EVALUATION OF THE EFFECT OF Detarium microcarpum (ETHYL ACETATE FRACTION) STEM BARK ON HAEMOGLOBIN POLYMERIZATION (in vitro)

BY

CHIKUNIE OSOBUAGBAMIGBE PRAISE

BMS1600160

A PROJECT SUBMITTED TO THE DEPARTMENT OF MEDICAL BIOCHEMISTRY, SCHOOL OF BASIC MEDICAL SCIENCES, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF BACHELOR OF SCIENCE, BSc. (HONS) MEDICAL BIOCHEMISTRY, OF THE UNIVERSITY OF BENIN, BENIN CITY.

JULY, 2021

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CERTIFICATION PAGE

We the undersigned hereby certify that Chikunie Osobuagbamigbe Praise carried out this work, in the department of Medical Biochemistry, University of Benin, Benin City and we approve same as adequate in scope and quality for the award of Bachelors of Science Degree (B.sc) in Medical Biochemistry.

Signed:

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Mrs. Merit Ayevbuomwan **Date**

(Project Supervisor)

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Prof. A. A. Omonkhua **Date**

(Head of Department)

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External Supervisor **Date**

DEDICATION

This is dedicated to God Almighty the giver of life and knowledge, for giving me wisdom, strength and good health to complete this work. I also dedicate this work to my aunt, Mrs Okonoboh Patience and my parents Mr and Mrs Godwin Chikunie whose love, support and care has nurtured me all the way and whose encouragement made sure that I gave all it takes to finish that which I have started.

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I appreciate and exalt God for strengthening me and preserving my life from the beginning till the end of the research work.

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I want to also appreciate my siblings Favor, God power and Freedom Chikunie for their support and encouragement throughout the course of this work.

Thank you all for making me who I have become.

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LIST OF ABBREVIATIONS

Hb	Hemoglobin
EDTA	Ethylenediaminetetraacetic acid
D.Microcarpum	Detarium Microcarpum
HBB	Beta-globin
HbF	Fetal Hemoglobin
HbS	Sickle Hemoglobin
HbD	Hemoglobin D disease
SCD	Sickle Cell Disease/Disorder
p-hydroxybenzoic acid	para -hydroxybenzoic acid
WHO	World Health Organization
RBC	Red blood cells

ABSTRACT

Reports have shown that extracts of Detarium Microcarpum possess potent pharmacological properties. The aim of this research was to investigate the in vitro antisickling property of ethyl acetate extracts of Detarium Microcarpum stem bark. The stem bark was first ground to powder and soaked in ethyl acetate to obtain an ethyl acetate soluble fraction. The sickling of the red blood cells (RBCs) was introduced using sodium metabisulphite followed by treatment with ethyl acetate extract, Phosphate buffer saline and p-hydroxybenzoic acid. Sickling of red cells occur as a result of polymerization of deoxygenated HbS molecules, so that, they become stacked linearly. In vitro studies have revealed that plant extracts altered the polymerization of deoxyHbS molecules. Therefore, the present study was aimed at determining the effect of Detarium microcarpum stem back on haemoglobin polymerization. About 5ml of venous blood was collected from each Sickle cell patient with a sterile syringe. The blood samples were washed thrice with Phosphate Buffered Saline (PBS) using standard procedures and the resulting packed cell was used for haemoglobin polymerization assay. Detarium microcarpum is a legume tree shrub belonging to the family of **Fabacae**. Its roots, stem bark, leaves and fruits are known to possess medicinal properties. The in vitro haemoglobin polymerization properties of Ethyl Acetate (EA) fraction of D. microcarpum stem bark was evaluated using blood samples obtained from forty confirmed sickle cell disease patients using standard techniques. At the end of the research it was observed that D. microcarpum extract significantly ($p<0.05$) reduced polymerization of haemoglobin at t/90min with a reduction of about 46.05% when compared to the control (PBS+HbSS Blood sample) which was 90%. This was most significant ($p<0.05$) at t/40min which was 20% and t/90min which was 46.05% against the control which was 25% and 90%. A similar trend was also observed when D. microcarpum extract was compared to the standard (p-hydrozybenzoic acid; reference antisickling drug) with a significant

($p<0.05$) percentage reduction of 70% at t/90min when compared to the Control. This result shows an inhibition of haemoglobin polymerization in the test group treated with D. microcarpum. Conclusively, the study showed that the extract of Detarium Microcarpum exacerbated polymerization of deoxyHbS molecules in a concentration and time dependent manner. The Ethyl Acetate extract of Detarium Microcarpum demonstrated the most significant antisickling effect with a potential for use in the clinical management of Sickle Cell Disease (SCD).

