PHYTOCHEMISTRY AND ANTISICKLING EFFECTS OF LEAF AND STEM EXTRACTS OF *Costus afer*

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CERTIFICATION

This is to certify that this thesis entitled PHYTOCHEMISTRY AND **ANTISICKLING EFFECTS OF LEAF AND STEM EXTRACTS OF** *Costus Afer* was carried out by AMAEFULA BLESSING EZINNE (Reg No 20184138939) of the Department of Biochemistry, Federal University of Technology, Owerri Imo State

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ABSTRACT

The antisickling effect of the stem and leaf extracts of *Costus afer* was investigated to determine and certain effects. This work was approached by partitioning the extracts of *Costus afer* stem and leaf into fat-soluble (SFSF and LFSF), ethanol-soluble fraction (SESF and LESF), and methanol-soluble fraction (SMSF and LMSF). The activities of these extracts were compared with the activities of other nutrients which have been approved and documented. The phytochemical screening was qualitatively and quantitatively determined using standard GC-MS methods. The result shows the presence of twenty (20) different phytochemicals with a wide range of functions. The most abundant phytochemical in the samples is Isolupanine with the values; 31.380 µg/ml in leaf and 29.396 µg/ml in the stem. The amino acid profiles of the extracts revealed the presence of Phenylalanine, arginine, lysine, serine, glutamic acid, methionine, and others. Glutamic acid has the highest concentration with the following values; 10.14g/100g protein and 11.65g/100g protein in leaf and stem extracts respectively. The Haemoglobin polymerization inhibition and the relative percent inhibitions were estimated. The leaf methanol soluble (LMSF) has the highest percent inhibition of 81.90 ± 0.00^{g} on HbSS polymerization. While the lowest percent inhibition on polymerization of 22.78 ± 0.00^{b} was observed in SFSF. Amongst all the fractions of the samples, LESF was able to exhibit the highest percent improvement in the Fe^{2+}/Fe^{3+}, 282.94 ± 0.00^{g}. The total free amino acid values range from 432.55 ± 2.05^{a} mg/50g in the LMSF to $1,188.00 \pm 2.20^{e}$ mg/50g in the LFSF. The total vitamin C concentration of the samples expressed in mg/1 is; 297.59 ± 33.26 in the stem and 566.15± 87.10 for the leaf. Based on the free amino acid, the vitamin C concentrations, the inhibition of HbSS polymerization, and the improvement in the Fe^{2+}/Fe^{3+} ratio, the leaf and stem extracts would undoubtedly provide adequate nutritional and antisickling efficacy required for the management of sickle cell disease.

Keywords: Antisickling agent, *Costus afer*, polymerization, inhibition, extract, sickle cell disease.

CHAPTER ONE

INTRODUCTION

1.0 BACKGROUND OF STUDY

Abnormal haemoglobin, often known as haemoglobin S (HbS), is a hallmark of sickle cell disease (SCD), a hereditary blood disorder. The variety of illnesses that occur depends on the distribution of different haemoglobin genes; nonetheless, homozygous sickle cell (SS) disease is the most common genotype at birth in most countries. Serjeant (2013) suggested that this genotype frequently displays greater mortality in his study "Natural History of Sickle Cell Disease". This implies that the proportional number of sickle cell genotypes is influenced by both the age of individuals and the geographic distribution of particular genes. This illness affects millions of people worldwide, especially those with African ancestry (Aidoo *et al*., 2002). The study of defective haemoglobin and the methods by which the beta-6-glutamic acid in the gene producing the human beta-globin component is replaced by valine led to the development of molecular biology of the disease. When deoxygenated, this alteration significantly reduces the solubility of sickle cell haemoglobin (Hb S). Valine takes the role of glutamic acid when adenine is changed to thymine. In these circumstances, the biconcave disc-shaped erythrocyte deforms into a sickle cell as a result of the HbS molecules polymerizing to create intracellular fibers. The faulty sickle cell gene is inherited from one parent, and the gene for normal hemoglobin Hb A is inherited from the other parent, which results in the development of the sickle cell trait (Imaga, 2013). Falciparum malaria is less contagious, parasite numbers are lower, and mortality rates are lower in those with the sickle cell trait. They also have a relative resistance to the illness. The window in early childhood between the loss of passively acquired maternal immunity and the development of active immunity is when this survival advantage is most noticeable, but the length and timing of this window may differ between communities and be influenced by the pattern of malarial transmission (Serjeant, 2013). In their study, Serjeant (2013) noted that symptoms appear as -chain synthesis, which at birth produces 60% to 80% of the fetal hemoglobin (Hb F), is replaced by β-chain synthesis, resulting in rising amounts of the aberrant Hb S. Different people transition through this stage at different rates, but for some people, pathological levels of Hb S may be achieved by 8 to 10 weeks of birth, and issues that could be fatal may start to develop at that point. Dactylitis and mortality are two examples of the disease's early consequences, the study further demonstrates. These are brought on by the newborn's acute chest syndrome and abnormally poor splenic function, which makes them vulnerable to both acute splenic sequestration and severe septicemia. The complicated pathophysiology of sickle cell disease, which can include elements from infection, infarction, fat embolism, and pulmonary sequestration, has led to the

term "acute chest syndrome" becoming extensively used in the context of the condition. In such circumstances, incentive spirometry may be used to prevent this condition from developing. This syndrome may also follow hypoventilation that is linked to the pleuritic discomfort of avascular necrosis of the ribs or sternum. One of the most frequent causes of death in people over the age of two is acute chest syndrome (Serjeant, 2013). According to Balkaran *et al.* (1992), stroke incidence is 8% by the age of 14 and has a median age of onset of 6 years, with some occurrences occurring as early as the second year of life. Infarction of the brain caused by stenosis of significant cerebral arteries, especially those as large as the internal carotid artery, is the main pathology at this age. Small vessel occlusion, which distinguishes other illness presentations, is not the pathology's primary mechanism, which is poorly known. Chronic transfusion programs can stop many recurrent stroke episodes within three years of the initial event, according to research by Sarnaik *et al*. (1979) and Pegelow *et al*. (1995). Around this time, hypersplenism also begins to be evident. The disorder is distinguished by persistent splenic enlargement, significant red cell sequestration, significant bone marrow expansion, and a novel hematological equilibrium. Adolescence is the peak of clinical challenges, but it also marks the time when many patients are lost to follow-up after moving from pediatric to adult care. In addition to nocturnal enuresis, avascular necrosis of the femoral head, leg ulceration in some populations, priapism, and problems related to delayed growth and puberty, pregnancy, and the need for effective contraception, these difficulties also include an increased frequency of bone pain crises. In a Jamaican cohort research, Readett *et al.* (1990) discovered that by the age of 8 years, 45 % of children suffered bedwetting more frequently than or equivalent to twice weekly, as opposed to 19 % of normal (AA) controls. In addition, Serjeant (2013) noted that although nocturnal enuresis is not life threatening, it may have a significant impact on behavior and social functioning. Enuresis always goes away eventually, however in some people, it can last up to age 18 or 20. Due of the obvious stress brought on the patient and family members, the negative effects are more social and psychological than medical. The avascular necrosis of the bone marrow commonly causes the bone pain crisis, the adult form of dactylitis. Although the bilateral, symmetrical involvement and the ubiquitous precipitation by skin cooling make the vaso-occlusive crisis (VOC), which is typically thought to be caused by vaso-occlusion, difficult to explain. According to (Serjeant, 2013), bone pain crises peak in adolescence and early adulthood, especially in males, and subsequently decrease in severity and frequency beyond the age of 25 to 30. Increased haemoglobin levels and low HbF levels constitute triggering factors, whereas stress, infections, and exposure to the cold through the third trimester of pregnancy are risk factors. A second rise in the prevalence of stroke happens at age 25, this time largely hemorrhagic and with a bad prognosis. These include

subarachnoid hemorrhage, which is commonly caused by berry aneurysms, direct intracerebral hemorrhage, and intraventricular hemorrhage. When pulmonary fibrosis progresses, it may be accompanied by pulmonary hypertension, which further compromises the heart's ability to pump blood. Acute chest syndrome is an important clinical issue. It is notable that pulmonary hypertension, persistent hypertrophy, and intraventricular fibrosis all decrease the compliance of the left ventricular wall, congestive heart failure becomes more common. Declining renal function, which is accompanied by glomerular fibrosis that worsens with time and is connected with a fall in GFR, a reduction in erythropoietin levels, and a persistent decline in total hemoglobin, is a substantial cause of morbidity after the age of 40 (Morris *et al*., 1991). Patients with renal problems are often clinically silent, but the gradually declining haemoglobin level compromises heart function. Haemoglobin levels generally fall around the age of 40, further impairing heart function.

Many factors, both genetic and environmental, are likely to influence illness manifestation and outcome. High levels of Hb F, which are present in almost all patients from birth, preclude the vaso-occlusive and hemolytic characteristics of the disease in the first few months (Serjeant, 2013). Patients who maintain higher amounts of Hb F have less hemolysis and more moderate clinical courses, albeit the level of Hb F that persists varies widely between individuals and geographical locations. It is known that heterozygous α^{+}-thalassemia, a condition in which one of two closely related α-thalassemia genes is deleted, lowers intravascular sickling because mean cellular haemoglobin concentration (MCHC) is a factor in Hb S polymerization (Seakins *et al*., 1973). Any subsequent reduction in sickling may be counterbalanced by an increase in total haemoglobin. Thus, thalassemia may offer an intriguing paradigm to discriminate between the detrimental consequences of a higher hematocrit impeding blood flow in bigger vessels and the effects of suppressing sickling in tiny blood vessels. There is no denying the fact that environmental factors, such as climatic factors, skin cooling crises that produce bone pain, or seemingly unrelated events like contact with parvovirus B19 or *Streptococcus pneumoniae*, have a key impact in how sickle cell disease develops. In areas plagued by the disease, such as sub-Saharan Africa and central India, malaria continues to be a significant factor in determining morbidity and mortality. *Salmonella species* transmission rates in the general population may be reflected in susceptibility to other potentially dangerous illnesses. Nutrition, availability to public health measures like immunization, and socioeconomic status which may affect how easy the families can communicate, travel, and receive medical care are additional critical considerations. The significance of environmental factors and their underlying mechanisms has immense promise to enhance our understanding of and options for therapy for sickle cell disease.

The prevalence of malaria and other diseases, as well as the accessibility and complexity of healthcare and other services, greatly impact the causes of death. In comparison to the general population with African SS disease in the United States, where malaria is absent, the median survival is decreased by at least 20 years even in the most sophisticated conditions (Serjeant, 2013). According to Wright *et al*. (1997), at older ages, the selection of penicillin-resistant and/or non-vaccinated *Streptococcus pneumoniae* serotypes, as well as the emergence of *Salmonella* as a significant bacterium, may be altering the pattern of septicemias, which continue to be a major cause of death from acute chest syndrome. According to Serjeant (2013), determining the cause of death is critical to developing measures to prevent the causative pathologies, and a lack of available data, blood and virus cultures, and autopsy are important hurdles in Africa, Arabia, and India. However, Steinberg *et al*. (2003) and Bakanay *et al*. (2005) observed that in Jamaica, even with autopsy rates as high as 90%, comprehensive histology and blood cultures sometimes fail to detect a credible cause of death. Furthermore, the effects of modern long-term medications such as hydroxyurea and chronic transfusion programs on survival must be studied, and the difficulty in interpreting results is highlighted by the most recent data on mortality and hydroxyurea.

Indigenous traditional healers have been using a variety of plant medicines to manage sickle cell anaemia. There are various substances, including amino acids, particularly phenylalanine, that prevent sickling by altering the erythrocyte membrane, resulting in an increase in erythrocyte cell volume and thereby lowering intracellular haemoglobin content. Antioxidants are also significant dietary elements that aid in the management of sickle cell anaemia. Vitamin C and vitamin E levels are low in sickle cell patients (Imaga, 2013). The most widely used method for preventing or reversing sickling in vitro and in vivo is to use drugs or procedures that directly bind covalently to the haemoglobin molecule, bind non-covalently to this molecule, and impact the cell membrane. Many well-known antisickling medications, including potassium cyanate, have a relatively high level of toxicity. Furthermore, it is commonly accepted that none of the currently available treatments for sickle cell anaemia consistently produce positive results over extended times. Therefore, it would be extremely desirable to offer an active agent that is efficient in preventing sickling of erythrocytes in sickle cell patients while avoiding the toxicity associated with several prior art therapeutic medications. The focus of traditional or synthetic medications designed to date for the management of sickle cell disease is on symptomatic pain reduction and crisis management. Alternative therapy involving the use of phytomedicines has proven not only to reduce crisis but also reverse sickling. Some of these plants, according to healers, have had clinical successes.

One of the 150 species of sturdy, perennial, and rhizomatous plants of the genus *Costus* is the plant *Costus afer*. According to Edeoga and Okoli (2000), it is present in the forest belts of Senegal, South Africa, Guinea, Niger, Sierra Leone, Ghana, Cameroon, and Nigeria. The plant is also known as bush cane or ginger lily. In the work of Oliver (1960), it is referred to as "Okpete" or "Okpoto" in Igboland, "Kakizawa" in Hausa, "tete-egun" in Yoruba, and "Mbritem" in Efik. It is known as "Monkey sugar cane" in Cameroon. Flowers on it are white and yellow. Ezejiofor *et al*. (2013) stated when the phytochemical analysis was done on the stem, seeds, leaves, and rhizomes after harvesting, it detected the presence of several bioactive metabolites. The use of medicinal plants in the production of contemporary drugs and for traditional therapeutic reasons in the provision of primary health care is significant. *Costus afer* is a common medicinal plant used for both conventional medicine and a variety of sociocultural purposes, such as wrapping local food, making mats, curing coughs, and providing food for little ruminant animals, measles, malaria, eye problems, kyphosis, and other illnesses. The main components of *Costus afer* being employed in the production of modern medications include steroid sapogenins, saponin aferosides A–C, and dioscin. The Fruit was mashed and utilized as a cough suppressant (Omohua, 2011). Research by Boison *et al*. (2019) stated that the leaf and stem extracts have antihyperglycemic activity and antinociceptive effects. Hepatoprotective effect against alcohol-induced liver cirrhosis and against carbon tetrachloride-induced hepatoxicity has also been reported (Ayakeme *et al*., 2014). The dried aerial section is used to treat hypertension and the stem is employed as an enema for hemorrhoids and worms, an infusion of the inflorescence is administered to alleviate digestive issues, and consuming the leaves which are still in the development phase is thought to offer the weak, dehydrating sufferer vigor (Omokhua, 2011). These corroborate the findings by Boison *et al*. (2019) that 95 % if not all the parts of *Costus afer* are used to improve health conditions like malaria, measles, diabetes mellitus, arthritis, and digestive disorders.

