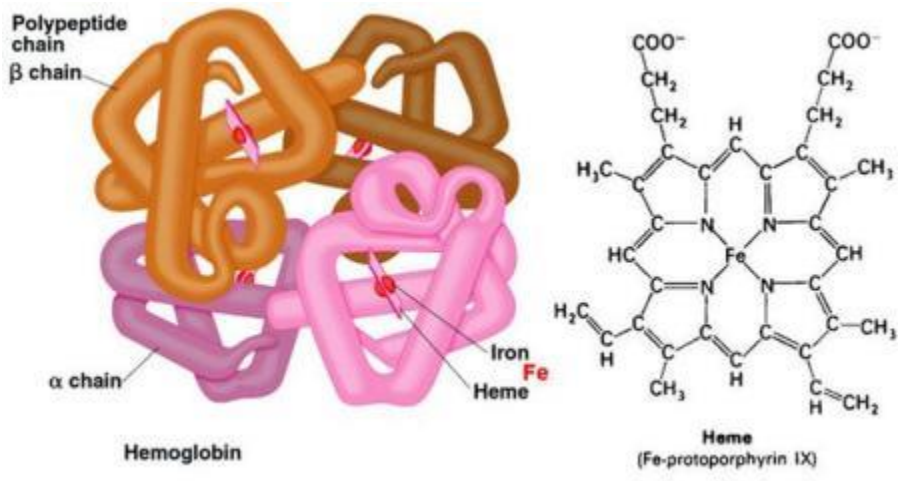

Figure 1: Structure of Haemoglobin (Sembulingam and Prema, 2012)

2.2.3.1 Haemoglobin A (HbA)

HbA is the principal Hb in a normal adult individual, it exists as a tetramer (which contains four subunit proteins), consisting of two α and two β subunits non-covalently bound, each made of 141 and 146 amino acid residues, respectively. This is denoted as $\alpha_2\beta_2$. The subunits are structurally similar and about the same size. Each subunit has a molecular weight of about 16,000 daltons and contains a buried heme group to which a single O_2 molecule binds (Sembulingam and Prema, 2012). Genes for the α-chain are found on chromosome 16 and those for the β-chain on chromosome 11. Haemoglobin is present in blood at concentrations of 13.5-18.0 gdl^{-1} in men and 11.5-16.0 gdl^{-1} in women. Each erythrocyte contains around 200-300 million molecules of haemoglobin (Sembulingam and Prema, 2012).

2.2.3.2 Haemoglobin S (HbS)

Haemoglobin S is an abnormal type of haemoglobin that can be inherited. In sickle haemoglobin (HbS), a missense mutation (GAG to GTG) causes the substitution of

valine for glutamic acid in the sixth position of the β globin chain. The gene for haemoglobin S has the highest frequency among people of African heritage (about 1 in 10) (Ademola and Jude, 2014). Replacement of negatively charged and hydrophilic glutamate by non charged and hydrophobic valine leads to instability and decreased solubility of the haemoglobin molecule when deoxygenated making it having the capacity to polymerize. HbS polymer injures the sickle erythrocyte, increases its density, increases its adhesivity, and shortens its life span leading to haemoglobin precipitation and haemolysis (Martin *et al*., 2019).

The polymerization of deoxy Hb S is the primary and indispensable event in the molecular pathogenesis of sickle cell disease (Forget and Bunn, 2013). The polymer has the form of an elongated rope-like fiber, which usually aligns with other fibers, forming a fascicle, and distorting the red cell into the classic crescent or sickle shape. HbS can undergo innumerable cycles of deoxygenation-induced polymerization and reoxygenation-induced depolymerization. However, the sickle erythrocyte membrane can withstand only a finite number of these cycles before it is irreversibly injured (Forget and Bunn, 2013).

Polymerization is not the only pathophysiologic event that accounts for all the pathophysiology of sickle cell disease. Downstream events following polymerization, including changes in red cell membrane structure and function, disordered red cell volume control, increased red cell adherence to vascular endothelium (Kotue, 2016), misregulation of vasoactivity, and inflammation contribute to sickle vaso-occlusion and hemolysis that are the hallmarks of sickle cell disease (Kato *et al*., 2007; Zhang *et al*., 2016). These are called pleiotropic effects, because they go beyond the immediate consequences of the abnormal gene.

2.2.3.3 Haemoglobin F (HbF)

HbF is a tetramer of α and γ globin chains. Adult Hb (or in the case of sickle cell anemia HbS) replaces HbF as a result of the switch from γ- to β-globin synthesis that occurs in fetuses (Zhang and Gerstein, 2004). The high fraction of HbF at birth masks the expression of SCD until HbS levels increase to 75% at approximately 6 months of age because of the inhibitory effect of Hb on HbS polymerization and cellular sickling (Idowu *et al*., 2011). The efficacy of HbF is due to its ability to dilute HbS concentrations below the threshold required for polymerization in erythrocytes. HbF also has a direct influence on Hb S polymer stability (Akinsheye *et al.*, 2011; Pace and Zein, 2006). Asymmetric hybrid molecules Hb F/S ($\alpha_2,\gamma\beta^S$) (Akinsheye *et al.*, 2011) are produced when HbF levels remain elevated to produce the observed clinical benefit. It was also reported that compound heterozygotes for sickle cell trait and hereditary persistence of HbF (HPFH) were clinically normal despite having a very high HbS concentration (Forget 2011). Foetal haemoglobin differs most from adult haemoglobin in that it is able to bind oxygen with greater affinity. This greater affinity for oxygen is explained by the lack of foetal haemoglobin's interaction with 2,3-bisphosphoglycerate (2,3-BPG or 2,3-DPG) (Obeagu *et al*., 2015). The expression of γ-globin is controlled by a developmentally regulated transcriptional program that is recapitulated during normal erythropoiesis in the adult bone marrow (Stamatoyannopoulos, 2005). Reactivation of fetal hemoglobin (HbF) expression is an important therapeutic option in patients with haemoglobin disorders (Kotue 2016).

Butyrate, several other Short chain fatty acids and their derivatives, 5-azacytidine (and decitabine), hydroxyurea, and erythropoietin were shown to induce HbF *in vivo* in patients with these disorders (Hassana and George, 2006). In spite of the

incomplete understanding of the mechanisms of induction of HbF by these agents, considerable progress has been made in developing these agents as drugs that induce HbF in patients with SCD. The increase in HbF levels was shown to be associated with decreased DNA methylation at the promoters of the γ-globin genes (*HBG1* and *HBG2*) (Akinsheye *et al.,* 2011). Butyrates, well-known inhibitors of histone deacetylases (HDAC), are being tested in clinical trials as inducers of HbF in patients with SCD and β-thalassemia (Atweh and Schechter, 2001). When administered by continuous infusion, arginine butyrate resulted in a significant increase in γ-globin synthesis, HbF levels and F cells in patients with SCD. However this increase in HbF levels was not sustained with continuous therapy (Ikuta *et al*., 2001). The leading hypothesis for the mechanism of induction of HbF by butyrate is that it increases the transcriptional activity of the γ-globin promoters by increasing the level of histone acetylation and a decrease in the level of DNA methylation at the promoters of the γ-globin genes (Weinberg *et al*., 2005). Butyrate was also shown to increase HbF production in patients with SCD by increasing the efficiency of translation of γ-globin mRNA (Weinberg *et al*., 2005). Butyrate was also shown to activate signaling through the soluble guanylate cyclase (Ikuta *et al*., 2001) and p38 MAP kinase (Pace *et al*., 2003) pathways. It is not yet clear how these signaling activities lead to the transcriptional activation of the γ-globin genes.

5-Azacytidine and decitabine inhibit DNA methylation at cytosine residues by DNA methyltransferases (DNMT). These two drugs serve as cytidine analogs that are incorporated into DNA, where they form covalent bonds with DNMT, leading to depletion of functional enzyme (Atweh and Schechter, 2001; Saunthararajah and DeSimone, 2004). Studies have also shown that decitabine induces a rapid and selective degradation of DNMT1 through the proteasomal protein-degradation

pathway (Ghoshal *et al*., 2005). Treatment with decitabine is also associated with increased histone acetylation at the promoters of γ-globin genes (Lavelle *et al*., 2006). This suggest that decitabine-induced DNA hypomethylation may result in secondary changes in histone acetylation, giving rise to an open chromatin structure that allows the binding of transcription factors, leading to de-repression and transcriptional activation of γ-globin gene expression in adult life.

Earlier studies had suggested the involvement of the erythroid transcription factors GATA-1 and NF-E2 in the induction of erythroid differentiation and activation of globin expression by butyrate (Andrews *et al*., 1993; Shivdasani and Orkin, 1995). Moreover, GATA-1 and NF-E2 were shown to be targets for histone acetyltransferases and their acetylation was shown to enhance their transcriptional activity. NF-E2 (Gavva *et al*., 2002) and GATA-2 (Ozawa *et al*., 2001) were also shown to be associated with HDAC. Although the effects of these interactions on the level of acetylation of these transcription factors are still not known, it is tempting to speculate that the enzymatic activity of butyrate as an HDAC inhibitor may increase the acetylation of these transcription factors and result in the induction of γ-globin gene expression. Induction of HbF by either DNMT inhibitors or HDAC inhibitors results in a change in both DNA structure (i.e., hypomethylated CpG sites) and chromatin configuration (i.e., hyperacetylated histones) (Bank 2006). More studies are necessary to fully elucidate the mechanisms of activation of fetal globin gene expression by butyrate.