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Humans have relied on nature throughout their ages to cater for their basic needs including medicines to cure a wide spectrum of diseases. Plants have formed the basis for sophisticated systems of traditional medicines. For therapeutic agents many of the presently known lead compounds are natural products or their derivatives. Ethnomedicinal studies play a vital role to discover new drugs from indigenous medicinal plants. Green pharmaceuticals are getting popularity and extraordinary importance because vast opportunities for new drug discoveries are provided by the unmatched availability of chemical diversity and natural products either as pure compounds or as homogenous plant extracts (Ghulam *et al.,* 2017). Therefore, in recent years the demand for herbal medicines and several natural products from a variety of plant species is consistently increasing. In spite of being an agricultural country and having different ecological regions, the medicinal plants of Pakistan have not been explored for their secondary metabolites which are responsible for treating different diseases. Although, huge importance of different extracts of medicinal plants have been reported for their different activities such as antimicrobial, anti-cancerous, antiviral and antioxidant but complete biochemical profiling of these medicinal plants is lacking (Ghulam *et al.,* 2017).

1.2 General Characteristics

Plants had been used for medicinal purposes long before recorded history. Ancient Chinese and Egyptian papyrus writings describe medicinal uses for plants as early as 3,000 BC. Indigenous cultures (such as African and Native American) used herbs in their healing rituals, while others developed traditional medical systems (such as Ayurveda and Traditional Chinese Medicine) in which herbal therapies were used (Odoh Uchenna Estella and Ene Chidiebere, 2020). Ethnomedicinal studies play a vital role to discover new drugs from indigenous medicinal plants and

green pharmaceuticals are getting popularity and extraordinary importance (Ghulam *et al.,* 2017), because vast chances for new drug discoveries are provided by the unrivaled availability of chemical diversity and natural products either as pure compounds or as homogenous plant extracts (Jigna *et al.,* 2007).

Detarium microcarpum is a plant indigenous to Africa, which occurs naturally in many African countries, particularly in savannah regions. Its leaves and fruits are used mainly as food and as folk medicine. It has anti-diabetic, antioxidant, and hepatitis C inhibitor properties and has been traditionally utilized in cancer treatment (Hinawi *et al.,* 2018).

Detarium microcarpum is an African tree belonging to the Fabaceae family (Dayamba *et al.,* 2016). It is a multipurpose species, with a wide range of uses due to its medicinal properties, edible fruit (eaten raw, cooked or made into flour with many uses of its own) and hardwood used as fuel-wood. Among the Ibo tribe of south eastern Nigeria, the plant known as "Ofo" is believed to be a "religious" tree which grows in God's own compound, symbolizing truth and honestly. It is the most investigated specie of the genus because of its popular use in African traditional medicine (Odoh and Ene, 2020). As far as development rate, the shoots of the storage compartment can achieve a tallness of 1.5–2 m in 1–2 years and are considerably more overwhelming than seedlings which on proximate develop to 0.6 m following 3 years and may achieve 1.5 m in 4 years. It blossoms amid the rainy season (July to September/November), yet the principle blooming period just keeps going up to 8 days. It proves to be fruitful from September – January/May and in November; the tree sheds its leaves and delivers new leaves in March (Mariod *et al.,* 2019). It is often used as a folk cure to treat diseases through its medicinal properties (Meda *et al.,* 2017). In Mali, D. microcarpum leaves are used as a fertiliser and, in Burkina Faso, sometimes used as masks. Its sweet fruit (length: 4 cm, width: 2.5 cm) acts as a sugar substitute, when the pulp powder is used in the preparation of cake and couscous and can be stored for 1–3 years at ambient temperature in jute bags. The seeds can be used to make frankincense, and women in some African countries wear

them in necklaces. D. microcarpum wood is widely used as firewood, which burns even when wet. In addition, Dinka women in Sudan heat the roots to use as a perfume (Dayamba *et al.,* 2011).