1.1 PROBLEM STATEMENT

Sickle cell disease is a common genetic disease in Africa and other continents of the world that affects the life expectancy of patients. Since its discovery by Herrick in 1910, no therapy has been proven with evidence to cure sickle cell disease without controversy, hence its prevalence. Medicine derived from plants in their original state which are known as phytomedicines have been under clinical research as remedy for sickle cell disease for decades. Some limited clinical trials have established the positive effects of phytomedicines both *in vivo* and *in vitro*. However, there has been little systematic appraisal of their benefits. In spite of the estimated increase in research for herbal remedies, progress has been slow. In some countries of Africa where advanced technologies are greatly lacking, use of phytomedicines in management of children with sickle cell disease has not been documented, despite a large presence of the sickle

cell disease patients (Lubega *et al*., 2021). This study focuses on the antisickling potential of leaves and stem of *Costus afer* as a phytomedical therapy against sickle cell disease. The availability, accessibility and low cost of *Costus afer* could be an effective and easier route in the use of phytomedicines against sickle disease.

1.2 AIM AND OBJECTIVES.

1.2.1 Aim of Study

The aim of this study is to investigate the antisickling potentials of stem and leaf extracts of *Costus afer*.

1.2.2 Objectives of study

1 Determine the qualitative and quantitative phytochemical profile of leaf and stem extracts of *Costus afer*

2 Determine the amino acid profile of stem and leaf extracts of *Costus afer*

3 Determine the free amino acid contents of stem and leaf extracts of *Costus afer*

4 Determine the ascorbic acid concentration of leaf and stem extracts of *Costus afer*

5 Determine the antisickling potentials of *Costus afer* stem and leaf extracts via;

i) Polymerization inhibition test

ii) Determination of Fe^{2+}/Fe^{3+} ratio

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Several polls have been carried out in response to ongoing research and studies on herbal remedies and pharmaceuticals for sickle cell disease. This was done through the important data gathering required for the primary research to be accomplished, in addition to providing information on a specific set of bioactive molecules that qualify herbal drugs as effective against sickle cell disease. Plant extracts are frequently used to treat sickle cell disease (SCD), which offers comfort when the condition is at its worst. It is clear that using the right blends of several plant extracts can reverse sickled erythrocytes. The use of a single plant, however, has not been documented. Sickle Cell Disease (SCD), Sickle Cell Anemia (SCA), and SCA/SCD Screening were the search terms used in the initial search of the databases Medline, PubMed Elsevier, and Google Scholar. The majority of these publications are full-length research projects or research articles. A summary of the prior research investigations gives a general idea of the research issue.

2.11 Sickle Cell Trait (SCT) and Sickle Cell Disease (SCD): an overview

Recently, Ashorobi *et al*. (2002) reaffirm that only a single abnormal haemoglobin beta gene allele is present in individuals with sickle cell traits (SCT), which is a harmless illness. This explains why they have improved quality of life, no vaso-oclusive crises, and a death rate that is comparable to the presence of two abnormal haemoglobin beta gene alleles in the affected person. The benign nature of the sickle cell trait means that it typically has no clinical repercussion. Patients with sickle cell trait do not experience crises, unlike sickle cell illness. Although they might have Hb SS in their family history, sickle cell trait patients may manifest symtoms during any crisis like those of persons with normal haemoglobin. They, in other words are usually asymptomatic. Sickle cell trait, however, can be linked to severe morbidity and death, according to (Tsaras *et al.,* 2009). Sickle cell trait is closely linked to renal medullary carcinoma, which is uncommon yet often lethal. As a result, sickle cell trait may not be entirely benign, and patients who experience any of these problems should be treated immediately. Sickle cell trait, unlike sickle cell illness, does not result in a vaso-occlusive crisis. If they are exposed to circumstances that encourage sickling, those with sickle cell trait could appear with the same symptoms as people with sickle cell anaemia. Severe hypoxia, dehydration, an increase in symptomatic outflow, hypothermia/hyperthermia, elevated 2, 3-DPG levels, and the discharge of inflammatory cells are among the conditions. Small capillaries, particularly those in the bones, will get blocked by sickled red blood cells as a result of sickle haemoglobin

(Hb SS). Other cells, such as platelets and inflammatory cells, work together to increase the adherence of red blood cells in addition to the sickling of the cells. The chest, heart, lunga, belly, kidneys, and extremities are just a few of the body organs where this could take place. Sickle cell trait and sickle cell disease are linked to a higher estimated glomerular filtration rate (eGFR) reduction in black individuals with a faster decline in sickle cell disease (Olaniran *et al.*, 2020).

Hb S, also referred to as sickle haemoglobin, is the cause of sickle cell trait. The point mutation on the beta globin chain replaces A with T at codon 6 of the beta haemoglobin chain resulting in valine. The valine-type haemoglobin makes red blood cells sickle when exposed to low oxygen levels. Hb S from one parent and Hb A from the other are heterozygous in sickle cell trait patients.

2.1.2 History of Sickle Cell Disease

In 1910, James Bryan Herrick, an American physician and clinical cardiologist worked on a special case of a black patient, Walter Noel, a student of Dentistry who had anaemia for three years. He observed the patient's blood and was able to describe red blood cells as crescent-shaped and this characterized the disease. However, Herrick was only able to report a fraction of the discovery. He was not sure at the time whether the blood condition was a disease or a manifestation of another disease. Regardless of his equivocal findings, Herrick invented the name "sickle-shaped" to describe the unusual morphology of this patient's RBC. However, in light of the patient's symptoms. An observer commented that the disease is a "great masquerader" in the 1950s. A historical perspective was given to this discovery when more details surrounding Herrick's report were uncovered by records from Chicago, Washington DC and Grenada (Savitt & Goldberg, 1989). Several comparable instances were recorded during the next 15 years, reinforcing the concept that this was a new disease entity and providing enough evidence for a preliminary clinical and pathological description. (Sydenstricker, 1924).

The study by Makani *et al*. (2011) explains that while the birth rate is high, about 250,000 children born in sub-Saharan Africa suffer sickle cell disease and Aygun and Odame (2012) stated that 50–80% of these infants die before the age of 5. A report from several studies confirms that even with a 20-30-year gap in overall survival rate compared to persons with no sign of SCD, babies have a chance to live to adulthood in nations where survival resources are easily available. (Telfer *et al*., 2007; Quinn *et al*., 2010; Elmariah *et al*., 2014; Gardner *et al*., 2016; Serjeant *et al*., 2018). The impact of sickle cell disease on the worldwide population was not recognized as substantial by the World Health Organization (WHO) until later in 2006, despite global statistics that show it to be the most serious public health concern among

hemoglobinopathies. In Africa at the outset of the disease, it was identified as "cold-season rheumatism" considering the symptoms associated with the condition, Piel *et al*. (2013) declared it to be genetically inheritable. Linus Pauling, the first to postulate in 1945 that the disease could be caused by a defect in the haemoglobin molecule, cited these fundamental investigations in his book "Molecular Disease and Evolution.". This hypothesis was supported in 1949 by the demonstration of sickle vs normal haemoglobin, movement differences using gel electrophoresis alongside a clarification of the inheritance pattern of this disease which is autosomal recessive. During the same period, Watson (1948) predicted the importance of fetal haemoglobin (Hb F) by suggesting that its presence could explain the longer period required for sickling of newborn RBC compared to those from women with "sicklemia." Vernon Ingram and colleagues revealed soon after that mutant sickle hemoglobin (Hb S) differed from normal hemoglobin A by a single amino acid. Ferrone (2004) examined the structure and physical properties of Hb S, which produced intracellular polymers with deoxygenation in his study "Polymerization and Sickle Cell Disease, a Molecular Review." These findings put SCD at the forefront of research on the molecular basis of human diseases

2.2 Etiology of Sickle Cell Disease

In his article on New England Journal of Medicine Perspective (NEJMP), Wailoo (2017) emphasized on the statement made by President Richard Nixon of United States. “It is a sad and shameful fact that the causes of this disease have been largely neglected throughout our history. We cannot rewrite this record of neglect, but we can reverse it.” Through sickle cell intervention programs that emerged after the US President signed the Sickle Cell Anaemia Act in 1972, significant advancements have been achieved. These innovations include the contentious ones that have been called into doubt. Some, nevertheless, were acknowledged and have fulfilled their promises. While others have fallen short of the expectations (Wailoo, 2017). The recognition of the role haemoglobin in sickling of sickle cell by Linus Paulings in the 20th century resulted in an important modification in the diagnosis and treatment of the disease. This finding subsequently changes SCD from a complex anomaly to the first molecular disease. The polypeptide subunits present in human haemoglobin are arranged in pairs. These subunits which are identical to each other, are each encoded by unique set of genes and this gives rise to its tetrameric molecular structure. The human alpha-like globin genes are located on chromosome 16, and the beta-like globin genes (ε, Gg, Ag, d, and b) are located on chromosome 11. As the fetus develops, gene order is expressed and this order arrangement of both the alpha-like and beta-like genes on the human chromosomes stays the same, interestingly. There is a reduction in Hb F level being transported in the blood during adulthood because human haemoglobin is only present as fetal haemoglobin (Hb F) before birth. After

birth, within the first year, Hb F is then gradually replaced by Hb A, a minor adult-type hemoglobin ($\alpha_2\beta_2$). Upon completion of the switch from Hb F to Hb A, patients with disorders of the beta-globin genes start manifesting the clinical features of their diseases. In light of this discovery, researchers have been enthusiastic about investigating the mechanism of action during this transition phase from Hb F to Hb A to significantly boost the Hb F level through therapeutic approaches.

Sickle cell disease is characterized by inability of an impaired erythrocyte to survive due to deformation in structure which is caused by a single amino acid substitution in the β-globin chain and the resultant haemoglobin S polymerizes (Sundd *et al*., 2019). When deformed red blood cells are present, its abnormalities are evident in clinical conditions such as haemolytic anaemia, cycles of micro vascular vaso-occlusion leading to end-organ ischemia-reperfusion injury and infarction. Sickled and destroyed erythrocytes that block blood vessels lead to gradual advancement to small- and large-vessel disease. This is majorly as a result of inflammation and redox instability. The synergistic effect of these molecular, cellular, and biophysical processes inevitably leads to acute and chronic pain and end-organ injury and failure in SCD. Rees *et al*. (2010) explained that the difference between the wild-type beta allele and the homozygous beta S (βS) allele (located on chromosome 11p15.5), which causes sickle cell anaemia is that thymine is substituted for adenine in codon 6 of the beta-globin gene in the latter. As a result of this single nucleotide polymorphism dbSNP Rs334 (T; T) glutamic acid residue (Glu) which is hydrophilic is replaced with a hydrophobic valine residue (Val) at the sixth position in the β-globin chain resulting in loss of charge. This consequently produces a mutated haemoglobin tetramer Hb S ($\alpha_2\beta s_2$) in the erythrocytes of individuals with sickle cell anemia (Bunn, 1997). Through series of molecular and cellular mechanisms, the mutated βS is inherited as a homozygous gene (HbSS) or even inherited alongside other mutations such as βC (HbSC), βD (HbSD), βO (HbSO/Arab), βE (HbSE), or a β-thalassemia allele (HbS/β-thal0 or HbS/β-thal+).

2.3 Incidence and Prevalence of Sickle Cell Disease in Africa

During the 2006 meeting in Geneva, Switzerland, World Health Organization (WHO), stated that the United Nations has recognized SCD as a global public health concern, and WHO recommended that 50% of member states will have established SCD control programs by 2020. The meeting revealed that neither has research on sickle cell disease in Africa received significant sponsorship nor has the required care for SCD sufferers in this continent received appropriate attention. This continent has 70% or more of the global population of SCD patient resident in it. Still, research on sickle disease has been centralized in the Northern region and

this has provided most advances in the understanding and management of this condition. This corroborates the report of Aliyu *et al*. (2008) that United Nations estimates that there are between 20 and 25 million people worldwide living with SCD, of which 12–15 million live in Africa. In their research work "Prevalence of sickle cell disease in a Northeastern region of the Democratic Republic of Congo: What impact on transfusion policy?", Agasa *et al*. (2010) observed that while Africa has the highest population of people living with this condition, there are certain areas in Africa with the densest population of SCD sufferers. The prevalence rate ranges between 10% and 40% of the population of people in areas between 15^0 North and 20^0 of the latitude and this has been recorded as the highest prevalence rate around the globe. In 2010, Rwezaula reported results of a study of records of over 2000 newborns at a hospital in Tanzania (Rwezaula, 2010). Aygun & Odame (2012) suggested immunity to malaria parasite, *Plamodium falciparum* acquired by individuals who are resident in Tropical regions of Africa is a valid reason for high incidence of SCT in those areas. However, being that SCD and SCT affect individuals differently, the former does not confer the same level of immunity to its sufferers (Komba *et al*., 2009; Rahimy *et al*., 2003). The theory of some acquired immunity by SCT individuals was adapted from a geostatistical mapping study conducted in tropical Africa. But this theory could only have a strong base for the results obtained in Africa as it did not consider incidence rates in other parts of the world (Aidoo *et al*., 2002; Piel *et al*., 2010). However, Weatherall, (2011); Wonkam *et al*. (2011) explained that high incidence rates of SCT and SCD in North America and the Caribbean and even other parts in the globe is attributed to the presence of residents who have migrated from Africa and other regions with high prevalence

2.4 Sickle Cell Disease Variants

Abnormal haemoglobins are formed as a result of deformed erythrocytes. This is the basis for the classification of different variants of SCD as a result of the varying complications associated with each.

The major groups include:

1. Sickle Haemoglobin SS disease (SS) or Sickle cell Anaemia
2. Sickle Haemoglobin-C disease (SC)
3. Sickle Haemoglobin-D (SD)
4. Sickle Haemoglobin beta-plus Thalassemia

2.4.1 Sickle Haemoglobin SS disease/ Sickle Cell Anaemia

Hemolysis and vaso-occlusive crises (VOC) are the two major conditions associated with sickle cell disease. Sickle Cell Anaemia (Hb-SS-Disease) clogs blood vessels which causes severe pain and also causes problems such as organ and tissue damage. That is why it is considered the most common sickle cell disease. Haemoglobin S (HbSS) is inherited by an offspring from both parents, the resultant sickle cell disease is sickle cell anaemia. This happens because β-globin subunits are replaced by haemoglobin S. Following this substitution, the abnormal haemoglobin S subunits and other haemoglobin molecules found in the red blood cell begin to form aggregate molecules. The adhesion exhibited by these molecules lead to blockage of blood vessels as the RBCs become distorted in shape. This distortion eventually impedes flow of blood through vessels as a result of long and rigid chains formed by the adhered molecules (Belfer *et al*., 2014).

Mangla *et al*. (2022) explained that susceptibility of sickle hemoglobin (Hb S) molecule to form rigid, elongated polymers can be attributed to the abnormality existent in the beta-globin gene. In the early stages of the sickling process, sickle erythrocytes cycle back and forth between the normal biconcave shape and the abnormal crescent shape (acquired at low oxygen pressure). The risk for hemolysis and VOC increases when this oscillatory transition produces erythrocytes with permanent sickle form. The series of conditions that characterize sickle erythrocyte are low affinity of Hb S to oxygen, physiologically high 2,3-diphosphoglycerate, and increased sphingokinase-1 activity. These inherent conditions alongside high concentration of Hb S and repeated damage to red blood cell (RBC) membrane accelerate the ability of the abnormal haemoglobin S to form polymers. This polymerization leads to loss of oxygen-affinity (deoxygenation) of RBCs. Kato *et al*. (2018), stated that the auto-oxidation of Hb S creates an imbalance in the amount of reactive oxygen species (ROS)present in the cells. This imbalance in the production and accumulation of ROS (which is exacerbated by increased expression of xanthine dehydrogenase and xanthine oxidase and decreased expression of NADPH oxidase) is a contributing factor to hemolysis, a condition that induces damage on cell membrane of the erythrocyte. Thereby releasing free hemoglobin (which scavenges nitrous oxide) and arginase-1 (which competes for L-arginine).