A potential mechanism by which hydroxyurea induces HbF has been hypothesized to involve the redox in-activation of a tyrosyl radical on ribonucleotide reductase (Pallis *et al*., 2014), an effect that can be mediated by NO (Tang *et al*., 2005) and

nitrovasodilators (Wood *et al*., 2006). Furthermore, Gladwin *et al*., (2003) demonstrated that soluble guanylyl cyclase (sGC) activators or analogs increased γ-globin gene expression in erythroleukemic cells and in primary human erythroblasts and that the activity of hemin and butyrate on γ-globin induction was prevented by inhibiting sGC or cGMP-dependent protein kinase. These show the participation of NO in the mechanism of HbF induction by hydroxyurea. The combination of erythropoietin and hydroxyurea has synergistic effects in increasing HbF in some patients (Atweh *et al*., 2001; Rodgers and Sauntharajah, 2001).

2.3 Antioxidants

An antioxidant is any substance that protects the body from damage caused by harmful molecules called free radicals (Halliwell, 2007). Free radicals are potentially damaging molecules released in the body through normal metabolic processes and are capable of attacking the healthy cells of the body, causing them to lose their structures and functions (Esra, 2014). Antioxidants have diverse physiological role in the body because they inhibit the process of oxidation even when they are present at relatively small concentration (Ergu, 2016). Antioxidants are critical for maintaining optimum health and well being (Kanchev and Kasaikina, 2013; Ginter *et al*., 2014) and they protect the body against the development of diseases caused by oxidative stress such as cancer (Halliwell, 2007), Parkinson's disease, Alzheimer's disease (Valko *et al*., 2007), myocardial infarction (Ramond *et al*., 2011) and Sickle Cell Disease (Amer *et al*., 2006).

Antioxidant protection system in humans involves a variety of components, both endogenous and exogenous in origin that function interactively and synergistically to neutralize free radicals (Peter, 2007; Bouayed *et al*., 2009). Endogenous (enzymatic

and non enzymatic) antioxidants are produced in the body such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH), among others (Valko *et al*., 2007). The main source of exogenous antioxidants such as vitamins, flavonoids, carotenoids, trace elements etc. is the diet (Andre *et al*., 2010), but can also be synthetic compounds, like butylhydroxyanisole, butylhydroxytoluene, gallates, etc. (Litescu, 2011). Synthetic antioxidants are chemically synthesized (do not occur in nature) and are added to food as preservatives to help prevent lipid peroxidation (Saikat and Raja, 2011).

2.3.1 Dietary Antioxidants

Studies demonstrate that an antioxidant rich diet has a very positive health impact (Wills *et al*., 2009; Sin *et al*., 2013). Natural antioxidants are constituents of many fruits and vegetables (Halvorsen *et al*., 2006) and they have attracted a great deal of public and scientific attention (Diwani *et al*., 2009). Dietary antioxidants such as vitamin C, vitamin E and carotenoids are well known and there is a surplus of publications related to their role in health (Boskou *et al*., 2005). Antioxidants through our diet play an important role in helping endogenous antioxidants for the neutralization of excess free radicals (WHO/ FAO, 2001). There has been an evidence of low circulating levels of antioxidant vitamins A, C and E in the plasma of HbSS patients (Nwaoguikpe and Braide, 2012). Vitamin C promotes the absorption of non-heme iron and is required for fighting infections. Researchers reported that on the administration of vitamin C to human sickle cell red cells in vitro, formation of dense cells is inhibited (Adewoye *et al*., 2008). Lachant and Kouichi (1986) and Jaja *et al*., (2008) proposed that vitamin C prevents in vitro heinz body formation in adult sickle red cells and normalizes the hemodynamic changes associated with positive adjustments.

Vitamin C is the most important water soluble antioxidant in extracellular fluids. It is capable of neutralizing Reactive oxygen species (ROS) in the aqueous phase before lipid peroxidation is initiated. Vitamin E is a major lipid soluble antioxidant (Paul and Sumit, 2002; Handan *et al*., 2007; Abdalla, 2009), and the most effective chain breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation (Bashir *et al*., 2004). β carotene; a provitamin A carotenoid effectively quenches singlet oxygen free radicals and prevent lipid peroxidation caused by oxygen and can intercept the propagation step of lipid peroxidation (Young and Low, 2001). In addition, several metalloenzymes such as glutathione peroxidase (Se), catalase (Fe) and superoxide dismutase (Cu, Zn and Mn) are also critical in protecting the internal cellular constituents from oxidative damage. Vitamins and minerals functioning as antioxidants are shown in the table below.

Table 1: Some Antioxidant Vitamins and Minerals

Nutrients	Component (location in cell)	Function
Vitamin C	Ascorbic acid (cytosol)	Reacts with several types of ROS/RNS
Vitamin E	α-tocopherol (membranes)	Breaks fatty acid peroxidation chain reactions
β-carotene	β-carotene (membranes)	Prevents initiation of fatty acids peroxidation chain reaction
Selenium	Glutathione peroxidase (cytosol)	An enzyme that converts hydrogen peroxide to water
Copper and Zinc	Superoxide dismutase (cytosol)	An enzyme that converts superoxide to hydrogen peroxide
Manganese and Zinc	Superoxide dismutase (mitochondria)	An enzyme that converts superoxide to hydrogen peroxide
Copper	Ceruloplasmin (water phase)	An antioxidant protein, may prevent copper and iron from participating in oxidation reactions
Iron	Catalase (cytosol)	An enzyme (primarily in liver) that converts hydrogen peroxide to water

Source: McDowell *et al*., 2007

2.4 Carrot

Carrot (*Daucus carota*) is the most important crop of *Apiaceae* family, it is usually orange in colour, though purple, red, white, and yellow varieties exist (Abdel-Aal *et al*., 2013). Carrots were first used for medical purposes and gradually used as food (Carlos and Dias, 2014). It is a root vegetable that has worldwide distribution with carotenoids, flavonoids, polyacetylenes, vitamins, and minerals, all of which possess numerous nutritional and health benefits (Joao, 2014). Most of the benefits of carrots can be attributed to their beta-carotene and fiber content (Krishan *et al*., 2012).

Carotenoids widely distributed in orange carrots are potent antioxidants which can neutralize the effect of free radicals (Leja *et al*., 2013). It is a good source of antioxidant agents and also rich in β carotene, Vitamin C, Vitamin K, vitamin B8, thiamin (B1), riboflavin (B2), pyridoxine (B6), folate, pantothenic acid, potassium, iron, copper, and manganese (Ching and Mohamed, 2001; Horbowicz *et al*., 2008; USDHHS and USDA, 2010). Carrots have a unique combination of three flavonoids: kaempferol, quercetin and luteolin which play an important role as antioxidants (Ching and Mohamed, 2001) and are also rich in other phenols, including chlorogenic, caffeic and p-hydroxybenzoicacids along with numerous cinnamic acid derivates (Goncalves *et al*., 2010). Bioactive polyacetylenes, such as falcarinol and falcarindiol are found in carrots. The concentration of falcarinol in fresh carrots depends on carrot tissue cultivar and water stress (Lund and White, 1990). Falcarinol is the most bioactive phytochemical of the carrot polyacetylenes (Pferschy-Wenzig *et al*., 2009).

2.4.1 Health Benefits of Carrot

2.4.1.1 Antioxidant, Anticarcinogen, and Immunoenhancer Benefits

Carotenoids widely distributed in orange carrots are potent antioxidants which can neutralize the effect of free radicals. Beta-carotene is the major carotene that is present in these roots (Sun *et al*., 2001). It is one of the powerful natural antioxidants which helps protect the body from harmful free radical injury. Carotenoids and polyphenols are phytochemicals that are responsible for the antioxidant activity of carrots. They protect human body against cardiovascular disease, arteriosclerosis and cancer (Ciccone *et al*., 2013). Zhang and Hamauzuet (2004) reported that flavonoids and phenolic derivatives, present in carrot roots play also an important role as antioxidants. They also exert anticarcinogenic activities (Zaini *et al*., 2011), reduce inflammatory insult, and modulate immune response (Dias, 2012).

2.4.1.2 Nephroprotective and Hepatoprotective Benefits

Renal ischemia reperfusion caused significant impairment of kidney function, the renoprotective activity of *Daucus carota* root extract was studied in renal ischemia reperfusion injury in rats and it was found to minimize this effect probably by the free radical scavenging activity (Mital *et al*., 2011). Ebeid *et al*., (2015) observed that carrot extract help to protect liver from acute injury by the toxic effects of environmental chemicals.

2.4.1.3 Anti-Diabetic, Cardiovascular Disease and Cholesterol Effect

A research by Coyne *et al*., (2005) demonstrated a significant association between vitamin A-rich carotenoids and diabetes status. This finding suggests that carrot and

vitamin A-rich carotenoids might help diabetics to manage their condition. A study conducted by Chau *et al*., (2004) confirmed the strong relationship between dietary fiber intake and lower risk of type 2 diabetes. In another study where lyophilized carrot enriched diet was administered to mice, it was observed that carrot ingestion decreased lipemia, improved antioxidant status in mice and increased the level of vitamin E and myocardial cells (Nicolle *et al*., 2003). The results suggested that carrot intake may exert a protective effect against cardiovascular disease linked to atherosclerosis. The effect may be due to the synergistic action of dietary fiber and antioxidant polyphenols in carrot (Nicolle *et al*., 2003).