D. microcarpum fruit are source of vitamin C (3.2 mg/100 g) and contain about 4.8 g/100 g protein. In addition, the fruit contains up to 64.5 g/100 g sugar. The major soluble sugars in alcoholic extracts of dehulled flour are sucrose, raffinose, glucose, fructose, and stachyose (Hinawi *et al.,* 2018). Lamien-Meda *et al.,* (2008) reported that D. microcarpum fruit contains the highest proportion of antioxidants, flavonoids, and total phenols among 14 types of African fruits (Hinawi *et al.,* 2018).

A preliminary screening of pharmacognostic studies on the stem bark revealed the presence of saponins, tannins, flavonoids, alkaloids, cardiac glycosides and carbohydrates as bioactive compounds. A co-chromatographic examination reported the existence of catechol, gallic corrosive, phloroglucinol, pyragallol and quercetin (Sani *et al.,* 2014).

1.3 Aim of Study

To investigate and validate the anti-sickling potential of the plant (Detarium microcarpum) in the management of sickle cell anaemia.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Sickle Cell Disease

Sickle cell disease (SCD) is a hereditary chronic hemolytic anemia with numerous clinical consequences (Janet *et al.,* 2012). Sickle cell disease (SCD) is an umbrella term that defines a group of inherited diseases (including sickle cell anaemia (SCA), HbSC and HbSβ-thalassaemia, characterized by mutations in the gene encoding the haemoglobin subunit β (HBB) (Gregory J. Kato *et al.,* 2018**).** Sickle cell disease (SCD) is a monogenic disorder that afflflicts approximately 100,000 Americans and millions of people worldwide. It is characterized by hemolytic anemia, vaso-occlusive crises, relentless end-organ injury, and premature death (Sargam *et al.,* 2018). Sickle cell disease (SCD) is a hereditary chronic hemolytic anemia with numerous clinical consequences. Intravascular sickling of red blood cells leads to multiorgan dysfunction. Sickle cell disease (SCD; drepanocytosis) is the most common of the genetic diseases and is a significant public health problem. Most commonly seen in the tropics in sub-Saharan Africa, the Middle East, and parts of India, as a result of the changing demographics in Europe and North America, SCD can now be seen virtually everywhere. The incidence of SCD varies by state, race and ethnicity (Gregory J. Kato *et al.,* 2018). In the United States the incidence is about 1:5000, whereas the incidence in African American children is about 1 in 500. (Janet *et al.,* 2012).

Sickle cell trait is characterized by the inheritance of a normal hemoglobin gene (HbA) from 1 parent and an abnormal, mutated 1-globin gene, the sickle hemoglobin gene (HbS), from the other parent. In sickle cell disease, 2 abnormal allemorphic hemoglobin genes are inherited, of which at least 1 must be the sickle hemoglobin. In the homozygous sickle cell disease (HbSS), both abnormal hemoglobins are HbS. A normal adult hemoglobin is made from a combination of 2 -globin protein chains with 2-globin chains and heme. The 1-globin gene is located on the short arm of chromosome

11. Approximately 150 diseases have been linked to this same chromosome 11 (Geoffrey *et al.,* 2009).

Because of a point mutation in the β-globin gene, abnormal hemoglobin molecules are created with a hydrophobic motif that is exposed in its deoxygenated state. Once exposed, the β1 and β2 chains of separate hemoglobin molecules bind and polymerize. This crystallization process disrupts the cellular architecture and promotes cellular dehydration with its associated physical and oxidative stresses. Thus, at low oxygen states, the red blood cell's hemoglobin precipitates into long crystals that cause it to elongate, morphologically switching into a "sickled" red blood cell (Janet I. Malowany *et al.,* 2012). Reperfusion injury contributes to sickle pathophysiology builds on the hypothesis that the sticky, stiff, oxidizing sickle red cell provokes inflammation as it obstructs blood flow (Orah, 2000).