2.4.2 Sickle Haemoglobin-C disease (SC)

In their research "The distribution of haemoglobin C and its prevalence in newborns in Africa", Piel *et al*. (2013) stated that a change in position 6 of the beta-globin chain (β6Glu-Lys) births a unique variant of sickle cell disease known as Haemoglobin C (Hb C). Structurally, it is one of the commonest among the variants of SCD in human populations just like haemoglobin S

whose amino acid substitution occurs at the same position (Hb S; β6Glu-Val), and haemoglobin E (Hb E, β_2-6-Glu-Lys) (Itano & Neel, 1950). Its less severe medical concerns regardless, Haemoglobin C (Hb C) diagnosis should be done on individuals in a population. In addition, genetic counselling is another strategy to employ to forestall transfer of this variant and other haemoglobinopathies from parents to offspring. Hb C can exist as a heterozygous allele in (AC) individuals, and as such, sufferers are asymptomatic to this trait. Earlier studies revealed that the less severe clinical symptoms such as mild haemolytic anaemia are evident in individuals who have inherited the homozygous allele (CC) for this trait. This stems from the reduced solubility of the red blood cells which can lead to crystal formation (Charache *et al*., 1967). Hb C shows clinical manifestation only in the presence of or in a synergistic effect with HbSC (sickle-haemoglobin C disease). This synergy is associated with chronic haemolytic anaemia and some sickle cell episodes that occur periodically. When in combination with β-thalassaemia (haemoglobin C-β thalassaemia), the resultant effect is moderate haemolytic anaemia with splenomegaly (Bain, 2006).

It has been found by Modiano *et al*. (2001); Hedrick, (2004) that in individuals where the trait exists as homozygous allele (CC), Hb C confers a total immunity against *Plasmodium falciparum*. This is not the case in heterozygous (AC) individuals. Initially, the data that provides statistics which suggest that Hb C has better fitness than Hb S, was however, obtained from a geographical area with fewer Hb C individuals than the geographical area used for Hb S (which had a good number of Hb S individuals). In light of human migration rates, data provided captures a more significant number for comparison (Piel *et al*., 2010).

A retrospective study by (Travassos *et al*., 2015) was performed in Morrocco for 12 years on haemoglobin C disease cases. The results revealed that the mean age of the population sample at the time of diagnosis was 38 years. Diagnosis done during the study detected heterozygous A/C (75%), homozygous C/C (8%), double heterozygous S/C (9%), C/beta +/- Thal (6%), C/ O - Arab (2%) to be the different causative factors. Results of further biochemical tests carried out in the population study corroborate the results (Ouzzif *et al*., 2019).

Karna *et al*. (2023) emphasized that haemoglobin C variant of SCD presents nothing short of a normal life expectancy with proper growth and development for individuals with this trait. In most patients, no treatment is required. Anaemia is a common symptom experienced in haemoglobin C and it occurs as result of a reduction in the number of red blood cells. Folic acid is administered as a management therapy (when there is a significant reduction in its reserves in the body) because it produces new red blood cells that help to alleviate the

symptoms. The immunity against malaria provided by Haemoglobin C explains its prevalence rate in Atlantic West Africa and Southeast Asia. Additionally, a wide range of people in Africa, South and Central America, Southern Europe, and Asia have hemoglobin C.

In their study, Mullins *et al*. (2018), explained that using complete exchange blood transfusions during cardiopulmonary bypass surgery makes the procedure reasonably safe and improves outcomes for patients with sickle cell disease and hemoglobin C

2.4.3 Sickle Haemoglobin-D (SD)

Hb-D Punjab, the Haemoglobin D variant of SCD has estimated frequency of 2% in Punjab region Northwest Indian. Hence, its name. However, in another part of India, Gujurat region of Western India its prevalence rate drops by one half (Torres *et al*., 2015).

In a co-inheritance with haemoglobin A (normal haemoglobin) as a heterozygous trait, the clinical complications of haemoglobin D is masked and results in maintained hematological integrity and zero clinical complications. The homozygous form (Hb DD) is rare but can also be inherited with no significant clinical symptom (Adekile *et al*, 2010; Taghavi *et al*, 2011). However, clinical symptoms such as mild microcytic and hypochromic anaemia, manifest in individuals who have the heterozygous combination of Hb-D Punjab and beta-thalassemia (Naoum *et al*., 2002). Like in homozygous haemoglobin S (Hb SS) patients, severe clinical complications such as VOS can be evident in individuals with double heterozygous Hb S/D.

2.4.4 Sickle beta-plus Thalassemia

Small deletions, a type of point mutation, that occur on genes coding for synthesis of blood transport protein (haemoglobin) leads to development of an abnormal haemoglobin known as beta-plus Thalassemia. This mutation reduces the amount of adult haemoglobin produced in the beta-globin unit and consequently leads to a reduction in the number of red blood cells in circulation (Galanello *et al*., 2010)

The structure of haemoglobin is affected differently in sickle cell thalassemia. The effects on the different globin units give rise to two categories of sickle cell thalassemia; α- and β-thalassemia. In a study that investigated the prevalence and genetic analysis of α- and β-thalassemia and sickle cell anaemia in South Western Iran, Nezhad *et al*. (2018) observed that people who are resident in Mediterranean, Africa, and South Asia have a high tendency to suffer β-thalassemia than its counterpart α-thalassemia Individuals living with β-thalassemia experience varying clinical complications including symptomatic conditions such as those seen in SCA and asymptomatic conditions that require close observation(Weatherall *et al*.,

2001).Among people of African race, the prevalence of β^+ mutation may lead to co-inheritance of β-thalassemia with the mild type β^+ mutation. This explains its mild effect is evident in the Hb concentration ranging from 5 g/dl to a level within the normal range.

Knowledge of family history, Hb A2 and Hb F levels determination and full blood count analysis can be used as a diagnostic yardstick to detect Hb S/beta-thalassemia.

2.5 Sickle Cell Disease Complications

The most dangerous side effect of autosomal recessive sickle cell disease is the inability of haemoglobin molecules to carry out special function of oxygen transport throughout the body. Tanabe *et al*. (2019) reports an increase in emergency room visits, the number of children admitted to hospitals, and deaths from sickle cell disease complications. However, an evidence of improved survival rate in children is seen in the reduced frequency of hospital admission and emergency visits among children while it is higher in adults.

The most notable sickle cell complication that affects the organ is spleen damage. Spleen and other organ damage occur when movement of sickled cells is obstructed as a result of impaired flow of blood through the capillaries, arterioles. Acute and chronic pain, priapism, acute chest syndrome, stroke, infection and sepsis, avascular necrosis and psychosocial complications are other examples of complications associated with sickle cell disease (Levenson *et al*., 2008; Zarrouk *et al*., 2006).

2.5.1 Priapism

A survey conducted in five hospitals located in UK and Nigeria reveals that an estimated 46 out of 130 sickle cell sufferers who are male have a reported history of priapism which is the most physical evidence of SCD for patients in this group. (Adeyoju *et al*., 2002). An erection that lasts for approximately 4 hours either fully or partially and includes either of the following; ischemic, stuttering, and non-ischemic is called priapism (Broderick, 2011). Sickle cell disease male patients who experience untreated frequent episodes of priapism most likely suffercorporal fibrosis and erectile dysfunction. In most cases, it likely works in conjunction with some complications such as pulmonary hypertension, leg ulceration, and overall risk of death which are characteristic of blood vessels (Crane, 2011). Erection in males occurs primarily through nitric oxide availability in circulation. In sickle cell patients, priaprism also develops partly as a result of lack of moderate regulation of the nitric oxide pathway and its downstream signaling (Crane & Bennet, 2011).

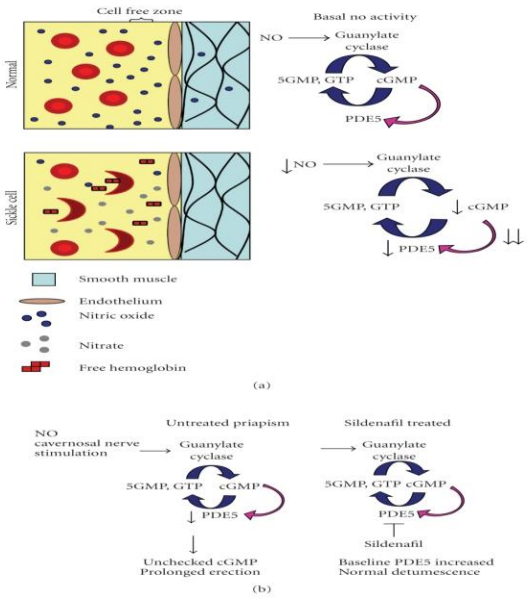

Plate 1: Nitric oxides signaling in normal red blood cell and sickle red blood cell from Rother *et al*., 2005

Haemoglobin in healthy red blood cells scavenge free nitric oxides (NO) and convert them to nitrates (NO_3^-) and methaemoglobin as result of their reaction with oxyhaemoglobin. In the presence of deoxyhaemoglobin, nitric oxides are converted to iron-nitrosylhaemoglobin (Reiter et al., 2002). The ability of haemoglobin in red blood cells to scavenge free NO is reduced drastically by the compartmentalization of haemoglobin in their pockets. This creates a haemoglobin-free zone adjacent to the endothelium and as a result, the scavenging activity of haemoglobin in red blood cells is less than 2% of free haemoglobin (Liao, 2002). In sickle cell patients, the level of free haemoglobin is higher when compared to normal volunteers (plate 1) and this upsets NO signaling pathway by exacerbated NO scavenging (Reiter, 2002).

2.5.2 Acute and Chronic Pain

The most frequent consequence and presentation of sickle cell disease is pain. Due to the fact that the pain can last for a longer period and can range from minor to serious problems. Fetal haemoglobin levels are high at infancy and masks many sickle cell complications. However, there is a likelihood that pain may manifest in infant (Aich *et al*., 2015). Research has shown that two forms of pain; acute and chronic are associated with SCD. Patients who are younger than 18 years are most prone to acute pain. While chronic pain is common among sickle cell adult patients (Dampier *et al*., 2014; Smith *et al*., 2008). SCD patients are quick to respond to any slight change in temperature (heat and cold) as well as reduced force to grip objects firmly. These represent pain in SCD (Kenyon *et al*., 2015). Management therapies for acute and chronic pain relies on the knowledge of action mechanism of the pain characteristics. Injury to the nerves acute and chronic pain as nerve fibres are activated. When nerve fibres are activated,

ROS accumulate in circulation n, the blood vessels are inflamed and this leads to impaired function and acute pain. Opoids are known to alienate pains associated with sickle cell disease. Despite its detrimental effects on patient's behaviour, organs and tissues, opioid is proven to be the best remedy against pain crisis in SCD (Gupta *et al*., 2015).

It is vital to understand the specific mechanisms that specifically describe the origin of sickle cell disease to develop therapies targeted towards satisfactory treatment of chronic and acute (vaso-occlusive) pain. Vascular dysfunction, inflammation and oxidative stress can cause acute pain through the activation of nerve fibers. Meanwhile, cells lining the blood vessels are one-cell thick and this feature allows the exchange of substances (especially oxygen) between the vessels and surrounding body tissues. When these cells are frequently activated for this function, inflammation may occur leading to chronic pain in the nerves (Aich *et al*., 2015).

2.5.3 Acute Chest Syndrome (ACS)

Acute chest syndrome is a group of disease conditions whose characteristics include chest pain, cough, pyrexia, hypoxia (low oxygen level) and pulmonary infiltrates. The causative agents of acute chest syndrome have different mechanisms of action. Increased sickle red cells adhesion to very small vessels of the lungs in absence of sufficient oxygen is the most suggested of these mechanisms of action (Jain *et al*., 2017). Pulmonary fat embolism and infarction are other factors that greatly contribute to the susceptibility of an individual to ACS. Children who are younger, having the Hb SS or beta thalassemia variant genotype and low level of Hb F in circulation have an increased chance of developing ACS (Jain *et al*., 2017). As explained by Platt, (2000), under hypoxia in the air sac area of the lungs, sickled red blood cells will begin to stick more to each, the white blood cells and the thin layer of cells lining the blood vessels. The result of this adhesion is vaso-occlusion and reduced oxygen state in tissues. In patients that suffer ACS, the concentration of the adhesion receptor molecules in blood plasma is usually significantly high and it confirms the abnormal body changes that cause ACS. The number of by-products released during nitric oxide metabolism reduces significantly (Stuart & Setty, 1999). A report suggests that just before acute chest syndrome occurs in patients, episodes of vaso-occlusion happen as an early indicator after which ACS manifests between the first three days after admission in ED (Vichinsky *et al*., 2000).

2.5.4 Avascular Necrosis (AVN)

Bones in the hip and neck regions of the body require frequent supply of oxygenated blood just like other areas of the body. However, under some health conditions such as sickle cell disease, supply of blood to these bones is limited and the effect is, avascular necrosis. This major complication associated with SCD, subjects the bones to an oxygen-deficient state. This

unavailability of oxygen causes loss of bone cells which eventually leads to gradual narrowing of joints and most significantly, bone collapse. Once this happens, necrosis comes afterwards because of deprived oxidative phosphorylation (Sanders, 2018). Traditional therapies (surgeries) that are less aggressive have been reported to be effective against avascular necrosis during its initial phase. These traditional surgeries are considered ineffective when AVN progresses to the advanced stage. For this, a contemporary and more vigorous medical approach for extended period is required. (Hernigou *et al*., 2003; Matos *et al*., 2012; Akinyoola *et al*., 2009).

Avascular necrosis affects individuals of all ages. It is however, most prevalent (about 50 %) in older people of 35 years and beyond. The prevalence rate reduces from 8.7 to 1 2.4 % among individuals who are 21 years of ages. It further reduces to 3 % in younger children under the age of 15. These statistics conclude the report of Akinyoola *et al*. (2009) that explains the age-dependence of AVN prevalence in sickle cell disease patients.

2.5.5 Sepsis

Health conditions such as impairment in spleen function, irregular adaptive immunity and deficiency in immune function linked with malnutrition are reasons individuals who suffer sickle cell disease are prone to infections and disease. When the body begins to react to some of these infections and also self-destruct its cells, tissues and organs, it is called sepsis (Dominik *et al*., 2020).

One of the organs of the lymphatic system, spleen, is responsible for filtering out abnormal, old, damaged and dead red blood cells from the plasma. In addition to this, spleen confers immune function on the lymphatic system by producing white blood cells which fight disease and infections. Sickling of red blood cells is characteristic to SCD and these deformed cells cluster together to create an obstruction in the sinusoids that is commonly experienced by SCD patients. Blood flow may be diverted via shunts found within the spleen and this will lead to circumventing the required filtration processes. Specialised white blood cells called macrophages that engulf deformed RBCs are affected by this obstruction and this eventually leads to poor phagocytosis of particles such as pathogens, thereby weakening the immune function in SCD patient (Booth *et al*., 2010).