2.4.1.4 Wound Healing Effect

The antioxidant and antimicrobial activities of ethanolic extract of carrot root, mainly flavonoids and phenolic derivates, may be involved in increased curative property. Animals treated with topical cream of ethanolic extract of carrot root, formulated at different concentrations, showed significant decreases in wound area, epithelization period and scar width when compared to control group animals in an excision wound model (Patil *et al*., 2012).

2.5 Cabbage

Cabbage is a leafy green biennial vegetable of *Brassicaceae* family, round or oval in shape, consisting of soft light green or whitish inner leaves covered with harder and dark green outer leaves (Boulger, 2014). Cabbage is a unique source of several types of phytonutrients (glucosinolates) (Ambrosone and Tang, 2009). Cabbage is an excellent source of Vitamin K, Vitamin C and Vitamin B6. It is also a very good source of Manganese, dietary fiber, Potassium, Vitamin B1, Folate and Copper

(USDA, 2014). Additionally, cabbage is a good source of Choline, Phosphorus, Vitamin B2, Magnesium, Manganese, Calcium, Selenium, Iron, Pantothenic acid, Protein and Niacin (Dinkova-Kostova and Kostov, 2012). It contains the antioxidants Choline, Beta-Carotene, Lutein, and Zeaxanthin as well as the flavonoids; Kaempferol, Quercetin, and Apigenin (Anna, 2005). Its overall antioxidant activity is largely due to its phenol and polyphenol content (Rohit *et al*., 2015). The antioxidants and vitamins in it have the potential to prevent and treat malignant diseases (Murillo and Mehta, 2001).

2.5.1 Health Benefits of Cabbage

2.5.1.1 Immunity and Digestion

Cabbage stimulates the production of tumor necrosis factor α (TNF) and interleukin-1 (IL-1); important players in antitumorial, antiviral, immunoregulatory and inflammatory responses (Komatsu, 1997). Glucosinolates and their breakdown products in cabbage and its rich glutamine content show clear benefits in optimizing immune function (Taipale, 2000). Glutamine is found abundantly in raw cabbage (Oguwike *et al*., 2014). Hickman, (1998) suggests that glutamine is essential to the health and maintenance of the intestinal tract. The epithelial cells of the intestinal lumen are renewed every 4-5 days as such a high level of cell proliferation is required to maintain homeostasis (Vanderflier and Clevers, 2009). Glutamine influences a number of signaling pathways that regulate cell cycle regulation and proliferation. Cabbage is very rich in fiber which helps the body retain water and it maintains the bulkiness of the food as it moves through the bowels. Thus, it is a good remedy for constipation and other digestion related problems (Oguwike *et al*., 2014).

2.5.1.2 Anticancer Benefits

The uniqueness of cabbage in cancer prevention is due to its richness in anti-inflammatory glucosinolates and antioxidants (Ambrosone and Tang, 2009). Glucosinolates are cabbage's overriding factor with regard to anticancer benefits. Glucosinolate hydrolysis products alter the metabolism or activity of sex hormones in ways that could inhibit the development of hormone-sensitive cancers. Isothiocyanates and indoles derived from the hydrolysis of glucosinolates, such as sulforaphane and indole-3-carbinol (I3C), have been implicated in a variety of anticarcinogenic mechanisms. In some cases, they help regulate inflammation while in others, they improve the body's detoxification system (Holst and Williamson, 2004). Epidemiological studies indicate that human exposure to isothiocyanates and indoles through cruciferous vegetable consumption may decrease cancer risk, but the protective effects may be influenced by individual genetic variation (polymorphisms) in the metabolism and elimination of isothiocyanates from the body (Zhang, 2004). Polyphenols; the main phytonutrient antioxidants in cabbage and vitamin C are partly responsible for its cancer prevention benefits. Chronic oxidative stress and chronic inflammation are risk factors for development of cancer. Polyphenols also provide anti-inflammatory benefits (Sami *et al*., 2013).

2.5.1.3 Heart Health

Consumption of fruits and vegetables has been inversely associated with a decreased risk of cardiovascular disease (CVD), most likely due to the abundance and variety of bioactive compounds present (Nothlings *et al*, 2008). The use of polyphenolics, including anthocyanins, has been important in the treatment of chronic disease, such as CVD (Vauzour *et al*., 2010). The high polyphenol content in cabbage might also

reduce the risk of cardiovascular disease by preventing platelet buildup and reducing blood pressure, inhibiting the inflammatory process, the endothelial dysfunction and Nitric oxide (NO) production (Megan, 2016). Clinical and experimental studies have shown that oxidative stress is associated with the pathogenesis of cardiovascular processes. In addition, the heart is an organ with low concentrations of antioxidant in the body, which leads to increased ROS production, so it is most prone to tissue and cells damage, mainly in macromolecules, as DNA, proteins and cell lipids (Qin *et al*., 2003).

Cabbage intake can lower total cholesterol through the process of binding with bile acids. Bile acids are specialized molecules that aid in the digestion and absorption of fat, and when they are present in the digestive tract, fiber-related nutrients in cabbage can bind together with them for eventual excretion. Since the liver needs to replace the excreted bile acids and it uses cholesterol as a basic building block to produce bile acids, the cholesterol level drops down. Whenever this process takes place, the liver needs to replace the excreted bile acids by drawing upon existing supply of cholesterol, and as a result cholesterol level drops down (Talwinder *et al*., 2012).

2.5.1.4 Anti inflammatory Benefits

Regulation of inflammatory system can become compromised, and the problem of chronic inflammation can be experienced especially when combined with oxidative stress when there is insufficient intake of anti inflammatory nutrients (Sami *et al*., 2013). Chronic inflammation is a risk factor for development of cancer (Sami *et al*., 2013). All types of cabbage contain significant amounts of polyphenols and glutamine that provide anti inflammatory benefits (Rohit *et al*., 2015). The isothiocyanates (ITCs) made from cabbage's glucosinolates help regulate inflammation by altering the activity of messaging molecules within our body's inflammatory system (Heiss *et al*.,

2001). Cabbage is a good source of vitamin C which helps in fastening the healing of wounds (Rohit *et al*., 2015).

2.6 HONEY

Honey has a long history of human consumption as source of nutrients and medicine (Bogdanov *et al*., 2008). It is the oldest and only unique natural sweetener and unprocessed food consumed by man (Ouchemoukh *et al*., 2010). Honey is a mixture of sugar (mainly fructose about 38.5% and glucose about 31.0%) and other compounds such as vitamin C, phenol compounds, and enzymes that have antioxidant properties (Jaganathan and Mandal, 2009). It also contains trace amount of minerals (K, Ca, Ti, Cr, Mn, Fe, Ni, Cu, Zn, Se, Br, and Rb). Potassium is the most abundant, followed by calcium and iron (Adebiyi *et al*., 2004). The antioxidant activity of honey is generally attributed to its phenolic compounds and flavonoids (Beretta *et al*., 2007; Van den borg *et al*., 2008; Khalil *et al*., 2011). Honey also exhibits antimicrobial, antiviral, antiparasitic, antimutagenic, and anti inflamatory properties (Bogdanov *et al*., 2008). Its simple sugars are absorbed directly into the blood stream without undergoing digestion (MAAREC, 2004).

2.6.1 Health Benefits of Honey

2.6.1.1 Antioxidant Property of Honey

The antioxidant activity of honey is strongly correlated with the content of total phenolics (Chua *et al*., 2013) and the color of honey (Khalil *et al*., 2011). Honey contains flavonoids such as apigenin, pinocembrin, kaempferol, quercetin, galangin, chrysin and hesperetin and phenolic acids such as ellagic, caffeic, coumaric, and ferolic acids (Hussein *et al*., 2011; Alvarez-Suarez *et al*., 2013). Dark honey has a

higher total phenolic content and consequently, a higher antioxidant capacity (Blasa *et al*., 2006; Khalil *et al*., 2011). Antioxidant properties of honey act as an antidepressant during high emotional, physical and intellectual stress (Jaganathan and Mandal, 2009). Free radicals of oxygen are a natural byproduct of metabolism that cause cellular damage and breakdown of structure of DNA, antioxidants bind these molecules preventing their harmful effects. Honey unlike synthetic compounds, represents a natural product with no known side effects which can be harmful to health (Jaganathan and Mandal, 2009). Honey also inhibited oxidative stress which may be partly responsible for its neuroprotective activity (Shimazawa, *et al*., 2005). A wide range of phenolic compounds are present in honey which has promising effect in the treatment of cardiovascular diseases (Bogdanov *et al*., 2008).