Sickle cell disease (SCD) is a group of inherited disorders caused by mutations in HBB, which encodes haemoglobin subunit β. The incidence is estimated to be between 300,000 and 400,000 neonates globally each year, the majority in sub-Saharan Africa. Haemoglobin molecules that include mutant sickle β- globin subunits can polymerize; erythrocytes that contain mostly haemoglobin polymers assume a sickled form and are prone to haemolysis. Sickle cell anaemia (SCA) is the consequence of abnormal haemoglobin production due to an inherited point mutation in the β-globin gene. The resulting haemoglobin tetramer is poorly soluble when deoxygenated, and when this is prolonged, intracellular gelation of sickle haemoglobin occurs, followed by haemoglobin polymerization. If many cycles of sickling and unsickling occur, the red cell membrane will be disrupted leading to haemolysis and vaso-occlusive events (Andrea *et al.,* 2019).

2.1.1 Prognosis and epidemiology

There has been a recent explosion in the interest among investigators in both the hematology academic community and in the pharmaceutical industry to develop new treatments for sickle cell disease, as indicated by the large number of active clinical protocols (William and Franklin, 2017). Sickle cell anemia, a disease having both hemolytic and vaso-occlusive components, has been subjected to intense scrutiny at the clinical, cellular, biochemical, and molecular levels (Robert, 2021).

In a diagnostic laboratory the identification and characterization of HbS in patients with sickle cell anemia is routinely performed using gel electrophoresis and/or an automated cation exchange chromatographic method. Based on the net reduction in negative charge on the HbS molecules compared to HbA tetramers, caused by the replacement of a negatively charged glutamic acid residue by a neutral valine, HbA migrates faster and ahead of HbS in alkaline gel electrophoresis. However, co-migration of HbS along with other hemoglobin variants such as HbD, HbQ, Hb Lepore etc. often leads to ambiguity in its identification using this method (Amit *et al.,* 2020).

The prevalence of sickle cell disease is higher in Sub- Saharan regions of Africa and over four million cases of SCD have been reported in Nigeria, with an annual birth of one hundred and fifty thousand (150,000) babies. Reports have shown that close to eighty percent (80%) of children with SCD die before the age of five owing to a lack of adequate medical attention (Ayevbuomwan *et al.,* 2020; Oke 2014).

The sickle cell prevalence level decrease to between 1% and 2% in northern Africa and less than 1% in southern Africa. In countries such as Cameroon, Republic of Congo, Gabon and Nigeria, the prevalence is between 20% and 30% while in some parts of Uganda, it is high as 45%. In countries where the trait prevalence is above 20%, the disease affects about 20% of the population. The geographic distribution of the sickle cell trait is very similar to that of malaria. The sickle cell trait has a partial protective effect against malaria, and this may explain why it has been maintained at

such high prevalence level in tropical Africa. Those who inherit the gene from both parents do not have this protection. In addition, they suffer from severe effects of sickle cell disorder and may die before they reach reproductive age (WHO, 2015)

2.1.2 Pathophysiology.

The classical view of sickle cell anemia has always focused on the primary genetic defect — the abnormal sickle hemoglobin that polymerizes when deoxygenated. Polymerization within the red cell causes it to deform, to become rigid, to obstruct blood flow, and to produce acute and chronic tissue damage because of poor perfusion (Orah S. Platt, 2000). The sickle hemoglobin polymer and perhaps also high concentrations of unpolymerized oxidized HbS damage the erythrocyte and its membrane. Compared with normal erythrocytes, sickle erythrocytes vary in many different ways. This is a result of membrane damage and the heterogeneous cellular distribution of fetal hemoglobin (HbF). HbF concentrations vary among patients with sickle cell anemia and among erythrocytes of each individual (Martin H. Steinberg, 2008). The sickling process within red cells occurs as direct consequence of the substitution of a single nucleotide (A to T) in the codon for amino acid. The change converts a glutamic acid codon (GAG) to a valine codon (GTG). However, valine is a hydrophobic amino acid. During deoxygenation, valine hydrophobicity attracts hydrophobic regions of adjacent β-chains facilitating the polymerization of haemoglobin molecules. Formation of Hb aggregates is a thermodynamically unstable event,10 and under particular conditions, the number of molecules aggregating significantly increases and reaches a critical mass, known as the critical nucleus, after which point addition of further molecules to this compound generates a stable complex (Andrea *et al.,* 2019).