2.5.6 Organ Damage

When proper diagnosis is not done in SCD patients as they advance in age, multiple and frequently occurring kidney injuries result in renal failure. More than one organ can be affected

simultaneously with the brain and the kidney as major targets. While several factors cause this organ damage, deficiency in nitric oxide and destruction of red blood cells are the main culprits (Sharpe &Thien, 2014). Creatinine is a chemical waste from creatine metabolism. It is excreted by healthy kidneys alongside other waste substances. In kidney function tests to determine injury on the kidneys in sickle cell disease patients, high creatinine levels in the blood is an indicator of poor kidney function. However, this may not be reflected in kidney injuries from SCD episodes (Lebensburger *et al*., 2016). The kidneys suffer in a deprived oxygen state when many RBCs are deformed (Ataga *et al*., 2014).

Blood supply to the parts of the brain can also be cut reducing the availability of oxygen in the brain leading to stroke. Every cell, including the brain cells requires oxygen to generate energy. Gradual cell death occurs in the brain causing an injury to the nervous system. The three main types of stroke seen in children and adult SCD patients are; Ischemic stroke, hemorrhagic stroke, and silent stroke (Tanabe *et al*., 2019). Ischemic stroke results in seizures, headaches, epilepsy. These occur when blood supply to the cerebrum is inefficient (Keman *et al*., 2014). When compared to general population, adults who suffer SCD have a higher case of ischemic and hemorrhagic strokes (Talahama *et al*., 2014). Ischemic stroke and hemorrhagic stroke can occur side by side (Kossorotoff *et al*., 2015). Rather than increased dilatation of arteries which is considered a risk factor for hemorrhagic stroke, others include a low level of haemoglobin, blood transfusion that was done in the last 14 days, and high steady-state leukocyte (Strousse *et al*., 2006). It is common for children living with SCD to experience silent stroke with a prevalence rate of approximately 27 % before 6 years of age before age. In older children of about 14 years, the prevalence rate is estimated to be 37%. Silent stroke is also known to occur differently in HbSβ^{+}-thalassemia and sickle haemoglobin individuals with prevalence rate of 3% to 38% and 5 % to 31% of patients respectively (DeBaun *et al*., 2012). In a cohort study of individuals with sickle cell disease, Prengler *et al*. (2005) suggested that seizures caused by SCD occur when there are certain diseases that affect the blood vessels and reduces blood flow through the brain.

2.6 Sickle Cell Disease Therapies

Even after Herrick diagnosed sickle cell disease in 1910, people with the disorder were medically unnoticed until nearly a century ago. Having had comparable symptoms and raised newborn mortality, other infectious diseases frequently went undetected while sickle cell disease was present. With clear plans to stop further transmission of the characteristic, general awareness campaign is the first step in controlling SCD. It has been demonstrated that genetic counseling and carrier screening are effective methods for preventing the propagation of

thalassemia and other haemoglobinopathies. The authors of "Sickle Cell Disease: New Opportunities and Challenges in Africa" (Makani *et al*., 2011) noted that infections, particularly malaria, have been recognized as the main driver of the sickle cell emergencies among Nigerian sufferers. Therefore, in all cases, for sickle cell crisis among patients living in locations susceptible to plasmodium infection, mosquito control and antimicrobial administration for malaria are advised. Recent improvements in pain management and gene therapy are a part of a long history of modest advancement that has been under persistent threat. The life expectancy of those with sickle cell disease was increased, nonetheless, through theA use of antibiotics. Given their potential to lead to drug addiction in patients, there were certain societal considerations surrounding their use of pain relievers like opiates (Wailoo *et al*., 2017). Folic acid and preventive antimalarials are hence typical drugs. In places where pneumococcal infection is widespread, proactive antibiotics such as oral penicillin from 2-3 months of age until at least age five may also be used with antioxidants aspirin and other medications.

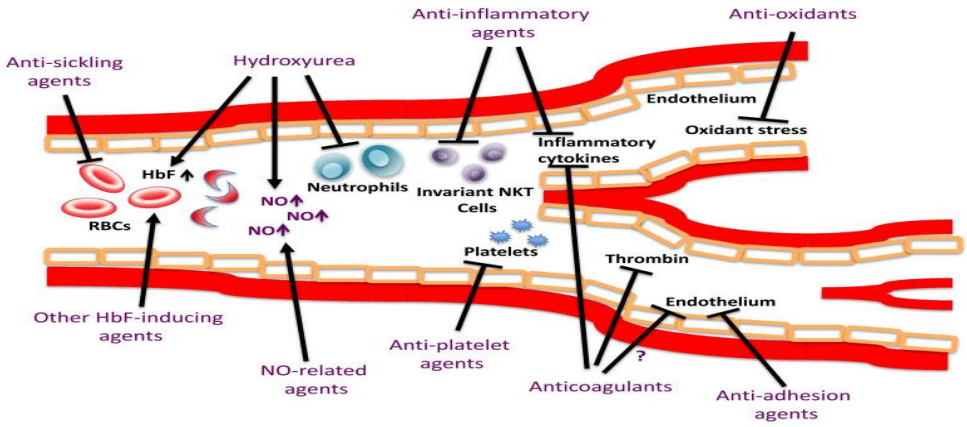

Plate 2: Sickle cell disease therapies target sites adapted from Ataga and Stocker 2015

2.6.1 Hematopoietic stem cell transplant (HSCT)

Hematopoietic stem cell transplant, often known as bone marrow transplant, is the delivery or replacement of weak blood stem cells that are found in red bone marrow with healthy cells in individuals with failing or deficient bone marrow. Hematopoietic stem cells (HSCs) are distinguished by their capacity to regenerate themselves and specialize into most mature blood cells (Bryder *et al*., 2006). Hematopoietic stem cell transplantation (HSCT) was first investigated in humans in the 1950s based on qualitative studies in mice models that demonstrated that infusion of healthy bone marrow materials into myelo-suppressed bone marrow might stimulate the restoration of functionality for the individual receiving the

transplant. Hatzmichael and Tuthill (2010) explained that hematopoietic stem cell transplantations (HSCTs) have been adopted for the treatment of cancer cells affecting the lymphocytes, metabolic defects present at birth, Leukemia performed each year for the treatment of disorders which include lymphoma, immune-deficiency illnesses, hemoglobinopathies and myelodysplastic and myeloproliferative syndromes yearly. Currently, hematopoietic stem cell transplant (HSCT) has been incorporated as part of the sickle cell disease therapeutic management strategies Walters *et al.* (2010) described the ability of infused healthy bone marrow to reinstate function in patients' diagnosed organs and to establish donor-derived erythropoiesis if administered early. According to Gluckman *et al*. (2017). 1,000 individuals who had sickle cell disease and visited the hospital between 1986 and 201 received transplants from their different siblings who were matched for HLA. The result of these transplants in patients who were in the children and adult category shows an outstanding recovery. The overall survival rate appreciated to about 93 %. Myeloablastive Chemotherapy (MAC) was used on 87 % of the visiting patients. While Reduced Intensity Chemotherapy (RIC) was used on the remaining 13 %. Both were utilized based on ability to eliminate weak blood stem cells and also prevent patient-host graft rejection initiated by the patient's immune system of during transplant (Salinas & Thein, 2020).

From the outcome of their research, Walters *et al*. (2001) concluded that individuals with SCD can recover from the disease even if some fragments of their own bone marrow remain after the transplant. This can only be possible if the outcome of the transplant gives a good and stable amalgamation of the two different cells. This corroborates the statement of Fitzhugh *et al*. (2017) "Donors could be Hb AA or Hb AS, and to reverse the sickle hematological genotype, the myeloid donor chimerism has to be >20%". The challenge faced with this SCD treatment approach is identifying and accessing donors with whose HLA matched with patients'.

2.6.2 Use of Sickle Haemoglobin (HbS) Polymerization Inhibitors

The factor which causes the onset of SCD and maintains its progression during the course is the polymerization of sickle haemoglobin in an oxygen-deficient state. When these polymers are formed, the red blood cells lose their elasticity and flexible formation, creating an avalanche of processes one of which is, the obstruction in the flow of blood through the vessel. In this reversible process, oxygenation returns the red blood cells to deformable state as the polymers formed by the sickle haemoglobin (Hb S) degenerates. The life cell membrane integrity is affected by the sickling and unsickling and consequently reduces the life span of red blood cell from120 days to 10days.

Eaton and Bunn (2017) explained that rather than focusing on medical complications that stem from sickling process of the RBCs, therapeutic approaches that work with the principle of inhibiting polymerization provide a more convincing solution.

Elevated concentration in the cells has reported by Ware, (2015) to be effective in SCD management by its masking and diluting effect on the intracellular of sickle haemoglobin. Another mechanism of action leveraged by fetal haemoglobin is its anit-polymerisation effect conferred on it by its tetrameric hybrid structure which does not form polymers. There are promising outcomes presented by pharmacologically active materials such as 5-azacytidine, hydroxyurea (HU), butyrate and Decitabine through their ability to initiate fetal hemoglobin (Hb F, $\alpha 2\gamma 2$) synthesis. One limiting factor of Hydroxyurea activity is that the there is no uniformity in increase in fetal haemoglobin in the RBCs. While the increase in Hb F is not active in some RBCs, in other cells where it is observed, the increase is uneven (Ware &McGann, 2015). There is a second option according to DeSimone *et al*. (2002) for SCD disease individuals not responsive to hydroxyurea treatment. Decitabine is administered to such patients to raise their intracellular fetal haemoglobin which produces a substantial effect. An area with many SCD patients Sub Saharan Africa was investigated in recent studies by Opoka *et al*. (2017); Tshilolo *et al*. (2019) and results show that no complications from hydroxyurea administration was recorded in this area compared to other areas. In countries with little or no access to good health care and resources, hydroxyurea is considered the best therapeutic option. Despite the quality and quantity of research that have been conducted on therapeutic approach of hydroxyurea, its long-term effect in forestalling destruction of the end organ is yet to be discovered (Nevitt *et al*., 2017; Luzzatto & Makani, 2019). However, DeBaun, 2014 has reported a complication associated with hydroxyurea treatment on fertility of male patients as contradicting evidence.

Hassanah and George Atweh, (2016) explained that addition of methyl group to the cytosine residues of the DNA structure by a specific transferase is prevented when 5-Azacytidine and Decitabine are infused in the DNA molecule. The binding of these pharmacological agents (which have the same properties as cytidine) creates a chemical covalent bond between the transferase and the agents, thereby leading to a loss in functionality of the enzyme.

In their article "Treatment of Sickle Cell Disease by Increasing Oxygen Affinity of Haemoglobin". Henry *et al*. (2021) stated that in 2019, a pharmacological agent, voxelotor, was certified for use by FDA. Thereby leading to a loss in functionality of the enzyme. This approval projected the idea that pharmacological agents that increase the attraction of oxygen molecule to haemoglobin of RBCs can be used as a strategy to treat sickle cell disease. The

existing knowledge that sickle RBCs have impaired attraction for oxygen molecule incited the approval of voxelotor as the only pharmacological agent with the potential to cure SCD in children and individuals 18 years old and beyond. When administered to patients, voxelotor reduces the formation of sickle cells in RBCs by increasing the number of Hb S molecules (that do not form polymers) in their relaxed with great attraction for oxygen (Strader *et al*., 2019).As reported, it is believed by many that in as much as haemoglobin exists in tense (T) and relaxed state (R), the distinct relaxed states (R2, R3, RR2, RR3 etc.) are in a dynamic equilibrium with each other working as a group to perform the function of oxygen transport. (Jenkins *et al*., 2009). This supports the evidence from research by Abdulmalik *et al*. (2011) that the bond that stabilizes the relaxed state (R) to promote oxygen attraction by Hb S occurs between aromatic aldehydes and quaternary R2.

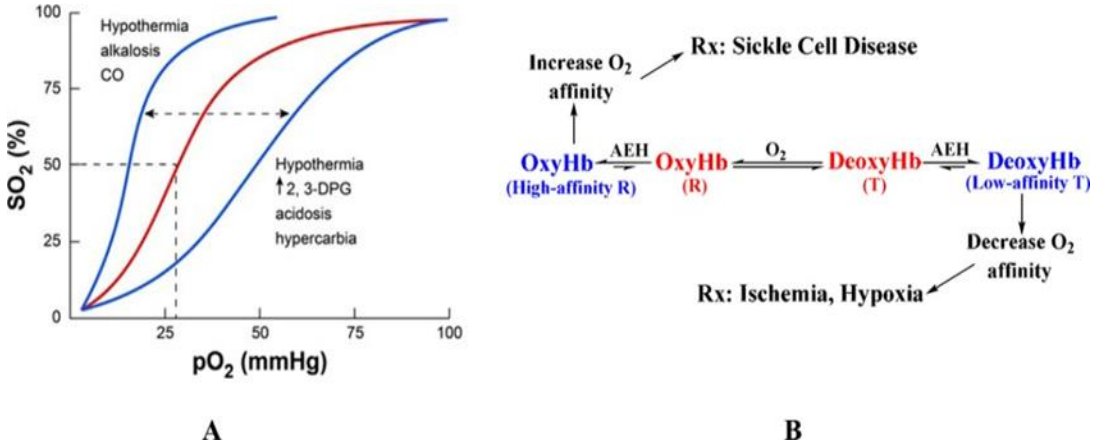

Plate 3: Oxygen equilibrium curve showing the direction of shift towards the tense or relaxed state.

Voxelotor (Oxbryta/GBT440) binds specifically to the N-terminal valine of the α-chain of Hb S to stabilize the oxygenated haemoglobin state (Strader *et al*., 2019), thus reducing the predisposition to sickling.

The realization that the polymerization of haemoglobin in sickle cell disease is intensified by the low oxygen (O_2) affinity of Hb S as a result of unusually high concentration of 2,3-Diphosphoglycerate in sickle erythrocytes, birthed another deliberate line of action to treat sickle cell disease by increasing the affinity of Hb S for oxygen sufficiently to prevent premature release of oxygen, but not so extensively as to compromise tissue oxygenation (Safo *et al*., 2014).