2.6.1.2 Antibacterial and Wound Healing Property of Honey

The antimicrobial activity of honey is due to its high sugar content, low pH and content of hydrogen peroxide and non-peroxide components i.e., the presence of phytochemical components like methylglyoxal (MGO) (Basualdo *et al*., 2007; Mavric *et al*., 2008). Also, honey is hygroscopic, which means that it can draw moisture out of the environment and dehydrate bacteria. The healing property of honey is due to the fact that it offers antibacterial activity, maintains a moist wound condition, and its high viscosity helps to provide a protective barrier to prevent infection and do not result in bacteria becoming resistant (Lu *et al*., 2014). Unheated honey has some broad-spectrum antibacterial activity when tested against pathogenic bacteria, oral bacteria as well as food spoilage bacteria (Lusby *et al*., 2005). Honey has been reported to have antibacterial activity against various bacterial species including *Escherichia coli*, *Corynebacterium diphtheriae*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *Pasteurella multocida*, *Yersinia*

enterocolitica, *Proteus species*, *Pseudomonas aeruginosa*, *Salmonella diarrhea*, *Salmonella typhi*, *Shigella dysentery*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Streptococcus mutans*, *Strep. Pneumonia* and *Streptococcus pyogenes* (Bansal *et al*., 2005; Visavadia, 2006; Estevinho *et al*., 2008; Abhishek *et al*., 2010). It was found that local application of raw honey on infected wounds reduced redness, swelling, time for complete resolution of lesion and time for eradication of bacterial infection due to *S. aureus* or *Klebsiella sp*. Its potency was comparable to that of local antibiotics (Al-waili, 2004).

2.6.1.3 Anti inflammatory Property of Honey

Honey such as Yemeni sidr honey (Alzubier and Okechukwu, 2011), Gelam honey (Kassim *et al*., 2012), Manuka honey (Keenan *et al*., 2012), Tualang honey (Ahmed and Othman, 2013), and Buckwheat honey (Van den Borg *et al*., 2008) exhibit anti inflammatory effect. Honey reduces the activities of cyclooxygenase-1 and cyclooxygenase-2, thus showing anti inflammatory effects (Hussein *et al*., 2013) and demonstrates immunomodulatory activities (Reyes-Gordillo *et al*., 2007). Ingestion of diluted natural honey showed reduction effect on concentrations of prostaglandins and thromboxane B2 in plasma (Al-Waili and Boni, 2003). Kassim *et al*., (2012) found that Gelam honey inhibits lipopolysaccharide-induced endotoxemia in rats through the induction of heme oxygenase-1 and the inhibition of Cytokines, Nitric oxide (NO), and high-mobility group protein B1. They found that honey reduced cytokine and NO levels while increasing heme oxygenase-1 levels. These observations support the hypothesis that honey can be used as a natural compound for the treatment of a wide range of inflammatory diseases. A reduction in inflammation was observed when honey was applied on localized swelling, redness, pain, and heat associated with inflammation (Jackson *et al*., 2006).

Honey has an anti inflammatory action free from adverse side effects associated with other forms of treatments (Ahmed *et al*., 2003) such as suppression of immune response and tissue growth, formation of ulcers in stomach etc. Though the exact mechanism of action is still unknown, research has focused on the antibacterial property of honey as a cause. Honey is hygroscopic in nature which means it has a dehydrating effect that inhibits bacterial growth, which contributes to its anti inflammatory and wound healing properties.

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials

All chemicals and reagents used were of analytical grades and were obtained from standard suppliers. The list of equipment, glass wares and chemicals used in this research work are presented in appendix II.

3.1.2 Samples

Fresh carrot and cabbage were purchased from Sokoto Central Market and identified at the Botany Unit, Department of Biological Sciences, Usmanu Danfodiyo University, Sokoto. Voucher no. 0183 and 0174 were issued. Honey was bought from Department of Biological Sciences, Usmanu Danfodiyo University, Sokoto.

3.1.3 Sickle Cell Anaemia Subjects

The subjects were twenty four (24) SCA patients attending the Sickle Cell Clinic of Usmanu Danfodiyo University Teaching Hospital (UDUTH), Sokoto (13 male and 11 female) and 15 patients of the sickle cell clinic of Specialist Hospital, Sokoto (7 male and 8 female) within the ages of 3-15 years were used for this study.

The number of patients recruited was arrived at using the formula (Araoye, 2004).

$$n = Z^2pq/d^2$$

Where:

n= the desired sample when the population is greater than 10,000

z= the desired normal deviate usually set at 1.96 which corresponds to the 95% confidence level

p= the prevalence rate of sickle cell anaemia patients in Nigeria which is 2% (WHO, 2013)

$$q = 1 - p = 1 - 0.02 = 0.98$$

d = degree of accuracy desire usually set at 0.04

$$n = (1.96)^2 \times 0.02 \times 0.98/(0.04)^2$$

Therefore n = 47

$$Attrition\ rate\ of\ 10\% = \frac{47 \times 10}{100} = 4.7$$

n = 54

3.1.3.1 Informed Consent/Assent and Protection of Patients

Parental consent/assent and ethical clearance from the authorities of Usmanu Danfodiyo University Teaching Hospital, Sokoto and Specialist Hospital, Sokoto were obtained before the patients were recruited for the study. All the patients and their parents were duly briefed of the nature of the study.

3.1.3.2 Inclusion Criteria

Sickle cell anaemia (HbSS) patients confirmed by Hb electrophoresis in alkaline/acid media within the ages of 3-15 years of both sexes were used for this study.

3.1.3.3 Exclusion Criteria

Patients that were excluded from the study were sickle cell anaemia patients with acute symptoms that lead to hospital admission 4 weeks prior to recruitment and those that received blood transfusions in the previous 3 months.

3.2 Methods

3.2.1. Processing of Vegetables

Fresh carrot and cabbage were washed with water, the carrot was scraped using a knife, and then both carrot and cabbage were grated separately. One-third of the grated vegetables were freeze dried and the remaining was dried in an electric oven at 40^0C. The dried vegetables were then grinded using an electric blender and sieved using 150μm sieve. The powder obtained was used for the preparation of Sicklo-N200.

3.2.2 Preparation of Sicklo-N200

The formula for preparing a single dose of 2.5g is as follows:

Ingredients	Quantity
Carrot	0.6 g
Cabbage	0.3 g
Honey	1.6 g
Microcrystalline cellulose	0.8 g
Sodium starch glycolate	0.035 g
Sodium benzoate	0.025 g
Carboxymethyl cellulose	0.054 g
Aspartame	0.035 g
Sunset yellow	0.0015 g
Orange flavour	0.005 ml

Granules preparation: The process of granulation was carried out for the carrot, cabbage and honey. Microcrystalline cellulose and sodium starch glycolate were the pharmaceutical excipients used for granulation and sodium benzoate was used as a preservative.

Sicklo-N200 was prepared by mixing the granules with the remaining excipients and sealed in nylon.

Figure 2: Prepared Sicklo-N200 (unpackaged)

Figure 3: Packaged Sicklo-N200

(photographs from personal camera)

The placebo was prepared by mixing the ingredients above excluding the active ingredients in the same proportion as Sicklo-N200 and sealed in nylon.

3.2.3 Experimental Design

The recruited patients (39) were grouped into 3 groups of 13 patients each as follows:

Group 1: Control subjects were administered placebo daily in addition to the routine drugs (for 12 weeks).

Group 2: Patients were administered 2.5g of Sicklo-N200 daily in addition to the routine drugs (for 12 weeks).

Group 3: Patients were administered 5.0g of Sicklo-N200 daily in addition to the routine drugs (for 12 weeks).

The routine drugs administered to the patients are folic acid and paludrine (Proguanil).

Baseline analyses (haematological and biochemical) were performed before the commencement of the supplementation and at 4 weeks intervals during the research period of 12 weeks.

3.2.4 Blood Sample Collection

Venous blood sample (4-5 mls) was collected from each of the patients using syringe and was placed in both labelled EDTA and plain bottles. The blood sample in the EDTA bottle was used for the haematological analyses which were carried out immediately after collection and the blood in the plain bottle was centrifuged for 5 min at 2000 rpm. The serum obtained was stored in sterile serum bottles at -20^0C until used for biochemical analyses.

3.2.5 Full Blood Count

Determination of packed cell volume (PCV), red blood cells (RBC) count, white blood cells (WBC), Hemoglobin, MPV, PDW, PCT, MCV, MCH, MCHC, RDW, and platelets were done using Automated Blood cell Counter (Theml *et al.*, 2004).

Principle

This method relies on the change in conductance evoked by cells passing through a small aperture. The aperture size differs for RBC, WBC, and platelets. The change in conductance results in an electric impulse that can be detected and recorded.

Procedure

The machine was turned on and put on standby mode. The uncoagulated blood samples were placed in the machine and a blood collecting tube was dipped into the blood. The machine was put on START, the blood sample was sucked into the unit and measurement immediately started. After analysis, the results were printed out.

3.2.6 Determination of Foetal Haemoglobin Level

Foetal haemoglobin was determined by the method of Pembrey *et al*., (1972).

Principle

Foetal haemoglobin determination is based on its resistance to denaturation at alkaline pH**.** HbF shows a decreased rate of alkaline denaturation, while other haemoglobins will be denatured within a minute of exposure to an alkali solution. The absorbance of foetal haemoglobin is measured at 415 nm.