As this progress, the reaction becomes autocatalytic and heterogeneous nucleation occurs on the surface of pre-existing polymers, leading to robust polymer generation, causing erythrocytes sickling. Polymerization progression is affected by oxygenation, 3-diphosphoglcerate (2,3-DPG)

concentration,14 pH, temperature, saline concentration and carbon monoxide(Andrea Piccin *et al.,* 2019).

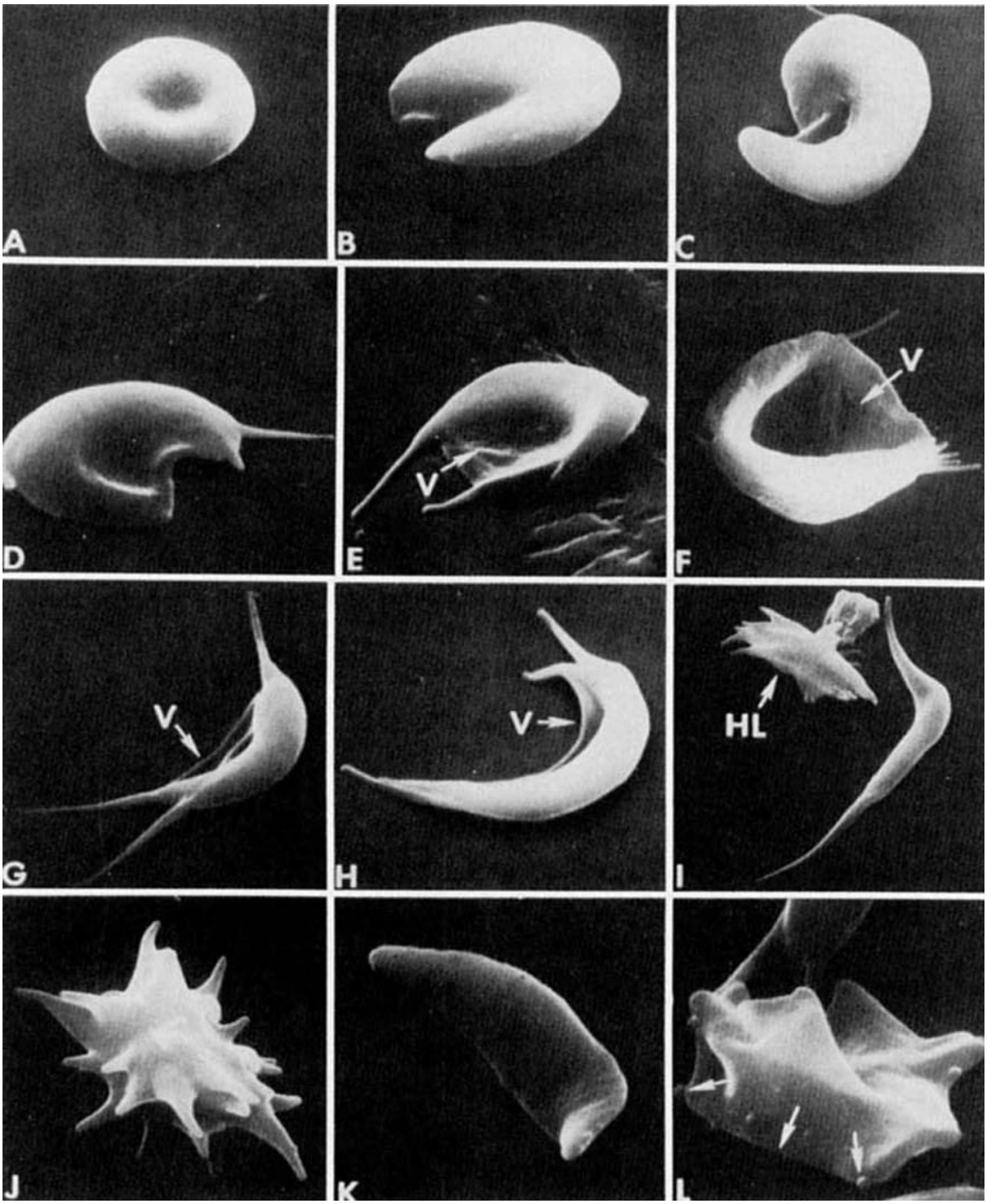


Figure 2.1 Scanning electron micrographs of sickle red cells. [From White (1974).] All cells (B-I,) are deoxygcnated except for the cell in A, which is oxygenated and has the normal biconcave disk shape. (Scc Whire (1974) for further description of the niicrographs and the abbreviations therein.