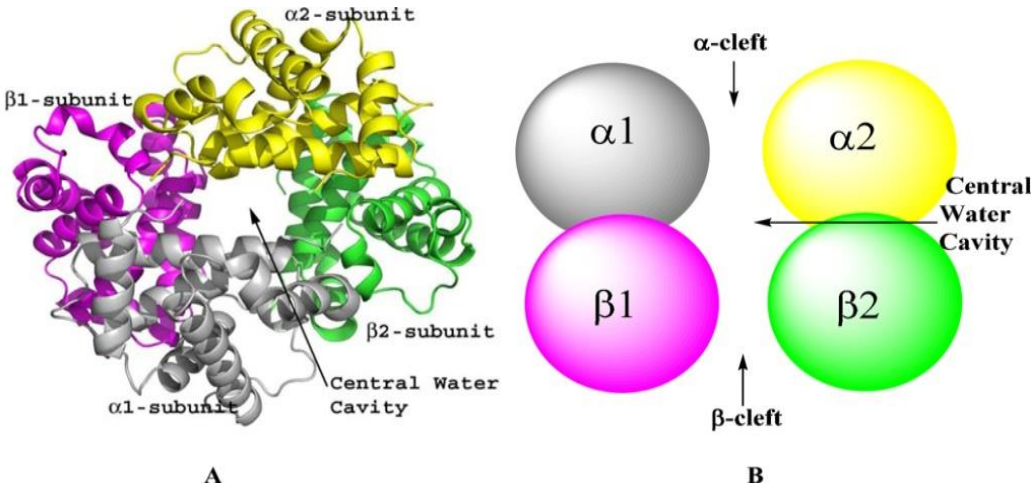

Plate 4: Ribbon diagram (A) of α1, α2, β1 and β2 subunits and spherical diagram (A) of α1, α2, β1 and β2 subunits

2,3- Phosphoglycerate is an allosteric effector of haemoglobin whose increase in concentration shifts the oxygen equilibrium curve to the tense state (Castilho *et al.,* 2003). This shift towards the tense state reduces the affinity of cells for oxygen which would lead to polymerization. Eaton and Bunn, (2017) explained that because of the critical role of 2,3-phosphoglycerate on oxygen affinity of sickle haemoglobin, there is a convincing reasoning for the development of drugs that target the enzymatic pathway responsible for its remarkably high (5 mM) concentration in red blood cells

2.6.3 Pharmacological Agents that target Vaso-occlusion

Vaso occlusion (VOC) is the obstruction of blood circulation in arterioles, venules and capillaries by sticky sickle red blood cells which causes deficiency in oxygen supply to target organs. The short-term effect of this obstruction is pain and hemolysis in long-term. The trigger for vaso-occlusive crisis is yet to be understood. But cold and dehydration are possible triggers. Patients with sickle cell anaemia may experience VOC several times per year.

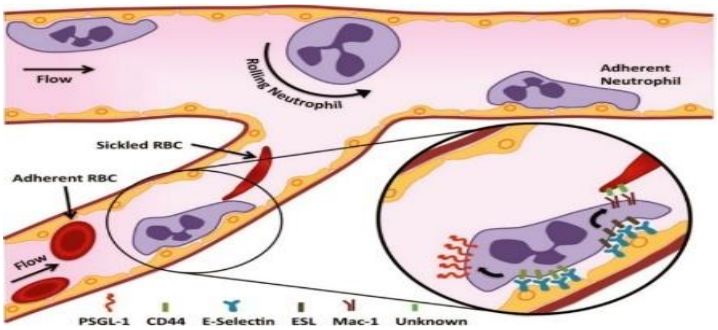

Plate 5: Interaction of adhesion molecules with endothelial cells From Manwani and Frenette 2013

The molecules present on the surface of red blood cells (e.g., VCAM-1) promote cell-cell and cell-matrix attraction in abnormal conditions such as SCD. In normal conditions, these molecules are inert and therefore do not mediate adherence of RBCs to other cells. In SCD, these molecules are continually displayed and made active progressively thereby facilitating the adherence of RBCs and the supporting platelets and leukocytes to the walls of the endothelial cells (ECs). Leukocytes and platelets that have been activated alongside the adhesion molecules also raise the chance of developing VOCs (Nasimuzzaman & Malik, 2019; Sundd *et al*., 2019; Telen *et al*., 2019). When VCAM-, for example, is now directly reacting with endothelial cells of the inner lining of the blood vessels, the effect may be production of radicals and activation of the transcription factor NF-κB by the endothelial cell. On the surface of the endothelial cells are present E-selectin, VCAM-1, and ICAM-1 whose gene transcription are regulated by NF-κB. Gutsaeva *et al*. (2011) suggests that E-selectin and P-selectin are directly involved in vascular occlusion crisis.

Chang *et al*. (2010) analysed the biological activity of Rivipansel (GMI-1070) new pharmacological agent with the latent ability to treat VO complications that occur in SCD patients. Amongst other adhesion molecules, the major substrate for the P-selectin inhibitor, GMI- 1070 isE-selectin. GMI- 1070 controls and prevents endothelium adherence of RBCs and also erythrocyte reaction with leucocytes brought about by E-selectin. By this, blood flow through tiny vessels is maximized as there will be a few or no sticky molecules on the endothelial cells.

L-glutamine has the potential to scavenge free radical species by producing glutathione, a known antioxidant and the cofactors nicotinamide adenine dinucleotide NAD(H), nicotinamide adenine dinucleotide phosphate NADP(H) and nitric oxides which can also act as antioxidants in the body. Food and Drug Administration has recently certified L-glutamine safe for consumption to help stop acute complications which SCD patients are susceptible to as reported (Sadaf & Quinn 2014). However, some clinical concerns ensued as reported by Quinn (2018) where individuals who experienced kidney dysfunction as a result of SCD were adversely affected by glutamine administration. When sickle hemoglobin (Hb S) degenerates, the fragments release superoxide, an ROS, from a non-enzyme catalyzed reaction. However, the degenerated fragments can be used as substrates by NADPH oxidase to the same product. ROS are unstable in structure and easily attack the cell membrane of living cells including RBCs. Membrane integrity is lost and this in part explains the different complications that happen in the SCD course (Goodman, 2004). Plasma content of the destroyed red blood cells is lost,

leaving oxidizing haemoglobin and iron chelates as the remaining components (Aslan *et al*., 2000). Supporting literature from Amer *et al*. (2006), summarizes that more ROS are generated from RBCs of individuals with SCD than in individuals with normal RBCs.

The FDA-approved form of glutamine, free glutamine (Endari) is usually prescribed as oral administration by dissolving the powder in prescribed amount of liquid to form a suspension or by mixing with food and taken twice daily. Glutamine also exists in more stable forms such as L-glycyl-L-glutamine, L-arginyl-L-glutamine, and L-alanyl-L-glutamine which are all dipeptides (Durante, 2019).

2.6.4 Phytomedicine

Oniyangi *et al*. (2018) defined phytomedicine as plant extracts got from plants in their original form which are used as traditional remedies for diseases. Research focused on identifying antisickling properties of native plants extracts have yielded positive results. Use of phytomedicines is at the top of the list of SCD management therapies in vogue and it is expected that other subsequent findings will include therapies that are innocuous and within reach for SCD patients. Uwakwe and Nwaoguikpe (2008) stated that the synergistic effect of *Xylopia aethiopica* and *Monodora myristica*, in addition to a prescribed course for food like fruits, fish and legumes, may provide an effective alternative for management of sickle cell disease and the complete range of abnormalities associated with it. The formation polymers by deoxygenated sickle haemoglobin molecules are delayed by long strands of polymers of Hb S that form a 3D structure resistant to flow of blood (Iyamu *et al*., 2002). Pharmacological agents whose mechanism of action promotes this delay before polymerization might be considered ameliorative in sickle cell disease crisis, because the tendency to form sickle cell by Hb S is reduced (Imaga, 2013). As reported, Niprisan (Nix-0699) formulated by National Institute for Pharmaceutical Research and Development was extracted in the dry form from different parts of five naturally occurring plants including; seeds of *Piper guineense*, flower buds of *Euglenia caryophyllata*, the stem region of *Pterocarpus osun*, leaf stalk of *Sorghum bicolor* and trona (Obodozie *et al*., 2009). Its chemical components, 5-hydroxymethyl-2-furfural, and MX-1520 are substances with the potential to restructure sickle haemoglobin within the cells thereby preventing the sickle formation process from occurring (Abdulmalik *et al*., 2005; Zhang *et al*., 2004; Iyamu *et al*; 2003; Wambebe *et al*., 2001). Ciklavit, a pharmaceutical grade agent was formulated by Prof Ekeke and Dr. Nwaoguikpe primarily from extracts of *Cajanus cajan* and some essential amino acids, vitamins and minerals. It was reported to provide relief from pain caused by SCD and may promote hepatoxicity in SCD patients (Akinsulie *et al.*, 2005). The mineral content of Ciklavit is essential in preventing oxidative stress (Blann *et al*.,

2003). This supported by literature from Nagalla and Ballas (2010) which suggests that the components of Ciklavit can be used to supplement the diet of SCD children and beyond to meet their nutritional requirement. Its mechanism of action as reported, is targeted at inducing Hb F rather than arginase inhibition (Taiwo & Imaga, 2017). *Allium cepa* (Onion), *Allium sativum (*Garlic) and *Telferia occidentalis* (Ugu) common consumables native to Nigeria. The results of the extracts of these three vegetables as reported by Nwaoguikpe (2009) shows the capacity to inhibit haemoglobin polymerization and also improve the Fe^{2+}/Fe^{3+} ratio. Both of which are pathophysiological in SCD. It was reported by (Obichi *et al*., 2015) that *Solenostemon monostachyus* contains phytochemicals such as tannins, saponins, alkaloids, flavonoids which act as potential free radical scavenging agents to help in preventing VOCs in SCD. Nwaoguikpe *et al*. (2010) in their study, observed inhibition of sickle cell polymerization and the improvement of the Fe^{2+}/Fe^{3+} ratio of HbSS when *Aloe vera extracts* were administered. The amino acids are all essential amino acids present in the extract in large quantities and were confirmed to be participants in sickling prevention process.

Antioxidants (phenolic acids) were reportedly present in methanol extract of *Carica papaya* leaf. Results from research by Imaga (2009) explains that suspensions containing Hb SS were treated before sickling process with *Carica papaya* leaf extract. 0–5 % of Hb SS formed the sickle shape at 40 minutes when subjected to severe oxygen- deficient condition. While under the same treatment condition, as high as 60 % sickling was recorded for suspensions containing Hb SS cells that were not pretreated. These results confer antisickling property on *Carica papaya* leaf extract. Nwaoguikpe, (2010) investigated the therapeutic effect of two varieties of tiger nut (*Cyperus esculentus*) extracts on SCD. It was observed to be of medical relevance in conditions like kwashiorkor and anemia.

2.7 Growth and Development in Sickle Cell Patients

Practices including hydroxyurea therapy, treatment given to prevent penicillium infection, augmentation of folate, and early screenings are done as a medical treatment for children with sickle cell anaemia (Serjeant, 2001). While the prevalence of the disease and the number of deaths that occur has reduced significantly as a result of these clinical therapies, growth deficiency is still a major medical concern among children with SCD. Kramer *et al*. (1980) in their study of pre- and postnatal growth and development in SCA, observed that children who suffer from SCA were relatively smaller and experienced growth delay reaching puberty as opposed to what was observed in healthy children. Although it begins late in both gender, developmental delays affect how SCA patients respond to sexual feelings and reproduction. Bone age is a major determinant of sexual maturity in both healthy individuals and SCA

patients. In SCA patients, deficiency in bone mass is common and it explains the delay in pubertal development. For example, in female individuals with healthy RBCs, menarche starts at around 10-14 years of age. In female patients sickle cell patients bone maturation is impaired and subsequently poor bone mass, menarche is delayed women (Veríssimo *et al*., 2007). When the endocrine system responsible for secreting hormones including sex hormones is dysfunctional and destruction of RBCs requires frequent use of energy and protein reserves to make new cells, the ripple effect is delayed growth and development. Increased heart function to pump enough oxygenated blood is also experienced. Lack of adequate nutrients always requires high energy consumption from reserves. It is generally accepted that there are Irregularities in the different body measurements that manifest from childhood in sickle patients. According to Zemel *et al*. (2007), there have been arguments concerning the relationship between haemoglobin levels and anthropometric measures in individuals living with SCD.

2.8 Gender Differences in Sickle Cell Disease

Gender does not play any role in the occurrence of SCD in the general population because it is inherited as two mutated genes, one from each parent. However, the idea that mortality and morbidity rate in SCD individuals is gender-sensitive is corroborated by a report Platt *et al.* (2018). The study shows that death usually occurs around an average of 42 years for male, while the females stand a better chance to live up to 48 years. Nitric oxide production is also higher in females with SCD than in males because the female sex hormone (oestrogen, specifically) enhances the synthesis of nitric oxide which controls when and how VCAM-1 is expressed (Kim-Shapiro & Gladwin, 2018). Sickle cell disease male patients are more prone to VOCs than females because the absence of oestrogen causes a slower rate of nitric oxide synthesis and faster consumption rate in males. NO also plays a role in regulating the transcription of fetal haemoglobin and may likely explain the gender-dependence of Hb F expression (which is higher in females) in SCD patients. Gladwin *et al*. (2003) suggests that medical care for SCD patients, males particularly, should include treatments whose mechanism of action either improves nitric oxide activity on living cells or inhibits its consumption

2.9 Costus afer

2.9.1 Taxonomy and Morphology

Costus afer alongside other species *Costus igneus, Costus pictus, Costus arabicus,* etc. are plants that are in a family classification called *Costaceae* with a common genus *Costus* most times referred to as Spiral Ginger. Approximately 175 species of this genus have been identified as distributed throughout the world. Botanically, the genus has long fleshy stems

with tuberous rootstock. The arrangement of the leaves is in spiral form. As part of the flower which is closely packed, the calyx is positioned

as like short tube, with its shape like an inverted cone. The different parts of the corolla are short, large, sub equal and laced over each other

Plate 6: (a) Stems of *Costus afer* **(b) Leaves of** *Costus afer*

2.9.2 Nutritional values and composition of *Costus afer*

Trace elements are the dietary elements that are needed in minute quantities for the proper growth. Ukana *et al*. (2012) evaluated the trace elements in the stem of *C. afer* and concluded that it has rich content of potassium, sodium, calcium, magnesium and phosphorus. The stem, leaf and rhizomes of *Costus afer* can be consumed as food While the rhizome is rich in carbohydrate, starch, amylase, protein and lipid, the stem of *C. afer* provides a significant amount of liquid, carbohydrate, unrefined fat, protein, fibre. Proximate analysis was conducted on the different parts and vitamins identified include B (1, 2, 3, 6, and 12), E, and C (Dike, 2009). Saturated and unsaturated fatty acids were both detected and made up of 78 % and 22 % of the oil respectively (Ekpe *et al*., 2018). Some phytochemicals such as saponins aferosides A-C, as well as diosein and parphyllin and flavonoid glycoside kaempterol 3-0∞-L-rhamnopyranoside. were also detected (Aweke, 2007)

2.9.3 Therapeutic uses of *Costus afer*

Around the globe, especially in Africa, where cultural practices are common, different phytomedicines are used for different purposes (Hamilton, 2003). The anti-diabetic, anti-inflammatory and anti-arthritic activities of *C. afer* make the plant treasured in South-East and South-West Nigeria (Soladoye & Oyesiku, 2008).

2.9.3.1 Anti-diabetic Effect

Armelle *et al*. (2016) reported that carbohydrate tolerance tests which involve starch and maltose tolerance tests were conducted on two groups of test animals fully fed with carbohydrate and varying dosages of *C.afer* extract respectively. For the two groups of test animals, metformin was used as a standard for *C.afer*. *C. afer* leaf extract was able to inhibit uptake of glucose by the cell almost as much as metformin would do. It has been previously

observed that more free radical species are created by tissues in a hyperglycemic state. This usually occurs when there is an increase in addition of glucose to protein without proper enzymatic control. Exposed glucose reacts with oxygen in the air, and modification in the metabolic pathway of polyol (Araki *et al*., 2010).