Procedure

To 2.8 ml of cyanmethaemoglobin (HiCN) solution (0.6 ml of haemolysate to 10 ml Drabkin's solution) in a test tube, 0.2 ml of 1.2 M NaOH was added and mixed thoroughly. The mixture was allowed to stand for exactly 2 mins, and then 2.0 ml of saturated ammonium sulphate was added. After vigorous

mixing, the denatured material was allowed to precipitate for 10 mins then filtered through a Whatman no 42 filter paper. Absorbance of filtrate was read at 415 nm. A control solution was prepared by mixing 1.4 ml of the cyanmethaemoglobin, 1.6 ml of distilled water and 2 ml of saturated ammonium sulphate. The solution was diluted 1:10 with distilled water and absorbance was read at 415 nm. The percentange of HbF was calculated as

$$percentage\ \text{HbF} = \frac{Atest \times 100}{Acontrol \times 4}$$

Where Atest = Absorbance of test, Acontrol = Absorbance of control, 100 = Conversion to % and 4 = Dilution factor of HiCN

3.2.7 Determination of Haemoglobin Polymerisation Rate

The method of Noguchi and Schechter, (1985) was used for HbSS polymerisation experiment.

Principle

HbSS polymerisation was assessed by the turbidity of the polymerising mixture at 700 nm using 2 % solution of Sodium metabisulphite as a reductant or deoxygenating agent. The level of polymerisation was ascertained by monitoring the absorbance of the assay mixture.

Procedure

Preparation of erythrocyte haemolysate

Erythrocytes were isolated from whole blood by centrifuging at 2000 rpm for 10 minutes. Sickle cells were sedimented while the plasma was siphoned out carefully

using Pasteur pipette. The erythrocytes were washed three times with a volume of isotonic saline (0.9%) equivalent to the siphoned plasma. The resulting erythrocytes were then suspended in a volume of isotonic saline equivalent to the siphoned plasma. The erythrocyte suspension was freeze thawed in a freezer to release a haemolysate, which was used for the haemoglobin polymerisation experiment.

Polymerisation studies of erythrocyte haemolysate

A portion of 0.1 ml of the erythrocyte haemolysate containing HbS was placed into a test tube followed by addition of 0.1 ml of isotonic saline and 4.8 ml of freshly prepared 2 % sodium metabisulphite. The mixture was mixed thoroughly on a vortex mixer. The change in absorbance of the assay mixture was recorded at a regular interval of 1 s for 10 mins at 700 nm. The rate of haemoglobin polymerisation was estimated by calculating the slope of a plot of absorbance against time.

3.2.8 Determination of Fe^{2+}/Fe^{3+} Ratio

The Fe^{2+}/Fe^{3+} ratio was determined by the method of Davidson and Henry (1974).

Principle

The approach employs the established procedure of lysing whole blood in distilled water and then determining the absorbance of haemoglobin (Hb) and methaemoglobin (mHb) at their characteristic wavelengths of 540 nm and 630 nm, respectively**.**

Procedure

Normal saline (0.02 ml) was added to 5.0 ml of distilled water and 0.02 ml of whole blood (HbSS) and incubated in a test tube at 27^0C for 1 hour. The percent Hb (% Hb)

and (% mHb) was determined spectrophotometrically at 630 nm and 540 nm respectively.

$$Fe^{2+}/Fe^{3+} \text{ ratio} = \frac{\boldsymbol{Abs\ at\ 540nm}}{\boldsymbol{Abs\ at\ 630nm}}$$

3.2.9 Estimation of Serum Vitamin A

The concentration of vitamin A was estimated by the method of Rutkowski *et al.*, (2006).

Principle

Vitamin A is transported as complexes with lipoprotein. The bonds are broken with the addition of potassium hydroxide (KOH) solution and the retinol extracted with xylene. The retinol has an absorption peak at 335 nm and can be determined by reading the extinction at this wave length, the sample is then irradiated with ultraviolet light to destroy the retinal present and absorbance read again at the same wave length. The differences in absorbance before and after irradiation correspond to the amount of retinol present.

Procedure

One milliliter (1 ml) of the serum was placed into a centrifugal test tube and 1 ml of KOH solution was added. The tube was shaken vigorously for 1 minute. The tube was heated in a water bath (60^{o}C, 20 minutes), then cooled down in cold water. One millilitre (1 ml) of xylene was added and the test tube was shaken vigorously again for 1 minute. The mixture was centrifuged for 10 minutes at 4000 rpm, the supernatant was transferred to a glass test tube and the absorbance of the solution was read at 335 nm against xylene. This gave the absorbance of retinol and any other material soluble in xylene (A_1). The xylene extract was irradiated to UV light for 30

minutes, until a steady absorbance (A_2) is obtained at 335 nm. This gave the absorbance of the unwanted material only as the entire retinol was bleached. The absorbance of retinol was obtained by subtracting the second absorbance (A_2) from the initial absorbance (A_1). The concentration (C_x) of serum vitamin A (µmol/l) in the sample was calculated using the formula:

$$C_X = (A_1 - A_2).22.23$$

Where: 22.23 is the multiplier on basis of the absorption coefficient of 1% solution of vitamin A (as the retinol form) in xylene at 335 nm in a measuring cuvette about thickness = 1 cm.

3.2.10 Estimation of Serum Vitamin C

The concentration of vitamin C (ascorbic acid) was estimated by the method of Omaye *et al*., (1979).

Principle

Ascorbic acid is oxidized by copper II ion to form dehydroascorbic acid, which reacts with acidic 2,4-dinitrophenylhydrazine to form a red osazone which is measured spectrophotometrically at 520 nm. A typical calibration plot was made and used to determine the concentration of ascorbic acid in the investigated samples.

Procedure

To 0.5 ml of serum, 2 ml of freshly prepared 6% metaphosphoric acid was added, mixed thoroughly and centrifuged for 10 minutes at 3500 rpm. Similarly, blank and standard were prepared but instead of serum, 0.5 ml of distilled water and standard were added respectively. To 1.2 ml of supernatant, 0.4 ml of DTC reagent (5 ml of

5% thiourea solution + 5 ml of 0.6 % copper sulphate + 10 ml DNPH reagent) was added, mixed and incubated in a water bath at 37^0C for 3 hours. The test tubes were transferred into ice bath for 10 mins. Two millilitres (2 ml) of 12 M sulphuric acid was added and mixed. The absorbance was read at 520 nm after zeroing the instrument with blank.

$$Concentration\ of\ vit\ C = \frac{Absorbance\ of\ test}{Absorbance\ of\ standard} \times Concentration\ of\ standard$$

3.2.11 Estimation of Serum Vitamin E

Vitamin E (tocopherol) was estimated by the method of Desai and Lewis (1984).

Principle: Vitamin E reduces ferric ion to ferrous ion which then form a red coloured complex with αα-dipyridyl reagent and measured at 520nm. The absorbance of this colour complex is directly proportional to the concentration of vitamin E.

Procedure: 1.5 ml of serum, 1.5 ml of standard vitamin E and 1.5 ml of distilled water were dispensed in to test tubes labeled as test, standard, and blank respectively. 1.5 ml of ethanol was added to test and blank. 1.0 ml of xylene was added was added in to all the tubes.The contents were mixed and centrifuged at 2500 rpm for 10 minutes, 1ml of xylene layer was pipetted into another set of identically labeled test tubes and1 ml of αα-dipyridyl reagent was added to the test tubes. The contents of the test tubes were mixed and the absorbance of sample and standard was read against blank at 460 nm. After 3 minutes 0.33 ml of ferric chloride was added to the test tubes and another absorbance was taken at 520 nm.

$$Conc.of\ Vit.E = \frac{Abs.of\ sample\ at\ 520nm - Abs.of\ sample\ at\ 460nm \times Conc.of\ std.}{Abs.of\ std.at\ 520nm}$$

3.2.12 Data Analysis

The results were expressed as mean ± standard error of mean. The differences between the variables across the groups were compared using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison. P value < 0.05 was considered statistically different. Statistical package GraphPad Instat, California, USA, Version 3.1 was used for the analysis.

CHAPTER FOUR

4.0 Results

The effect of Sicklo-N200 on foetal haemoglobin, haemoglobin polymerisation rate and Fe^{2+}/Fe^{3+} ratio in the SCA patients are presented in Table 2. From the observed values of treatment with 2.5 g of Sicklo-N200, the level of foetal Hb increased significantly ($P<0.05$) after 8 weeks, but the increase was not significant ($P>0.05$) at the 4^{th} week. The 5.0 g group gave a foetal Hb value of 1.36 ± 0.101%, 1.37 ± 0.119%, 2.86 ± 0.235% and 3.11 ± 0.235% at 0^{th}, 4^{th}, 8^{th} and12^{th} week respectively. The increase was found to be significant ($P<0.05$) at the 8^{th} and 12^{th} week when compared to baseline (0^{th} week).

The haemoglobin polymerisation rate of 2.5g group was decreased from baseline (0 week) to the 4^{th} week then to the 8^{th} week in a non significant ($P>0.05$) pattern. However, the 12^{th} week was found to be significantly ($P<0.05$) reduced when compared to baseline. The 5.0g group has significantly ($P<0.05$) reduced Hb polymerisation rate starting from the 8^{th} week down to the 12^{th} week, but the reduction at the 4^{th} week was not significant ($P>0.05$) when compared to baseline.

The ratio of the Ferric to Ferrous (Fe^{2+}/Fe^{3+}) of SCA patients administered 2.5 g Sicklo-N200 ranged from 0.9893 ± 0.051 to 1.0992 ± 0.007 and those administered with 5.0g ranged from 0.9983 ± 0.000 to 1.0973 ± 0.012. Both treatment groups showed significant ($P<0.05$) increase starting from the 8^{th} week through to the 12^{th} week.