2.1.3 Signs and symptoms

The symptoms of sickle cell disease do not appear until several months after birth when most of the fetal hemoglobin (HbF) is replaced by HbS (William and H. Franklin, 2017).

The overall height, weight, and body mass index are significantly decreased in children with SCD. Inadequate nutrition along with increased caloric requirements during the growth phase may be important considerations because normal height is often achieved by adulthood. Endocrine abnormalities, such as primary hypogonadism, hypopituitarism, and hypothalamic insufficiency, may also be involved (Ballas *et al.,* 2010).

This disease is a group of related disorders where sickle hemoglobin is the principal hemoglobin species. All have varying degrees of chronic hemolytic anemia, vasculopathy, vasoocclusive disease, acute and chronic organ damage, and shortened life span (Martin, 2008).

2.1.4 Complications

The hallmark for sickle cell is the sickle cell crisis. It is the most common reason for hospitalization in sickle cell patient, some of the complications include.

Vaso-occlusive Crisis

Vaso-occlusive events are found in varying degrees in all disease genotypes, some genotypes are clinically more severe than others. This is due in large part to variation in the cellular concentration of HbS and the propensity for polymer formation, which is highly dependent on HbS concentration. Following HbS deoxygenation and a delay of milliseconds to seconds, depending on the intracellular concentration of HbS, the HbS polymer appears in the sickle erythrocyte (Martin, 2008).

Hemolytic Anemia

When erythrocyte deformability is compromised by sickling, the erythrocyte membrane is disrupted, releasing free Hb polymers into the circulation. In normal conditions, free Hb is removed from the circulation with globin chain dimers binding to haptoglobin. This complex is subsequently phagocytosed and destroyed by macrophages. Free ferric haem binds haemopexin and undergoes degradation in the liver. However, free plasma Hb may impair the circulating levels of nitric oxide (NO). This is better known as "free-Hb scavenging effect," where Hb transforms NO into nitrate (NO3−) (Rother *et al.,* 2005; Reiter *et al.,* 2002). Similarly, the release into the circulation of arginase from disrupted red cells reduces plasma arginine levels (the latter being an important NO precursor) further reducing NO level and further damaging the NO-sustained mechanisms of vasodilation. Furthermore, NO depletion facilitates platelet aggregation and vasoconstriction. Ferric haem enhances endothelial dysfunction through pro-inflammatory damage, favoring vaso-occlusion (Andrea *et al.,* 2019).

Red Blood Cell Alterations

In SCA, abnormal globin chains cluster along the cell membrane and alter cytoskeletal proteins such as band 3, ankyrin and spectrin B and 3 is a major integral membrane protein of erythrocytes.

Band 3 is essential for ensuring structural cytoskeletal organization, the transmembrane anion transport, the red cell volume regulation and CO2 removal. Damage of band 3 contributes to sickling processes. The deposition of clusters of globin chains along the cytoskeletal membrane promotes IgG deposition and triggers monocyte chemotaxis leading to removal from circulation of aged red cells (Bosman, 2004). Together with the concomitant changes in structure and function of band 3, these data suggest that sickle RBCs undergo a process of early senescence. Preliminary results show that this process is reversed upon vitamin E supplementation. Hierso et al showed a reduction of naturally occurring anti-band 3 autoantibodies during crises. Furthermore, the diminished anti-band 3 antibodies correlated negatively with the rise in plasma advanced oxidation protein products and

RBC caspase 3 activity. This clearly suggests a key role for band 3 in sickling crises (Andrea *et al.,* 2019).