2.9.3.2 Anti-inflammatory and Anti-arthritic Effects

In the global population, women are three times more prone to rheumatoid arthritis than men (Chopra &Abdel Nasser, 2008). Rheumatoid arthritis is the prevailing cause of long-term diseases or conditions in countries where standard of living is high (Mody, 2009). Synovial fluids around the joints are surrounded by tissues which can be attacked by the immune system resulting to severe pain and sometimes inability to move without pain. (Ahmed *et al*., 2005; Aletaha *et al*., 2010). Some undesirable effects have been associated with the use of artificially modified drugs such as steroidal and non-steroidal anti-inflammatory drugs which have been chosen as a treatment action to prevent the disease. This challenge in the use of synthetic drugs gave rise to the search for more improved remedies from plant sources (Drini, 2017). *C afer* leaf is used for many diseases and infections particularly, inflammation, rheumatism, arthritis, cough, hepatic disorders, helminthic, miscarriages, epileptic attack and hemorrhoids used as a medicinal herb (Iwu, 1993; Burkill,2000; Anaga *et al*., 2004). In a study, it was observed that edema caused by induction of carrageenan in lab rats decreased remarkably when chloroform and methanol extracts from the leaf and stem of *C.afer*. In other reports, the rhizome was notably observed to be effective against croton aldehyde-induced mouse ear edema (Moody & Okwagbe, 2003; Aweke, 2007). β-cells of the pancreas which secretes insulin for regulation of blood glucose level may be susceptible to destruction in the presence of unstable free radicals, secreted by macrophages, T-cells, and natural killer cells intended for body immunity (Kokil *et al*., 2010). In light of this information, plants products that can lower blood sugar and also scavenge free radicals should be considered medically effective for treatment of diabetes. The indigenes of Ogba community of Rivers State have since leveraged the synergistic antimalarial property of boiled *C.afer* (leaf) and *Alchornea cordiflora* (leaf and stem) and their ability to treat kyphosis (Nna *et al*., 2019; Omokhua, 2011). It has also been reported that in Aluu community of Rivers State, most cough, measles and malaria cases have been traditionally treated by oral administration of *C.afer* stem juice extracts (Omokhua, 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemical Reagents

Some reagents were provided at the Biochemistry laboratory of Federal University of Technology Owerri and others were procured. All biochemical reagents, chemicals and materials used in this research are of standard analytical grade and they include; ethanol, hexane, sodium sulphate, chloroform, methanol, hydrogen tetraoxosulphate (VI) acid, potassium sulphate, copper sulphate, selenium powder, boric acid, hydrochloric acid, 2,6-dichlorophenol indophenol, ninhydrin, sodium metabisulphite, acetone, phenylalanine, 0.9% NaCl.

3.1.2 Equipment

Gas chromatography: BUCK M910, Column: RESTEK 15meter MXT-1 column, Amino acid analyzer: Applied Biosystems PTH Amino Acid Analyzer, Spectrophotometer: Bench centrifuge, Soxhlet extractor, Thimble, Kjeldahl flask, rotary evaporator, Microwave oven, Analytical balance.

3.2 Method

3.2.1 Collection of Plant Sample

The stem and leaf of *Costus afer* were freshly harvested from the University of Port Harcourt forestry and authenticated by a crop scientist from the University of Port Harcourt as *Costus afer*.

3.2.2 Sample Preparation

The leaves of *Costus afer* were separated from the stem and roots. The stem was debarked and the roots discarded. The leaf sample was air dried for a period of 14days while the stem sample was air-dried for a period 30 days. The dried leaf and stem samples were ground into powder using manual home grinder before proper analysis.

3.2.3 Collection of Blood Sample

The blood sample used for the analysis was collected with consent and approval of confirmed HbSS patients who attend clinic at Federal Medical Centre Owerri. The sample collection was done by qualified personnel from the hematology unit after careful and detailed explanation of the purpose of intended research project on the general health of HbSS patients.

3.2.4 Preparation of Blood Sample

0.20mL of the whole blood samples were used for the Fe^{2+}/Fe^{3+} ratio, while the remaining portions were collected into citrate-anticoagulant tubes to prevent clotting. At a revolution of 3000rpm done for 10 minutes, the erythrocytes were separated from the whole blood sample. This was done using an efficient bench centrifuge and with help of a Pasteur pipette, the plasma portion was carefully siphoned. Immediately after this, using repeated inversion, the erythrocytes were suspended in a volume of normal saline (0.9% NaCl) equivalent to the siphoned plasma. The erythrocyte suspension was then frozen at 0^0C and subsequently thawed to produce a haemolysate for polymerization experiment.

3.2.5 Phytochemical Screening of Leaf and Stem Samples

With reference to Kelly and Nelson (2014), characterization and quantification by gas chromatography of flavonoids, the phytochemical analysis was performed. 1g each of ground leaf and stem samples were weighed and transferred into separate test tubes and 15mL of ethanol was added to each. The test tube was allowed to react in a water bath at 60^0C for 60 minutes. The product of the reaction contained in the test tube was transferred to a separating funnel after the reaction time. The test tube was carefully and successfully washed with 20mL of ethanol, 10mL of cold water, 10mL of hot water and 3mL of hexane and all were transferred to the separating funnel containing the reaction product. These extracts were combined and washed three times with 10mL of 10%v/v ethanol aqueous solution. The solution was dried with anhydrous sodium sulphate and the solvent was evaporated. The sample was solubilized in 1000µL of hexane of which 200µL was transferred to a vial for analysis.

The quantification of the phytochemicals was done with an initial oven temperature of 200 ^{0}C which was later heated to 330 ^{0}C at a rate of 3 ^{0}Cmin^{-1} and was kept at this temperature for 5 minutes while the detector operated at 320 ^{0}C. The phytochemicals were determined by the ratio between the area and mass of internal standard and the area of the identified phytochemicals. The concentrations of the different phytochemicals were expressed in µg/g. The analysis was done separately for the leaf and stem samples.

3.2.6 Amino Acid Profile Determination

This was done with the method as described by AOAC (2005) with some modifications. The samples were dried at 700^0C to constant weight, defatted, hydrolyzed, evaporated in a rotary evaporator and loaded into the applied biosystems PTH (phenyl thiohydantoin) amino acid analyzer.

Each sample was defatted using chloroform/methanol mixture of ratio 2:1. About 4g of the sample was put in extraction thimble or wrapped in filter paper and extracted for 15 hours in Soxhlet extraction apparatus (AOAC, 2006).

The nitrogen determination was done by micro Kjeldahl method according to (AOAC, 2005) slightly modified. 250mg of sample was placed in Kjeldahl flask and about 200mg of catalyst mixture (potassium sulphate, copper sulphate and selenium powder) was added. $10cm^3$ of sulphuric acid was added to the flask containing the sample and catalyst mixture. The flask was heated gently for few minutes until frothing ceased and the heat was increased to digest for 1 hour 20 minutes. The digest was allowed to cool and $100cm^3$ of distilled water was added to make a known volume of the digest. $10cm^3$ aliquot of dilute solution of the digest was distilled by pipetting the volume into distillation chamber of micro Kjeldahl distillation apparatus. 10cm3 of 40% sodium hydroxide solution was added and it was steam-distilled into 10 cm^3 of 4 % boric acid containing mixed indicator titrate with standard 0.01N or 0.02N hydrochloric acid to grey end point.

$$\%N = \frac{(a-b) \times 14.0057 \times c \times 100}{d \times e}$$

a = titre value for the sample

b = titre value for the blank

c = volume to which digest is made up with distilled water

e = weight of dried sample (mg)

1.266g of defatted sample (leaf) and 0.800g (stem) were each weighed into glass ampoule. 7mL of 6NHCl was added and oxygen was expelled by passing nitrogen into the ampoule (this is to avoid possible oxidation of some amino acids during hydrolysis e.g., methione and cystine). The glass ampoule was then sealed with Bunsen burner flame and put in an oven preset at 105 $^0C \pm 5^0C$ for 22 hours. The ampoule was allowed to cool before broken open at the tip and the content was filtered to remove humins. The filtrate was then evaporated to dryness using rotary evaporator. The residue was dissolved with 5ml to acetate buffer (pH 2.0) and stored in plastic specimen bottles, which were kept in the freezer.

60µL of hydrolysate was dispensed into the cartridge of the analyzer. An integrator attached to the Analyzer calculates the peak area proportional to the concentration of each of the amino acids. This analysis was done separately for the individual samples.

The concentration of each amino acid is calculated using the formula below;

Concentration (g/100g protein) =NH x Width@ NH/2 x Sstd x C

Where Sstd = N E_{std} x Mol. Weight x µ AA_{std}

$$C = \frac{\text{Dilution x 16}}{\text{Sample Wt (g) x N \%}} \div \frac{\text{NH x W (nLeu)}}{\text{Vol. Loaded}}$$

3.2.7 Haemoglobin Polymerization Inhibition Test

The haemoglobin polymerization inhibition experiment was done using the original methods of Noguchi & Scheter, 1978; Iwu *et al*., 1988 and Nwaoguikpe *et al*., 1999. The turbidity of the polymerization mixture at 700nm using 2% solution of sodium metabisulphite as reductant or deoxygenating agent (Iwu *et al*., 1988) was a factor used to assess the polymerization of sickle cell haemoglobin.

4.4mL of 2% sodium metabisulphite ($Na_2S_2O_3$), 0.5mL normal saline (0.9% NaCl) and 0.1mL haemoglobin were pipetted into a cuvette, shaken and absorbance read in a spectrophotometer at 700nm every 2 minutes for 30 minutes. This was used as control and distilled water was used as blank for all assays. 4.4mL of 2% $Na_2S_2O_3$, 0.5mL of each extract and 0.1ml sickle haemoglobin (HbSS) solution are pipetted into the cuvette, for the test assay and the readings taken as stated above. The rates of haemoglobin polymerization for the control, extracts or fractions were estimated by calculating the change in optical density (OD_{700nm}) versus time in minutes. The rates were equally expressed as percentages concerning control. Alternatively, the rate can be calculated from the formula;

$$Rp = \frac{\text{Final OD} - \text{Initial OD}}{\text{Time}} = \frac{\Delta OD}{T}$$

$$Rp = \frac{OD_F - OD_I}{T}$$

Where Rp = Rate of Polymerization

OD_F= Final Optical Density

OD_I = Initial Optical Density

T = Time of Assay in minutes

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

Fe^{2+}/Fe^{3+} ratio was determined by the methods of Henry, (1974), while the oxygen affinity of haemoglobin and methaemoglobin were measured at 540nm and 630nm respectively. 0.02mL whole blood was lysed in 5.0mL of distilled water and 0.02mL normal saline to represent the control. The absorbance of haemoglobin (Hb) and methaemoglobin (MetHb) were measured at 540nm and 630nm to determine the %Hb and %metHb respectively. To determine the effect of the extract on Fe^{2+}/Fe^{3+} ratio; 0.02mL of each extract was added to 5.0mL of distilled water and 0.02mL of blood added and incubated for 60minutes in a test tube.

3.2.9 Extraction of Fat-Soluble (FAS) Fraction

50g of grounded samples (leaf and stem) were soaked in 2000mL of chloroform for 24 hours to defat it and obtain the fat-soluble fraction by filtration. The residue was kept for methanol extraction and the filtrate was subsequently evaporated in *vacuo* and the resulting fat soluble (FAS) extract weighed and volume recorded.

3.2.10 Methanol Extraction Process

The residue from the chloroform extraction was dried and soaked in 200mL of methanol (MeOH) of analytical grade for 2 hours. The solvent was filtered and the filtrate subjected to evaporation in vacuo. The weight and volume of methanol extract were taken

3.2.11 Determination of the Ascorbic Acid (Vitamin C) of the Extracts

The determination of the ascorbic acid (vitamin c) of each extract was carried out using the methods of AOAC. 1g of the ascorbic acid standard was dissolved in 100mL of distilled water and $10cm^3$ of the ascorbic acid was acidified with 2-3 drops of dilute hydrochloric acid. A burette was filled with a solution 2,6 – dichlorophenol of 0.01%. The indophenols solution was run into the ascorbic acid solution in the burette until there is a permanent pink solution. If Xcm^3 of the indophenol is required, $1cm^3$ of indophenols solution is equivalent to10mg/x vitamin C.

Having standardized the indophenols solution, $10cm^3$ of the test solution (each extract) was taken and treated similarly. Ascorbic acid reduces the coloured 2,6 – dichlorophenol to its leuco form. The solution is colourless at partial oxidation of ascorbic acid. A pink colouration of the acidified solution at slight excess of the indophenols indicates the complete oxidation of ascorbic acid.

3.2.12 Determination of Total Free Amino Acid of Extracts

0.1% Ninhydrin in acetone was diluted with water in the ratio of 1:4. The fat-soluble extract was diluted with ethanol in a 1:5 ratios and the methanol-soluble extract was diluted with water

in a ratio of 1:1. 20uL of each of the diluted extracts was added to 4mL portions of the dilute Ninhydrin.