Table 2: Effect of Sicklo-N200 on Foetal Haemoglobin, Haemoglobin Polymerisation Rate and Fe^{2+}/Fe^{3+} Ratio of Sickle Cell Anaemia Patients.

Test	Dose(g)	Week			
		0	4	8	12
Foetal Haemoglobin (%)	PLA	3.48 ± 0.378	1.82 ± 0.197	4.01 ± 0.765	1.55±0.126
	2.5	1.11 ± 0.062[a]	1.43 ± 0.074	1.84 ± 0.101[b]	1.97±0.143[b]
	5.0	1.36 ± 0.101[a]	1.37 ± 0.119	2.86 ± 0.235[b]	3.11±0.235[b]
Hb Polymerisation rate	PLA	0.0235± 0.004	0.0145±0.002	0.0181±0.003	0.0396±0.004
	2.5	0.0281± 0.004[a]	0.0223 ± 0.003	0.0125 ±0.002	0.0072±0.000[b]
	5.0	0.0294 ±0.004[a]	0.0204 ±0.003	0.0058 ±0.000[b]	0.0025±0.000[b]
Fe^{2+}/Fe^{3+} ratio	PLA	1.0030± 0.003	1.0199± 0.010	1.0580± 0.014	1.0094±0.007
	2.5	0.9893 ±0.051[a]	1.0016 ±0.003	1.0601 ±0.010[b]	1.0992±0.007[b]
	5.0	0.9983± 0.000[a]	0.9986 ± 0.000	1.0487± 0.004[b]	1.0973±0.012[b]

Values are presented as mean ± SEM. Mean value with different superscript letters in rows are significantly different ($P<0.05$). PLA= Placebo

4.2 Effect of Sicklo-N200 on Red Blood Cell Indices of Sickle Cell Anaemia Patients

The result of the effect of varied doses of Sicklo-N200 on RBC indices of SCA patients are presented in Table 3. From the results, it was shown that patients administered 2.5g of Sicklo-N200 and placebo did not show any significant ($P<0.05$) increase in the RBC count throughout the period of treatment. However, patients administered 5g of Sicklo-N200 showed significant ($P<0.05$) increase from the 8^{th} to the 12^{th} week of treatment.

The treatment didn't induce a significant ($P<0.05$) increase in Hb and PCV values throughout the period of treatment. In the contrary MCV, MCH and MCHC was significantly ($p<0.05$) decreased in all the dose groups starting at the 8^{th} week when compared to baseline. Effect of the various doses of Sicklo-N200 induced a decrease in the RDW value during the period of treatment but it was not significant ($P>0.05$).

Table 3: Effect of Sicklo-N200 on Red Blood Cell Indices of Sickle Cell Anaemia Patients.

Test	Dose(g)	Week 0	4	8	12
RBC(10^{12}/L)	PLA	3.05±0.181	2.97±0.131	3.73±0.186	3.93±0.195
	2.5	2.92±0.166	2.82±0.156	3.44±0.219	3.45±0.147
	5.0	2.77±0.112[a]	2.81±0.097	3.76±0.154[b]	3.77±0.156[b]
Hb(g/dL)	PLA	8.57±0.404	8.09±0.285	8.08±0.292	8.24±0.287
	2.5	7.93±0.315	7.74±0.288	7.42±0.358	7.58±0.216
	5.0	7.75±0.214	7.81±0.172	7.97±0.193	7.64±0.239
PCV(%)	PLA	23.03±1.103	21.99±0.786	24.13±0.881	25.26±0.897
	2.5	21.18±0.158	20.99±0.814	22.04±1.103	23.33±0.667
	5.0	20.75±0.595	21.04±0.513	23.88±0.593	23.88±0.694
MCV(fL)	PLA	76.93±1.133[a]	74.90±0.981	65.90±1.076[b]	65.49±1.161[b]
	2.5	74.09±1.138[a]	76.35±1.500	65.82±1.096[b]	67.61±1.182
	5.0	76.25±1.209[a]	75.91±1.288	64.77±1.342[b]	64.58±1.329[b]
MCH(pg)	PLA	28.70±0.439[a]	27.51±0.324	22.08±0.382[b]	21.40±0.450[b]
	2.5	27.86±0.510[a]	28.24±0.667	22.24±0.425[b]	22.36±0.439[b]
	5.0	28.53±0.607[a]	28.26±0.543	21.69±0.485[b]	20.70±0.479[b]
MCHC(g/L)	PLA	372.92±1.490[a]	367.67±1.494	334.75±1.044[b]	326.18±1.370[b]
	2.5	375.69±1.462[a]	369.36±2.143	337.31±1.515[b]	330.46±1.343[b]
	5.0	373.80±2.242[a]	372.08±3.058	334.70±0.842[b]	319.50±1.788[b]
RDW(%)	PLA	16.54±0.202	15.76±0.193	15.86±0.175	15.74±0.159
	2.5	16.59±0.267	16.39±0.172	16.25±0.133	16.07±0.219
	5.0	16.78±0.237	16.35±0.242	16.04±0.248	16.10±0.262

Values are presented as mean ± SEM. Mean value with different superscript letters in rows are significantly different ($P<0.05$). PLA= Placebo, RBC= Red blood cell count, Hb= haemoglobin, PCV= Packed Cell Volume, MCV= Mean Corpuscular Volume, MCH= Mean Corpuscular Haemoglobin, MCHC= Mean Corpuscular Haemoglobin Concentration, RDW= RBC Distribution Width

4.3 Effect of Sicklo-N200 on White blood cells of Sickle Cell Anaemia Patients

Effect of different doses of Sicklo-N200 on white blood cells of SCA patients is presented on Table 4. A non-significant ($P>0.05$) decrease in WBCs count was observed in all the groups.

Table 4: Effect of Sicklo-N200 on White Blood Cells of Sickle Cell Anaemia Patients.

Test	Dose(g)	Week 0	4	8	12
	PLA	18.93±1.207	17.47±1.246	14.43±0.902	16.11±1.057
WBC(10^9/L)	2.5	14.65±0.627	14.60±0.949	12.92±0.767	11.96±0.472
	5.0	17.24±1.282	15.51±1.254	16.27±1.084	14.05±0.571
	PLA	8.67±0.627	6.55±0.429	5.77±2.43	5.81±0.481
LYM(10^9/L)	2.5	6.06±0.301	7.38±0.713	5.25±0.296	4.57±0.268
	5.0	7.14±0.557	7.37±0.642	6.06±0.308	5.87±0.343
	PLA	9.24±0.489	9.43±0.671	7.32±0.556	8.89±0.505
GRA(10^9/L)	2.5	7.54±0.390	6.02±0.369	6.58±0.643	6.37±0.333
	5.0	8.76±0.758	6.86±0.543	8.91±0.829	7.63±0.387
	PLA	1.41±0.092	1.49±0.087	1.34±0.082	1.41±0.098
MID(10^9/L)	2.5	1.10±0.075	1.20±0.081	1.09±0.083	1.01±0.049
	5.0	1.35±0.092	1.32±0.085	1.30±0.045	1.14±0.070

Values are presented as mean ± SEM. Mean value with different superscript letters in rows are significantly different ($P<0.05$). PLA= Placebo, WBC= White Blood Cell Count, LYM= Lymphocytes, GRA= Granulocytes, MID= Monocytes

4.4 Effect of Sicklo-N200 on Platelet Indices of Sickle Cell Anaemia Patients

The results of effect of Sicklo-N200 on platelet indices of SCA patients are presented in Table 5. From the results, the platelet counts decreased though non significantly ($P>0.05$) after treatment. The observed PCT, MPV and PDW values were not significantly ($P>0.05$) increased or decreased during the period of treatment.

Table 5: Effect of Sicklo-N200 on Platelet Indices of Sickle Cell Anaemia Patients.

Test	Dose(g)	Week 0	4	8	12
	PLA	353.40±25.070	294.90±19.477	294.30±21.585	322.20±29.861
PLT(10^9/L)	2.5	405.00±24.077	437.79±49.430	356.23±28.979	366.85±28.582
	5.0	442.22±16.228	377.25±26.268	405.60±24.169	410.70±29.057
	PLA	0.29±0.017	0.26±0.015	0.26±0.017	0.26±0.024
PCT(%)	2.5	0.32±0.019	0.39±0.034	0.33±0.026	0.31±0.024
	5.0	0.42±0.035	0.30±0.021	0.35±0.021	0.34±0.021
	PLA	8.43±0.171	8.96±0.150	8.71±0.170	8.08±0.197
MPV(fl)	2.5	7.97±0.134	8.20±0.136	8.49±0.139	8.26±0.141
	5.0	8.11±0.124	7.97±1.176	8.62±0.192	8.27±0.171
	PLA	14.71±0.394	14.91±0.430	14.26±0.254	14.51±0.383
PDW(%)	2.5	13.99±0.330	13.96±0.240	14.53±0.188	14.05±0.317
	5.0	12.93±0.218	13.64±0.247	14.01±0.334	13.87±0.321

Values are presented as mean ± SEM. Mean value with different superscript letters in rows are significantly different ($P<0.05$). PLA= Placebo, PLT= Platelet Count. PCT= Plateletcrit. MPV= Mean Platelet Volume. PDW= Platelet Distribution Width.