Cardiac complications

The chronic anemia in SCD leads to a compensatory increased cardiac output. This, in turn, leads to cardiomegaly and left ventricular hypertrophy with left ventricular dysfunction (Janet *et al.,* 2012). Acute myocardial infarction can occur, even without coronary artery disease, and is thus underdiagnosed in SCD (Janet *et al.,* 2012). An increase in oxygen requirements exceeding the oxygen carrying capacity may be involved in this setting. Cardiac arrhythmias and congestive heart failure have also been linked to premature death in SCD (Fitzhugh *et al.,* 2010).

Hepatobiliary complications

Liver pathology is common in SCD. Hepatomegaly has been documented in 90% of autopsy cases. Complications of SCD as well as its treatments can cause changes within the liver. Vaso-occlusive crises within the liver can lead to hepatic sequestration and intrahepatic cholestasis. Chronic hemolysis may lead to cholelithiasis, whereas frequent transfusions may lead to iron overload and viral hepatitis (Bauer *et al.,* 1980).

The effects of sickle cell anemia on the liver include intrasinusoidal sickling with proximal sinusoidal dilation, Kupffer cell hyperplasia with erythrophagocytosis, and hemosiderosis (Janet *et al.,* 2012). Focal necrosis, portal fifibrosis, regenerative nodules, and cirrhosis have also been described in postmortem examinations (Bauer *et al.,* 1980).

Vaso-occlusion can lead to sinusoidal obstruction and ischemia, resulting in acute sickle hepatic crises (Banerjee *et al.,* 2010). Similar to splenic sequestration, erythrocytes can be sequestered within the liver, leading to acute anemia. Hypovolemic shock is less likely because the liver does not distend as much as the spleen but can cause significant liver dysfunction. Intensive resuscitation along with the rapid physiological reversal of the sequestration has been shown to be fatal (Janet *et al.,* 2012).

2.1.5 Management

Folic acid and penicillin

Folic acid daily for life is recommended. From birth to five years of age, penicillin daily due to the immature immune system that makes them more prone to early childhood illnesses is also recommended.

Prophylactic Therapy

Three prophylactic measures have become widely accepted in the management of SCD; penicillin prophylaxis, immunization against pneumococcal infection and folate administration. The mortality rate due to Streptococcus pneumoniae pneumonia, sepsis, and meningitis was historically very high prior to the age of 6 years in children with SCD (Zakari *et al.,* 2008). This rate has been lowered tremendously by three maneuvers. The first is diagnostic screening for SCD in neonates, with immediate initiation of pencillin VK 125 mg twice daily, increased at the age of 3 years to 250 mg twice daily and continued until 5 years old. The second is immunization with heptavalent pneumococcal-conjugated vaccine at 2, 4, 6, and 12 months of age. The third is immunization with 23-valent pneumococcal polysaccharide vaccine at 2 and 5 years of age (American Academy of Pediatrics, 2000).

Red cell transfusion

The management of SCD continues to be supportive and includes hydration, pain relief, blood transfusion and psychosocial support. The majority of patients with SCD receive transfusions at some point in their life to reduce complications of the disease. The most common complication is a vaso-occlusive crisis, but transfusion has not been shown to be acutely beneficial for this indication (Danielson, 2002). Indications for acute simple transfusion include symptomatic anemia, aplastic crisis, splenic or hepatic sequestration, acute chest syndrome or acute multi-organ failure with severe anemia, and preparation of patients with homozygous SCD for major Surgery (Zakari *et al.,* 2008).

Hydroxyurea

Hydroxyurea is the most successful drug therapy for SCD. It is a cytotoxic and cytoreductive antimetabolite that acts via inhibition of DNA synthesis by inhibiting ribonucleotide reductase. Known pharmacologic effects of hydroxyurea that may contribute to this drug's efficacy in SCD include increased red cell content of hemoglobin F levels (which reduces the formation of hemoglobin S polymers), dose-related cytoreductive effects on neutrophils, increased water content of red cells, increased deformability and successful microvascular navigation of sickled cells and altered adhesion of red blood cells to endothelium by decreasing the expression of endothelial adhesion molecules. Treatment with hydroxyurea is associated with significant decreases in the yearly rate of painful crises, hospital admissions, and incidence of chest syndrome, priapism, hepatic sequestration, and blood transfusion requirements by as much as 50% (Zakari *et al.,* 2008).