4-20mg/mL of Phenylalanine was treated with 4mL portions of diluted ninhydrin. The resultant solution was heated to boiling for 5 minutes, cooled and absorbance taken from a spectrophotometer at 570nm using distilled water as a blank. A standard curve of absorbance against concentration was plotted and the values of concentration of free amino acids in the extracts were extrapolated from the plot.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

Results of all analysis are shown in tables

Table 1: Qualitative phytochemical analysis of n-hexane fraction of ethanol extracts of *Costus afer* leaf

Component	Presence or Absence in Sample
Dihydrocytisine	+
Ammodendrine	++
Spartein	+
Hydroxylupanine	++
Sapogenin	++
Tannin	+
Kaempferol	+
Ribalinidine	++
Anthocyanin	++
Flavone	++
Flavonone	+
Aphyllidine	++
Proanthocyanidin	+
Isolupanine	++
Epihedrine	++
Cyanogenic glycoside	+
Narigenin	++
Lectin	+

Key: ++ = Abundant + = trace

Table 2: Quantitative phytochemical analysis of n-hexane fraction of ethanol extracts of *Costus afer* leaf

Component	Concentration (µg/mL)
Dihydrocytisine	5.199
Ammodendrine	10.860
Spartein	7.910
Hydroxylupanine	5.574
Sapogenin	23.450
Tannin	9.648
Kaempferol	5.979
Ribalinidine	15.265
Anthocyanin	11.524
Flavone	12. 072
Flavonones	7.649
Aphyllidine	24.241
Proanthocyanidin	6.741
Isolupanine	31.380
Epihedrine	16.468
Cyanogenic glycosides	7.210
Narigenin	11.277

Table 3: Qualitative Phytochemical Analysis of n-hexane fraction of ethanol extracts of *Costus afer* stem

Component	Presence or Absence in Sample
Dihydrocytisine	+
Ammodendrine	+
Spartein	++
Catechin	+
Hydroxylupanine	+
Sapogenin	+
Tannin	+
Kaempferol	++
Cardiac glycoside	++
Ribalinidine	++
Anthocyanin	+
Flavone	++
Flavonone	+
Aphyllidine	+
Proanthocyanidin	+
Isolupanine	++
Epihedrine	+
Cyanogenic glycoside	+
Narigenin	+

Key: ++ = Abundant + = trace

Table 4: Quantitative phytochemical analysis of n-hexane fraction of ethanol extracts of *Costus afer* stem

Component	Concentration (µg/mL)
Dihydrocytisine	5.347
Ammodendrine	6.002
Spartein	10.121
Catechin	9.854
Hydroxylupanine	5.574
Sapogenin	5.192
Tannin	7.607
Kaempferol	15.401
Cardiac glycoside	2.907
Ribalinidine	15.114
Anthocyanin	5.717
Flavone	8.226
Flavonones	7.649
Aphyllidine	5.523
Proanthocyanidin	6.870
Isolupanine	29.296
Epihedrine	5.012
Cyanogenic glycosides	6.617
Narigenin	6.993

Table 5: Amino acid profile of fat-soluble extracts of *Costus afer* leaf

Amino Acid	Concentration g/100 g protein
Leucine	6.20
Lysine	3.63
Isoleucine	2.98
Phenylalanine	4.52
Tryptophan	0.92
Valine	4.30
Methionine	1.23
Proline	4.47
Arginine	5.00
Tyrosine	2.75
Histidine	2.11
Cystine	1.27
Alanine	4.44
Glutamic acid	10.14
Glycine	2.80
Threonine	3.00
Serine	3.21
Aspartic acid	7.32
Asparagine	5.30
Glutamine	6.21

Table 6: Amino acid profile of fat-soluble extracts of *Costus afer* stem

Amino Acid	Concentration g/100 g protein
Leucine	5.60
Lysine	4.64
Isoleucine	4.22
Phenylalanine	3.81
Tryptophan	0.73
Valine	4.10
Methionine	0.85
Proline	3.04
Arginine	4.90
Tyrosine	2.92
Histidine	3.00
Cystine	1.45
Alanine	3.07
Glutamic acid	11.65
Glycine	3.56

Table 7: The rates of polymerization, relative percent (%) polymerization and the relative percent (%) inhibition of HbSS of Extracts of *Costus afer* leaf and stem

Groups	Rate of Polymerization	Rel. (%) Polymerization	Rel. % Inhibition
I	0.008 ± 0.00^{a}	100.00 ± 0.00^{d}	0.00 ± 0.00^{a}
II	0.003 ± 0.00^{a}	43.04 ± 0.00^{b}	59.96 ± 0.00^{e}
III	0.001 ± 0.00^{a}	20.63 ± 0.00^{a}	81.90 ± 0.00^{g}
IV	0.003 ± 0.00^{a}	41.77 ± 0.00^{b}	58.24 ± 0.02^{d}
V	0.006 ± 0.00^{a}	77.22 ± 0.00^{c}	22.78 ± 0.00^{b}
VI	0.006 ± 0.00^{a}	73.42 ± 0.00^{c}	26.58 ± 0.00^{c}
VII	0.002 ± 0.00^{a}	25.32 ± 0.00^{a}	74.68 ± 0.00^{f}

Group values (Mean ± SD) with different Superscript(s) are significantly different at $p<0.05$, while groups with same superscript(s) are not.

Key: I = Control; **II =** Leaf Fat-Soluble Fraction (LFSF); **III** = Leaf Methanol-Soluble Fraction (LMSF); **IV** = Leaf Ethanol-Soluble Fraction (LESF); **V =**Stem Fat-Soluble Fraction (SFSF); **VI** = Stem Methanol-Soluble Fraction (SMSF); **VII =**Stem Ethanol-Soluble Fraction (SESF)

Table 8: *In vitro* effect of extracts of *Costus afer* stem on the Fe^{2+} / Fe^{3+} ratio of HbSS blood

Groups	%Hb (%)	%MetHb (%)	Fe^{2+}/Fe^{3+}	%Increase/Decrease (%)
I	83.60 ± 0.01^{b}	16.30 ± 0.17^{e}	5.13 ± 0.05^{b}	0.00 ± 0.00^{a}
II	92.63 ± 0.01^{f}	7.37 ± 0.01^{b}	12.56 ± 0.01^{f}	145.38 ± 0.95^{f}
III	89.31 ± 0.01^{c}	10.67 ± 0.01^{d}	8.37 ± 0.01^{c}	64.18 ± 0.11^{c}
IV	95.13 ± 0.01^{g}	4.87 ± 0.01^{a}	19.51 ± 0.02^{g}	282.94 ± 0.00^{g}
V	90.85 ± 0.01^{e}	9.15 ± 0.01^{c}	9.93 ± 0.01^{e}	94.64 ± 0.12^{e}
VI	89.44 ± 0.01^{d}	10.56 ± 0.01^{d}	8.47 ± 0.01^{d}	66.14 ± 0.11^{d}
VII	72.88 ± 0.01^{a}	27.12 ± 0.01^{f}	69 ± 0.00^{a}	47.24 ± 0.03^{b}

Group values (Mean ± SD) with different Superscript(s) are significantly different at $p<0.05$, while groups with same superscript(s) are not.

Key: I = Control; **II =** Leaf Fat-Soluble Fraction (LFSF); **III** = Leaf Methanol-Soluble Fraction (LMSF); **IV** = Leaf Ethanol-Soluble Fraction (LESF); **V =** Stem Fat-Soluble Fraction (SFF); **VI** = Stem Methanol-Soluble Fraction (SMSF); **VII =** Stem Ethanol-Soluble Fraction (SESF)

Table 9: Ascorbic Acid (Vitamin C) concentration of *Costus afer* leaf and stem extracts

Plant Part	Concentration (mg/100g)
Leaves	566.15 ± 87.10
Stem	297.59 ± 33.26

Values represent Mean ± Standard deviation from triplicate determination

Table 10: Total free amino acid concentrations of *Costus afer* leaf and stem extracts

Groups	Volume of Extract (ml)	Amino Acid Concentration (mg/ml)	Total FAA (permg/50g)
I	210.00 ± 0.00[a]	6.67 ± 0.06[f]	1,400.00 ±12.12[f]
II	205.00 ± 0.00[a]	2.11 ± 0.01[a]	432.55 ± 2.05[a]
III	215.00 ± 0.00[a]	5.10 ± 0.01[d]	1,097.22 ± 1.24[d]
IV	220.00 ± 0.00[a]	5.40 ± 0.01[e]	1,188.00 ± 2.20[e]
V	215.00 ± 0.00[a]	3.61 ± 0.01[b]	776.15 ± 2.15[b]
VI	220.00 ± 0.00[a]	4.51 ± 0.01[c]	992.93 ± 1.27[c]

Group values (Mean ± SD) with different Superscript(s) are significantly different at $p<0.05$, while groups with same superscript(s) are not.

Key: I = Leaf Fat-Soluble Fraction (LFSF); **II** = Leaf Methanol-Soluble Fraction (LMSF); **III**= Leaf Ethanol-Soluble Fraction (LESF); I**V =** Stem Fat-Soluble Fraction (SFSF); **V** = Stem Methanol-Soluble Fraction (SMSF); **VI =** Stem Ethanol-Soluble Fraction (SESF)

4.2 Discussion

The GC-MS phytochemical analysis of the leaf and stem n-hexane fraction of the ethanol extracts of *Costus afer* is displayed in Tables 1, 2, 3 and 4. The results in table 1 and 2 show that about 20 phytochemicals of different categories such as phenolics, terpenoids and alkaloids are contained in the extracts. Quantitatively, alkaloids are notably the most abundant. The phytochemical composition of the leaf and stem extracts are the same to a very large extent with slight variations in their concentrations as indicated in table 3 and 4. However, cardiac glycosides which have been reported to strengthen a failing heart and catechins which both have antimicrobial properties were present only in the extracts of the stem (Kepp*et al*., 2012). Although present in stem extract in trace amount, it was completely absent in the leaf extract. The result of the present study shows that *Costus afer* leaf and stem both have a high content of Isolupanine but more abundant in the leaf Isolupanine, the most abundant alkaloid present, prevents replication of viral particles in a host, especially SCD patients (Flora *et al*., 2018). Other secondary alkaloids present in both leaf and stem of *Costus afer* include: hydroxylupanine, ammodendrine, aphyllidine, spartein. A data by Deepak *et al*. (2016), confirms the biological activity of the alkaloids to include antimicrobial and anticancerous. Spartein, which was considerably found in the extract, prevents seizures and slows the induction of convulsive behavior (Fridha & Medina, 2016).

The amino acid profile of the two samples of *Costus afer* (leaf and stem,) were determined and the data were expressed as grams of amino acid per 100 g crude protein of sample. The nutritionally essential amino acids present in the stem and leaf extracts of *Costus afer* include histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. But their concentrations vary in both as indicated in tables 5 and 6 respectively. The nutritionally non-essential amino acids present include alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, proline, serine and tyrosine with varying concentrations in the two samples. Arginine, a basic non-essential amino was remarkably present at a concentration of 5.0g/mg in the leaf and 4.90g/mg in the stem. It contributes to prevention of polymer formation by sickle RBCs and improving the oxidant status of sickle RBCs (Nwaoguikpe, 1999; Elekwa *et al*., 2005; Nwaoguikpe *et al*., 2012). The amino acid profile of the two samples of *Costus afer* from this study show that glutamic acid had the highest concentration with values ranging from 10.14g/100g in the leaves to 11. 65/100g in stem. Tryptophan had the lowest value with values ranging from 0.73/100g in stems to 0.93 /100g in leaves. Phenylalanine, serine, lysine, arginine and asparagine have been reported as antisickling amino acids (Nwaoguikpe *et al*., 1999; Nwaoguikpe *et al*., 2012)

The results of polymerization inhibition activity tests in table 5 and 6 show that the methanol extract and ethanol extract of *Costus afer* leaf and stem respectively, exhibited a high % inhibition on HbSS polymerization (81.90% and 74.68% respectively) compared to other fractions with varying percent inhibitions. Supporting literature of Eaton and Bunn, (2017) explains that the root cause of pathology in sickle cell disease is polymerization of haemoglobin and agents that increases the delay time allow more cells to escape the blood circulation through the arterioles, capillaries and venules before fibers form. The ability of the different fractions of leaf and stem extracts of *Costus afer* to inhibit polymerization conforms to with previous research results or findings.

The iron contained in normal haemoglobin exists in reduced ferrous state (Fe^{2+}). When haemoglobin iron becomes oxidized to the ferric state (Fe^{3+}), it is no longer able to bind oxygen and is called methaemoglobin. In healthy individuals, red blood cells contain a cytochrome-b5-based enzymatic system which reverses this reaction and Fe is restored back to its original state (Tietz, 1975).

The results in table 7 show the significant improvement in the Fe^{2+}/Fe^{3+}ratio of all fractions of the samples. The ethanol soluble fraction of *Costus afer* leaf exhibits the highest percent increase in $Fe^{2+}/Fe^{3+}$and this improved the oxygen affinity of sickle haemoglobin. It is logical to conclude that the extracts, though at varying levels, can stabilize the erythrocyte by reducing the fragility of red blood cells.

Cell membrane of cells in SCD patients are more susceptible to membrane damage than cells in healthy individuals because the former produce more reactive oxygen species (ROS). Vitamin C is a powerful anti-oxidant capable of inhibiting ROS formation in a variety of situations by functioning as an electron donor to reduce molecular oxygen (Eric Allen *et al*., 2017). Table 7 shows two concentrations of vitamin C (ascorbic acid) in mg/100g in leaf and stem extracts of *Costus afer*. This proves that the samples have great tendency to inhibit cell membrane damage by ROS. Moreover, vitamin C is a known antisickling agent (Nwaoguikpe, 2009).

Table 8 shows varying concentration of TFAA (per mg/50g) of different extracts of *Costus afer*. The values range from 432.55 ± 2.05^{a}mg/50g in the LFAS to in the control $1,400.00 \pm 12.12^{f}$mg/50g. Antisickling action of phytomedicines may be attributed to the presence of amino acids as reported (Abraham *et al*., 1982). In another research Ogoda *et al*. (2002) explains that the presence of phenylalanine in *Cajanus cajan*, is believed to be the reason for their antisickling effect.

Conclusion

Bone marrow transplant and gene therapy remain the only cure for sickle cell disease. Besides the cost of undergoing such treatments, the procedures are not without risk. Some of the substantial risks include graft vs host disease (occurs during bone marrow transplant), negative effect of chemotherapy (which is required to kill old marrow cells before transplant or gene therapy is done).

When cure is not available, the goal of phytomedicines is to control the disease or act as treatment pattern with palliative intent through targeted therapies. This research has proven that *Costus afer* a phytomedicine that has been reportedly used to manage such chronic diseases as diabetes (Boison *et al*., 2019) contain substantial number of bioactive components. Therefore, it should be considered effective against sickle cell disease based on its evidence-based clinical relevance.

Recommendation

The only cure for sickle cell disease since the 21st century is bone marrow transplant. This procedure is expensive and donors who should be stable family members, are hard to come by. The high cost associated with modern drugs coupled with increasing poverty level in most African countries makes the use of phytomedicines a better alternative. Phytomedicines have increase the life expectancy of HbSS patients as reported from previous research (Ameh *et al*., 2012). Scientific evaluation of more accessible and available phytomedicines like *Costus afer* should be greatly considered.

Contribution to Knowledge

Research on sickle cell disease has for many decades provided effective management therapies using phytomedicine. This investigation, in addition to previous research on the traditional use of *Costus afer* for management of such disease as diabetes and malaria has found *Costus afer* beneficial in effectively managing sickle cell disease.