4.5 Effect of Sicklo-N200 on Serum Vitamin A, C and E of Sickle Cell Anaemia patients

The effect of Sicklo-N200 on serum vitamin A, C and E are presented in Table 6. The values of serum vitamin A observed every 4 weeks through the period of treatment were not consistently increasing or decreasing and they were not significant ($P>0.05$). Serum vitamin C and E levels increased significantly ($P<0.05$) in Sicklo-N200 treated groups at the 8^{th} week when compared to baseline (0 week).

Table 6: Effect of Sicklo-N200 on Serum Vitamin A, C and E of Sickle Cell Anaemia Patients.

Test	Dose(g)	Week 0	4	8	12
Vitamin A (µmol/L)	PLA	1.01 ± 0.174	1.62 ± 0.178	1.76 ± 0.172	1.45 ± 0.080
	2.5	1.90 ± 0.179	1.60 ± 0.178	1.76 ± 0.136	1.39 ± 0.111
	5.0	2.15 ±0 .354	1.16 ± 0.096	1.72 ± 0.094	1.77 ± 0.132
Vitamin C (mg/dl)	PLA	0.30 ± 0.014	0.18 ± 0.016	0.25 ± 0.030	0.32 ± 0.031
	2.5	0.23 ± 0.007[a]	0.18 ± 0.010	0.62 ± 0.038[b]	0.50 ± 0.031[b]
	5.0	0.27 ± 0.012[a]	0.19 ± 0.014	0.44 ± 0.030[b]	0.37 ± 0.031
Vitamin E (mg/dl)	PLA	0.92 ± 0.030	0.87 ± 0.026	0.78 ± 0.019	0.93 ± 0.010
	2.5	1.05 ± 0.078[a]	1.15 ± 0.035	2.06 ± 0.106[b]	2.15 ± 0.089[b]
	5.0	0.85 ± 0.040[a]	1.37 ± 0.078[b]	2.23 ± 0.024[b]	2.18 ± 0.038[b]

Values are presented as mean ± SEM. Mean value with different superscript letters in rows are significantly different ($P<0.05$). PLA= Placebo.

CHAPTER FIVE

5.0 Discussion, Conclusion and Recommendations

5.1 Discussion

Treatment with 2.5 g and 5.0 g of Sicklo-N200 induced an increase in foetal Hb during the period of treatment. The change was not significant ($P>0.05$) at the 4^{th} week but was found to be significant ($P<0.05$) at the 8^{th} and 12^{th} week when compared to baseline (0^{th} week). When level of HbF is high, they are uniformly dispersed within the red cell and retard the sickling process. Thus, coinherithance of sickle haemoglobin with hereditary persistence of foetal haemoglobin (HPFH) is associated with mild disease (Lal and Vinchinsky, 2011). Increased HbF production observed could be as a result of the inhibition of beta globin gene and stimulation of gamma globin chain synthesis by the short chain fatty acids which are metabolic products of anaerobic bacterial fermentation of dietary fibre. The level of HbF of patients that were given the placebo decreased non significantly ($P>0.05$) after treatment. Goldberg *et al*., (1990) reported an increase in the level of HbF and a reduction of the rate of haemolysis and intracellular polymerisation of HbS in patients with SCA when patients were treated with a combination of nutritional source of short chain fatty acids and hydroxyurea for 6 months. Significant higher levels of HbF was detected in sickle cell anaemia patients treated with hydroxyruea (HU) (Pallis *et al*., 2014; Wang *et al*., 2001) where the efficacy of HU to induce HbF synthesis was confirmed.

Samuel *et al*., (2019) reported that hydroxyurea increases the level of HbF in patients with SCA. Similarly, Sher *et al*., (1995) reported an increase in HbF in SCA patients treated with an infusion of arginine butyrate. Zimmerman *et al*., (2004) reports that an

increase in the level of HbF in sufficient quantities can ameliorate sickle cell disorder. HbF contains gamma globin chains instead of beta chains. Hence, it is not affected by the genetic defect that causes SCA. Increased levels of HbF decrease the tendency towards intracellular polymerisation of sickle Hb that characterizes the disease (Rohit *et al*., 2014) thereby reducing the rate of sickling. In this research, supplementation with Sicklo-N200 showed a similar increase with that observed with HU treatment which increases HbF and decreased platelets (Covas *et al*., 2004).

Treatment with both doses of Sicklo-N200 improved Fe^{2+}/Fe^{3+} ratio significantly ($P<0.05$) at 8 and 12 weeks, but the increase at 4 weeks was non significant ($P>0.05$) as well as the group that was given placebo had no siginificant ($P>0.05$) effect on Fe^{2+}/Fe^{3+} ratio. The rate of haemoglobin polymerisation of the group administered 2.5 g of Sicklo-N200 reduced though non-significantly up to 8 weeks, but was found to be significantly ($P<0.05$) reduced at the 12^{th} week when compared to the haemoglobin polymerisation rate at the 0^{th} week. The 5.0 g group was significantly ($P<0.05$) reduced starting from the 8^{th} week to the 12^{th} week. However, the group that was given placebo had no significant effect on haemoglobin polymerisation rate.

This is in line with the findings of Goldberg *et al*., (1992) where treatment with hydroxyurea resulted in increased oxygen affinity and decrease in haemoglobin polymerisation rate of Hb. Nwaoguikpe (2009); in an in vitro study reported that extracts of onion, garlic and *Telferia occidentalis* (ugu) exhibited high level potency in inhibiting sickle cell haemoglobin polymerisation, improvement in Fe^{2+}/Fe^{3+} ratio, and providing the SCA patients with adequate nutrients and phytochemicals for a stable healthy status. Sickling of red cells occur as a result of polymerisation of de-oxygenated HbS molecules that become stacked linearly in the vasculature. The ability of the sickle erythrocytes to revert to their normal morphology is due to

increased oxygen affinity. In this study, reduction in haemoglobin polymerisation rate over time stablilzes the erythtocyte by reducing the fragility of the red cells thereby reducing haemolysis. Sicklo-N200 was able to improve the Fe^{2+}/Fe^{3+} ratio, hence facilitating the conversion of methaemoglobin to haemoglobin and increasing the oxygen affinity of sickle cell haemoglobin which enables sickle erythrocytes to revert back to their normal morphology i.e. improving the oxidant status of sickle cell erythrocytes. This may be attributed to the vitamins in Sicklo-N200 which might have raised the minimum gelling concentration of HbSS such that the protein does not polymerise at physiological conditions.

There was a non significant ($P>0.05$) increase in RBC levels of the group that was administered 5 g of Sicklo-N200 at 4 weeks, extending the treatment to 8 and 12 weeks resulted in a significant ($P<0.05$) increase. The increase was non significant ($P>0.05$) in the groups that were administered 2.5 g of Sicklo-N200 and placebo. The RBC levels in this study is similar to the finding of Rukayya *et al*., (2018) who reported elevated level in RBC count in patients given camel milk for 6 weeks. The haemolysis that occurs as a result of rapid destruction of red blood cells in homozygous SCA patients accounts for the reduction of the erythrocyte count.

An insignificant ($P>0.05$) decrease in Hb level was observed in the group that was given 2.5 g Sicklo-N200 and placebo while an insignificant ($P>0.05$) increase was observed in the 5.0 g group. This result indicates that Sicklo-N200 does not have effect on haemoglobin level. The PCV levels of the patients administered 2.5 g and 5 g of Sicklo-N200 increased with an increase in the number of days of treatment. However, the increase was found to be non significant ($P>0.05$) within the duration of the research of 12 weeks. Patients that were given the placebo did not show any

significant increase in PCV. The non significant increase observed could be attributed to the period of exposure and to the choice of antisickling factors in Sicklo-N200.

The values of MCV, MCH and MCHC in all the experimental groups were observed to decrease significantly ($P<0.05$) after 8 weeks of treatment. Decrease in MCHC decreases the rate and degree of sikcling of red cells (Beutler, 2006). The values of MCV, MCH and MCHC in HbAA individuals are lower when compared to HbSS patients (Samuel *et al*., 2015; Charles *et al*., 2018). α-Thalassemia reduces MCHC and erythrocyte density thereby reducing the tendency of deoxy-HbS to polymerise (De Ceulaer *et al*., 1983). MCH mirrors MCV results; small red cells would have a lower value of MCH. The values of RDW decreased in all the groups as the period of treatment is extended though non significantly ($P>0.05$), low values indicate uniformity in size of RBCs.

Patients with SCA are known to have significantly higher mean total WBC and differential count than people with AA genotype (Omoti, 2005). This could be as a result of generation of a covert inflammatory response leading to the relaese of cytokine mediators, one of whose main function is increased neutrophils production by the bone marrow (Omoti, 2005). Treatment with 2.5 g and 5 g of Sicklo-N200 resulted in decrease of WBC counts from 14.65±0.627 to 11.96±0.472 and 17.24±1.282 to 14.05±0.571 respectively. The number of granulocytes of the groups that were administered 5 g and 2.5 g of Sicklo-N200 was 8.76±0.758 and 7.54±0.390 before the treatment and decreased to 7.63±0.387 and 6.37±0.333 respectively after completing the treatment which fall within the normal range ($2.00\text{-}7.80\times10^9$/L). The number of lymphocytes also decreased in both groups that received Sicklo-N200, though not to the normal range ($0.60\text{-}4.10\times10^9$/L).