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APPENDIX I

Haemoglobin polymerization Inhibition Test

Sample	Time(minutes)	Rate of Polymerization	ΔAbsorbance
HbSS (Control)	0	0.369	0.00
	2	0.371	0.002
	4	0.374	0.005
	6	0.381	0.012
	8	0.385	0.016
	10	0.396	0.027
	12	0.413	0.044
	14	0.435	0.066
	16	0.452	0.083
	18	0.476	0.107
	20	0.493	0.124
	22	0.512	0.143
	24	0.532	0.163
	26	0.565	0.196
	28	0.588	0.219
	30	0.606	0.237

$$\text{Rate of Polymerization (Rp)} = \frac{\text{Final OD} - \text{Initial OD}}{\text{Time}}$$

$$\text{Rp} = \frac{0.237 - 0.00}{30} = 0.0079$$

Sample	Time(minutes)	Rate of Polymerization	∆Absorbance
FAS (leaves)	0	0.196	0.00
	2	0.216	0.02
	4	0.232	0.036
	6	0.248	0.052
	8	0.257	0.061
	10	0.264	0.068
	12	0.267	0.071
	14	0.271	0.075
	16	0.276	0.080
	18	0.279	0.083
	20	0.284	0.088
	22	0.285	0.089
	24	0.290	0.094
	26	0.296	0.100
	28	0.299	0.103
	30	0.298	0.102

$$\text{Rate of Polymerization (Rp)} = \frac{\text{Final OD} - \text{Initial OD}}{\text{Time}}$$

$$\text{Rp} = \frac{0.102 - 0.00}{30} = 0.0034$$

Sample	Time(minutes)	Rate of Polymerization	∆Absorbance
MWS (leaves)	0	0.247	0.00
	2	0.253	0.006
	4	0.271	0.024
	6	0.277	0.030
	8	0.287	0.040
	10	0.292	0.045
	12	0.297	0.050
	14	0.304	0.057
	16	0.309	0.062
	18	0.310	0.063
	20	0.311	0.064
	22	0.311	0.064
	24	0.308	0.061
	26	0.300	0.053
	28	0.297	0.050
	30	0.296	0.043

$$\text{Rate of Polymerization (Rp)} = \frac{\text{Final OD} - \text{Initial OD}}{\text{Time}}$$

$$\text{Rp} = \frac{0.043 - 0.00}{30} = 0.00163$$

Sample	Time(minutes)	Rate of Polymerization	∆Absorbance
ESF (leaves)	0	0.524	0.00
	2	0.557	0.033
	4	0.578	0.054
	6	0.593	0.069
	8	0.602	0.078
	10	0.607	0.083
	12	0.611	0.087
	14	0.614	0.090
	16	0.615	0.091
	18	0.621	0.097
	20	0.622	0.098
	22	0.624	0.10
	24	0.625	0.101
	26	0.629	0.105
	28	0.622	0.098
	30	0.623	0.099

$$\text{Rate of Polymerization (Rp)} = \frac{\text{Final OD} - \text{Initial OD}}{\text{Time}}$$

$$\text{Rp} = \frac{0.099 - 0.00}{30} = 0.0033$$

Sample	Time(minutes)	Rate of Polymerization	∆Absorbance
FAS (stem)	0	0.637	0.00
	2	0.618	-0.0019
	4	0.613	-0.024
	6	0.617	-0.02
	8	0.632	-0.005
	10	0.636	-0.001
	12	0.648	0.011
	14	0.656	0.019
	16	0.670	0.033
	18	0.686	0.049
	20	0.702	0.065
	22	0.716	0.079
	24	0.739	0.102
	26	0.755	0.118
	28	0.787	0.150
	30	0.820	0.183

$$\text{Rate of Polymerization (Rp)} = \frac{\text{Final OD} - \text{Initial OD}}{\text{Time}}$$

$$\text{Rp} = \frac{0.183 - 0.00}{30} = 0.006$$

Sample	Time(minutes)	Rate of Polymerization	ΔAbsorbance
MWS (stem)	0	0.0574	0.00
	2	0.592	0.018
	4	0.606	0.032
	6	0.605	0.031
	8	0.591	0.017
	10	0.583	0.009
	12	0.593	0.019
	14	0.613	0.039
	16	0.638	0.064
	18	0.663	0.089
	20	0.683	0.109
	22	0.698	0.124
	24	0.713	0.139
	26	0.724	0.150
	28	0.737	0.163
	30	0.747	0.173

$$\text{Rate of Polymerization (Rp)} = \frac{\text{Final OD} - \text{Initial OD}}{\text{Time}}$$

$$\text{Rp} = \frac{0.173 - 0.00}{30} = 0.0058$$

Sample	Time(minutes)	Rate of Polymerization	ΔAbsorbance
ESF (stem)	0	0.233	0.00
	2	0.237	0.004
	4	0.244	0.011
	6	0.252	0.019
	8	0.260	0.027
	10	0.268	0.035
	12	0.271	0.038
	14	0.276	0.043
	16	0.282	0.049
	18	0.285	0.052
	20	0.288	0.055
	22	0.289	0.056
	24	0.290	0.057
	26	0.292	0.059
	28	0.292	0.059
	30	0.293	0.060

$$\text{Rate of Polymerization (Rp)} = \frac{\text{Final OD} - \text{Initial OD}}{\text{Time}}$$

$$\text{Rp} = \frac{0.06 - 0.00}{30} = 0.002$$

Determination Of Fe^{2+} /Fe^{3+}

HbSS Control @540nm = 0.210, 0.210, 0.211

@630nm= 0.093, 0.092, 0.093

$$\%Hb= \frac{(A540)^2}{(A540)^2 \text{ x } (A630)^2} x \frac{100}{1}$$

$$\%Hb \text{ (control)} = \frac{(0.210)^2}{(0.210)^2 \text{ x } (0.093)^2} \text{ x } \frac{100}{1}$$

$$\frac{0.0441}{0.0441 \text{ x } 0.008649} \text{ x } \frac{100}{1}$$

$$\frac{0.0441}{0.052749} \text{ x } \frac{100}{1}$$

$$= 83.60$$

$$\%mHb = \frac{(A630)^2}{(A630)^2 \text{ x } (A540)^2} \text{ x } \frac{100}{1}$$

$$\%mHb \text{ (control)} = \frac{(0.093)^2}{(0.093)^2 \text{ x } (0.210)^2} \text{ x } \frac{100}{1}$$

$$\frac{0.008649}{0.008649 \text{ x } 0.0441} \text{ x } \frac{100}{1}$$

$$\frac{0.008649}{0.052749} \text{ x } \frac{100}{1}$$

$$= 16.40$$

FAS (leaves) @540nm = 0.280, 0.279, 0.280

@630nm= 0.079, 0.078, 0.079

$$\%Hb \text{ FAS (leaves)} = \frac{(A540)^2}{(A540)^2 \text{ x } (A630)^2} \text{ x } \frac{100}{1}$$

$$\%Hb \text{ FAS (leaves)} = \frac{(0.280)^2}{(0.280)^2 \text{ x } (0.079)^2} \text{ x } \frac{100}{1}$$

$$\frac{0.0784}{0.0784 \text{ x } 0.006241} \text{ x } \frac{100}{1}$$

$$\frac{0.0784}{0.084641} \text{ x } \frac{100}{1}$$

= 92.63

%mHb = $\frac{(A630)^2}{(A630)^2 \text{ x } (A540)^2}$ x $\frac{100}{1}$

%mHb FAS (leaves) = $\frac{(0.079)^2}{(0.079)^2 \text{ x } (0.280)^2}$ x $\frac{100}{1}$

$\frac{0.006241}{0.006241 \text{ x } 0.0784}$ x $\frac{100}{1}$

$\frac{0.006241}{0.084641}$ x $\frac{100}{1}$

= 7.37

MWS (leaves) @540nm = 0.318, 0.318, 0.317

@630nm= 0.110, 0.110, 0.111

%Hb MWS (leaves) = $\frac{(A540)^2}{(A540)^2 \text{ x } (A630)^2}$ x $\frac{100}{1}$

%Hb MWS (leaves) = $\frac{(0.318)^2}{(0.318)^2 \text{ x } (0.110)^2}$ x $\frac{100}{1}$

$\frac{0.101124}{0.101124 \text{ x } 0.0121}$ x $\frac{100}{1}$

$\frac{0.101124}{0.113224}$ x $\frac{100}{1}$

= 89.31

%mHb = $\frac{(A630)^2}{(A630)^2 \text{ x } (A540)^2}$ x $\frac{100}{1}$

%mHb MWS (leaves) = $\frac{(0.110)^2}{(0.110)^2 \text{ x } (0.318)^2}$ x $\frac{100}{1}$

$\frac{0.0121}{0.0121 \text{ x } 0.101124}$ x $\frac{100}{1}$

$\frac{0.0121}{0.113224}$ x $\frac{100}{1}$

= 10.69

ESF (leaves) @540nm = 0.367, 0.366, 0.367

@630nm= 0.083, 0.083, 0.082

%Hb ESF (leaves) = $\frac{(A540)^2}{(A540)^2 \text{ x } (A630)^2}$ x $\frac{100}{1}$

%Hb ESF (leaves) = $\frac{(0.367)^2}{(0.367)^2 \text{ x } (0.083)^2}$ x $\frac{100}{1}$

$\frac{0.134689}{0.134689 \text{ x } 0.006889}$ x $\frac{100}{1}$

$\frac{0.134689}{0.141578}$ x $\frac{100}{1}$

= 95.13

%mHb = $\frac{(A630)^2}{(A630)^2 \text{ x } (A540)^2}$ x $\frac{100}{1}$

%mHb ESF (leaves) = $\frac{(0.083)^2}{(0.083)^2 \text{ x } (0.367)^2}$ x $\frac{100}{1}$

$\frac{0.006889}{0.006889 \text{ x } 0.134689}$ x $\frac{100}{1}$

$\frac{0.006889}{0.141578}$ x $\frac{100}{1}$

= 4.87

FAS (stem) @540nm = 0.271, 0.272, 0.271

@630nm= 0.086, 0.087, 0.086

%Hb FAS (stem) = $\frac{(A540)^2}{(A540)^2 \text{ x } (A630)^2}$ x $\frac{100}{1}$

%Hb FAS (stem) = $\frac{(0.271)^2}{(0.271)^2 \text{ x } (0.086)^2}$ x $\frac{100}{1}$

$\frac{0.073441}{0.073441 \text{ x } 0.007396}$ x $\frac{100}{1}$

$\frac{0.073441}{0.080839}$ x $\frac{100}{1}$

= 90.85

%mHb = $\frac{(A630)^2}{(A630)^2 \text{ x } (A540)^2}$ x $\frac{100}{1}$

%mHb FAS (stem) = $\frac{(0.086)^2}{(0.086)^2 \text{ x } (0.271)^2}$ x $\frac{100}{1}$

$\frac{0.007396}{0.007396 \text{ x } 0.073441}$ x $\frac{100}{1}$

$\frac{0.007396}{0.080839}$ x $\frac{100}{1}$

= 9.15

MWS (stem) @540nm = 0.326, 0.326, 0.326

@630nm= 0.112, 0.111, 0.112

%Hb MWS (stem) = $\frac{(A540)^2}{(A540)^2 \text{ x } (A630)^2}$ x $\frac{100}{1}$

%Hb MWS (stem) = $\frac{(0.326)^2}{(0.326)^2 \text{ x } (0.112)^2}$ x $\frac{100}{1}$

$\frac{0.106276}{0.106276 \text{ x } 0.012544}$ x $\frac{100}{1}$

$\frac{0.106276}{0.11882}$ x $\frac{100}{1}$

= 89.44

%mHb = $\frac{(A630)^2}{(A630)^2 \text{ x } (A540)^2}$ x $\frac{100}{1}$

%mHb MWS (stem) = $\frac{(0.112)^2}{(0.112)^2 \text{ x } (0.326)^2}$ x $\frac{100}{1}$

$\frac{0.012544}{0.012544 \text{ x } 0.106276}$ x $\frac{100}{1}$

$\frac{0.012544}{0.11882}$ x $\frac{100}{1}$

= 10.56

ESF (stem) @540nm = 0.241, 0.242, 0.241

@630nm= 0.147, 0.147, 0.147

%Hb ESF (stem) = $\frac{(A540)^2}{(A540)^2 \text{ x } (A630)^2}$ x $\frac{100}{1}$

%Hb ESF (stem) = $\frac{(0.241)^2}{(0.241)^2 \text{ x } (0.147)^2}$ x $\frac{100}{1}$

$$\frac{0.058081}{0.058081 \text{ x } 0.021609} \text{ x } \frac{100}{1}$$

$$\frac{0.058081}{0.07969} \text{ x } \frac{100}{1}$$

= 72,88

$$\%\text{mHb} = \frac{(A630)^2}{(A630)^2 \text{ x } (A540)^2} \text{ x } \frac{100}{1}$$

$$\%\text{mHb ESF (stem)} = \frac{(0.147)^2}{(0.147)^2 \text{ x } (0.241)^2} \text{ x } \frac{100}{1}$$

$$\frac{0.021609}{0.021609 \text{ x } 0.058081} \text{ x } \frac{100}{1}$$

$$\frac{0.021609}{0.07969} \text{ x } \frac{100}{1}$$

= 27.12

Formula for Conversion of Glutamate and Aspartate to Glutamine and Asparagine Respectively

$$\frac{4.2}{100} \text{ x } \frac{Glx}{1} = \text{Gln}$$

$$\frac{6.3}{100} \text{ x } \frac{Glx}{1} = \text{Glu}$$

Where Glx= 6.21

$$\frac{4.3}{100} \text{ x } \frac{Asx}{1} = \text{Asn}$$

$$\frac{5.3}{100} \text{ x } \frac{Asx}{1} = \text{Asp}$$

Where Asx = 5.30

Table 3: Results of Amino Acid Profile of Fat-Soluble Extracts of *Costus afer* Leaf

Amino Acid	Net height (mm)	NH/2 (mm)	Width@NH/2 (MM)	Sstd	Concentration g/100g protein
Leucine	106	53.00	1.00	5.91	6.20
Lysine	68.5	34.25	1.00	5.37	3.63
Isoleucine	45.5	22.75	1.00	6.63	2.98
Phenylalanine	25.5	12.75	1.00	17.96	4.52
Norleucine			1.00		Internal Standard
Tryptophan	17.5	8.75	1.00	5.32	0.92
Valine	73.5	36.75	1.00	5.92	4.30
Methionine	23	11.50	1.00	5.41	1.23
Proline	22	11.00	1.00	20.56	4.47
Arginine	29	14.50	1.00	17.42	5.00
Tyrosine	8	4.00	1.00	34.84	2.75
Histidine	33	16.50	1.00	6.47	2.11
Cystine	10.5	5.25	1.00	12.26	1.27
Alanine	58.5	29.25	1.00	7.68	4.44
Glutamic acid	67	33.25	1.00	15.33	10.14
Glycine	59	29.50	1.00	4.81	2.80
Threonine	54	27.00	1.00	5.62	3.00
Serine	59.5	29.75	1.00	5.47	3.21
Aspartic acid	118	59.00	1.00	6.28	7.32

Table 4: Results of Amino Acid Profile of Fat-Soluble Extracts of *Costus afer* Stem

Amino Acid	Net height (mm)	NH/2 (mm)	Width@NH/2 (MM)	Sstd	Concentration g/100g protein
Leucine	96	48.00	1.00	5.91	5.60
Lysine	87.5	43.75	1.00	5.37	4.64
Isoleucine	64.5	32.25	1.00	6.63	4.22
Phenylalanine	21.5	10.75	1.00	17.96	3.81
Norleucine			1.00		Internal Standard
Tryptophan	14	7	1.00	5.32	0.73
Valine	70	35.00	1.00	5.92	4.10
Methionine	16	8.00	1.00	5.41	0.85
Proline	15	7.50	1.00	20.56	3.04
Arginine	28.5	14.25	1.00	17.42	4.90
Tyrosine	8.5	4.25	1.00	34.84	2.92
Histidine	47	23.50	1.00	6.47	3.00
Cystine	12	6.00	1.00	12.26	1.45
Alanine	40.5	20.25	1.00	7.68	3.07
Glutamic acid	77	38.50	1.00	15.33	11.65
Glycine	75	37.50	1.00	4.81	3.56
Threonine	65	32.50	1.00	5.62	3.61
Serine	74.5	37.25	1.00	5.47	4.02
Aspartic acid	144	72.00	1.00	6.28	8.93