Results from the study conducted by Okpala, (2004) and Charles *et al*., (2018) revealed that as the disease progresses from mild to severe with hemolysis, patients have higher counts of WBC and PLTs. This increase could be because of some oxidative stress (Al-Basheer *et al*., 2015). The decrease in the levels of WBC differential observed in treated groups is an indication of an amelioration of the disesase. Another study among SCA children in Nigeria reported that leukocytosis and neutrophilia are related to disease severity (Ademola and Kuti, 2013). Hydroxyurea was reported to lower WBC count and thus improves the clinical outcome of SCA patients (Zimmerman *et al*., 2004). Similarly, the cytoreductive effect of HU was confirmed by the study of Pallis *et al*., (2014) which found an association between HU therapy and significant reductions in leukocyte, eosinophil, and monocyte counts. This effect can improve the inflammatory profile generally observed in SCA patients, which is mainly due to increases in eosinophil and monocyte counts.

The moderate leucocytosis observed in this study is in line with earlier studies which is a common feature of SCA and was thought to be due to chronic pain resulting in the redistribution of granulocytes from marginal pool to the circulating pool (Ahmed *et al*, 2006; Salawu *et al*., 2009; Akinbami *et al*., 2012). Those involved with the management of SCA patients should be aware of this variability to avoid confusing the disease with infection which leads to leucocytosis.

The normal range for platelets is 95-450 cells × 10^9/L, for MPV is 7.0-11.0 fl, for PDW is 10.0-18.0% and for PCT is 0.1-0.5% and all the values obtained before, during and after supplementation are within the normal range. The platelets and plateletcrit levels decreased non significantly in all the groups and a slight increase in the values of MPV and PDW was also observed in the groups administered both doses

of Sicklo-N200. The placebo group showed a non significant decrease in the values of the MPV and PDW after 12 weeks of treatment. This result shows that Sicklo-N200 has no effect on platelet indices which react to bleeding from blood vessel injury by clumping, thereby initiating a blood clot.

A significant increase in the level of vitamin C was observed in the groups treated with Sicklo-N200 after taking the supplementation for 8 weeks, but the significant increase extended to the 12^{th} week in the 2.5 g group. The level of vitamin E increased significantly in both 2.5 g and 5 g treated groups throughout the period of treatment. Sickle cell anemia patients have high levels of oxidative stress markers and low levels of antioxidant capacity (Kaddam *et al.,* 2017). Aminu *et al*., (2018) reported a significant increase in serum vitamin C and E after supplementation with camel milk. The changes in the values of serum vitamin A throughout the period of treatment were not consistently increasing or decreasing. Serum retinol is the most commonly used indicator of vitamin A status (Jared *et al*., 2004). However, retinol is unstable when exposed to heat or light which may account for the inconsistency observed. The antioxidant vitamins, (A, C and E) were found to be potent inhibitors of sickle cell haemoglobin polymerisation, and equally improved the oxidant status of sickle erythrocytes (Nwaoguikpe and Braide, 2012). The increase in the levels of serum vitamins C and E in the groups could be attributed to their diet and Sicklo-N200 which is rich in vitamins, minerals, fiber and polyphenols. It has been suggested that an increased intake of synthetic or natural forms of these vitamins could help reduce sickle cell anaemic crisis (Ayyub *et al*., 2003). Ohnishi *et al*., (2000) suggested that antioxidant vitamins should be given to sickle cell patients as a cocktail rather than individually. Sicklo-N200 also provided the huge energy demands of the bone marrow for RBC production which competes with the demands of a growing body.

5.2 Conclusion

Sicklo-N200 exhibited high level potency in increasing HbF level, inhibiting sickle cell haemoglobin polymerization, increasing Fe^{2+}/Fe^{3+} ratio, improving haematological parameters, increasing serum antioxidant vitamin levels and providing the SCA patients with adequate nutrients and phytochemicals for a stable health. The nutritional approach to the management of sickle cell disease is novel and remains the current and the most promising approach in the management of sickle cell anaemia. Potential anti sickling agents constituted from a mixture of food materials that interfere with the HbS polymerization level and the red cell membrane level may provide effective therapies for SCA. This study indicated that Sicklo-N200 is very beneficial in the management of sickle cell anaemia.

5.3 Recommendations

1. Molecular studies should be carried out to determine the expression level of gamma globin genes (HBG1 and HBG2) during the administration of Sicklo-N200 to SCA patients.

2. It may be worthwhile to further study the effect of Sicklo-N200 on antioxidant minerals and lactate dehydrogenase activity.

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Appendices

Appendix I

Preparation of Samples and Reagents

1.1 Lysing of blood sample

The red cells were washed 3 times in 0.9% saline and then lysed by adding to the packed cells 2 volumes of distilled water and 1 volume of carbon tetrachloride (CCl_4). The mixture was shook and centrifuged at 3000 rpm for 30 minutes. The clear haemoglobin solution was then pipetted.

1.2 Drabkin's solution

Drabkin's solution was prepared by dissolving 200mg of potassium fericyanide and 200mg potassium cyanide in distilled water. The solution was transferred into a volumetric flask and made up to 1 liter with distilled water

1.3 Saturated ammonium sulphate solution

Saturated ammonium sulphate solution was prepared by weighing 706g of ammonium sulphate in 1liter of distilled water. The solution was heated to dissolve and allowed to cool down to room temperature

1.4 6% Metaphosphoric acid

6% metaphosphoric acid was prepared by dissolving 6g of metaphosphoricacid in distilled water and the volume made up to 100 ml.

1.5 2% sodium metabisulphite

2% sodium metabisulphite was prepared by dissolving 2g of sodium metabisulphite in distilled water and the volume made up to 100 ml.

1.6 NaOH 1.2 M

4.8g of sodium hydroxide was dissolved in 100 ml distilled water.

Appendix II

List of Equipment

Equipment	Model	Manufacturer/Country
Automated blood cell counter	PCE-210E, V5.40	England
Centrifuge	800D	Kendo Lab, USA
Refrigerator	C1202	Thermocool Ltd.
Micropipette	Diapette	Teco diagnostic, USA
Spectrophotometer	SP 300	Optima, Germany
	A23615 DU	Japan
Water bath	GD 100	Grant Scientific Tech. Germany
Weighing balance	PC 44400	Mettler, England
Freeze dryer	BK-FD12PT	Japan
Oven	FN 055	India
Electric blender	MX 900	Japan

List of Chemicals/Reagents

Chemicals	Manufacturer	Grade
Ethanol	Hopkins and Williams	Analytical
α-Tocopherol	Lab Tech., India	Analytical
αα-Dipyridyl	BDH chemical, UK	Analytical
Xylene	BDH chemicals, UK	Analytical
Ammonium sulphate	May and Baker Ltd., England	Analytical
Sodium hydroxide	Loba chemie, India	Analytical
Sulphuric acid	BDH chemicals, UK	Analytical
Carbon tetrachloride	May and Baker Ltd.,England	Analytical
Potassium ferricyanide	BDH,UK	Analytical
Sodium metabisulfite	May and Baker Ltd., England	Analytical
Potassium cyanide	Loba chemie, India	Analytical
Potassium hydroxide	May and Baker Ltd., England	Analytical
Ferric chloride	Hopkins and Williams, England	Analytical
Copper sulphate	Sigma chemicals, USA	Analytical
Metaphosphoric acid	Sigma chemicals, USA	Analytical
2,4-dinitrophenylhydrazine	Sigma chemicals, USA	Analytical

List of Glass wares

Glass wares	Specification	Country
Beakers	Glass	England
Specimen bottles	Glass	England
Conical flasks	Glass	England
Test tubes	Glass	England
Measuring cylinder	Glass	England

Appendix III

USMANU DANFODIYO UNIVERSITY, SOKOTO

Consent to participate in a research study

Title of study: Effect of Sicklo-N200 in the Management of Sickle Cell Anaemia

Researcher:

Name: Sumayya A. Ayuba

Dept.: Biochemistry

Phone No.: 07039354675

Introduction

- You are being asked to participate in a research study "Effect of Sicklo-N200 in the Management of Sickle Cell Anaemia (SCA)".
- You were selected as a possible participant because you happen to be attending sickle cell anaemia clinic, Usmanu Danfodiyo University Teaching Hospital (UDUTH) Sokoto, Nigeria.
- I ask that you read this form and ask any question that you may have before agreeing to participate in this study.

Purpose of the study

- The purpose of this research is to find a nutritive therapy for the management of SCA using carrot, cabbage and honey which are cheap and readily available.

Description of the study procedures

- If you agree to participate in this research, you will be asked to do the following things: take the drug (suspension) once daily for 12 weeks in addition to your routine drugs and your blood sample will be taken for laboratory analysis.

Risks/discomforts of being in this study

- There is no foreseeable risk associated with this research study.

Confidentiality

- The records of this study will be kept strictly confidential.

- Your identity will not be disclosed at the presentation of this research work to the department.

Right to Refuse or Withdraw

- The decision to participate in this research is entirely up to you. You may refuse not to take part in the study at any time without affecting your relationship with the researcher.

Right to Ask Questions and Report Concerns

- You have the right to ask questions about this research and to have those questions answered by me before, during or after the research. If you have any further question about the study at any time, feel free to contact me on 07039354675.

Consent

- Your signature below indicates that you have decided to volunteer as a research participant for this study, and that you have read and understood the information provided above.

Name of participant:_____

Signature of participant/parent or guardian:_____Date:_____