2.2 Medicinal Plants as anti-sickling agents

The term of medicinal plants include a various types of plants used in herbalism and some of these plants have a medicinal activities. Medicinal plants have played an essential role in the development of human cultures. Many of the modern medicines are produced indirectly from medicinal plants, for example, aspirin. Analysis by in vitro method of *Zingiber officinale* roscoe (Ginger) was found to have anti-sickling effects as reported by Alabdallat *et al.,* (2016). The plant Cromolyn sodium also demonstrated high in vitro anti-sickling and was recommended for further in vivo investigations so as to be used for SCD therapy (British Journal of Haematology, 1998). Also, in vitro assays on *Achillea fragrantissima* leaves showed high anti-sickling inhibition of sickle cells (Nessim, 2016). An in vitro study was done on *Terminalia arjuna* and *Terminalia bellerica,* it was observed that the leaves of both plants showed significant anti-sickling effects and hence was recommended as a potential use in clinical management of SCD (Dilip *et al.,* 2017). The in vitro study on *Plumbago zeylanica* and *Uvaria chamae* roots was justified as a tradition medicinal recipe in the management

of SCD (Adejumo *et al.,* 2010).

Detarium microcarpum is a legume tree of tropical Africa, widely known for its ethno-medicinal potentials. Previous studies have reported its rich contents of phytochemicals which could be linked to the bioactive principle of the plant. Their interactions with 2, 3-bisphosphoglycerate mutase could inhibit 2, 3-bisphosphoglycerate synthesis, thus delaying enhanced delivery of oxygen to tissues which is twice the case seen in sickle cell anemia. This clearly shows the possible mechanism of action of D. microcarpum and further substantiates the antisickling potential of these compounds derived from D. microcarpum leaves and fruits (Ayevbuomwan *et al.,* 2020).

2.2.1 Characteristics of Medicinal Plants

Synergic medicine- The ingredients of plants all interact simultaneously, so their uses can complement or damage others or neutralize their possible negative effects.

Preventive medicine- It has been proven that the component of the plants also characterize by their ability to prevent the appearance of some diseases. This will help to reduce the use of the chemical remedies which will be used when the disease is already present i.e., reduce the side effect of synthetic treatment.

2.2.2 Classification of Medicinal Plants

Classification of medicinal plants is organized in different ways depending on the criteria used. In general, medicinal plants are arranged according to their active principles in their storage organs of plants, particularly roots, leaves, flowers, seeds and other parts of plant. These principles are valuable to mankind in the treatment of diseases (Adejumo *et al.,* 2010).

These medicinal plants consider as a rich resources of ingredients which can be used in drug development and synthesis. Besides that these plants play a critical role in the development of human cultures around the whole world. Moreover, some plants consider as important source of nutrition and as a result of that these plants recommended for their therapeutic values. These plants include sweet dattock, ginger, green tea, walnuts and some others plants. Other plants their

derivatives consider as important source for active ingredients which are used in aspirin and toothpastes. It has been estimated that about 13,000 species of plants have been employed for at least a century as traditional medicines by various cultures around the world (Andrea *et al.,* 2019).

These days the term Alternative Medicine became very common in western culture, it focus on the idea of using the plants for medicinal purpose. But the current belief that medicines which come in capsules or pills are the only medicines that we can trust and use. Even so most of these pills and capsules we take and use during our daily life came from plants. Medicinal plants frequently used as raw materials for extraction of active ingredients which used in the synthesis of different drugs. Like in case of laxatives, blood thinners, antibiotics and antimalarial medications, contain ingredients from plants (Dilip *et al.,* 2017).

2.2.3 Plants as a Basis of Some Important Drugs

Higher plants have been used as a source of drugs by mankind for several thousand years. In fact, ancient man was totally dependent on green plants for his day-to-day needs of medicament. With the development of modern medicine, synthetic drugs and antibiotics, the importance of plants as raw material for drugs decreased considerably. However, plants were used as a basis of some of the most important drugs, even in the modern system of medicine. With the advancement of synthetic organic chemistry most of the active constituents of plants used in medicine were synthesized. At one time it was thought that ultimately all the plant drugs would be obtained from synthetic sources (Dilip *et al.,* 2017).

2.3 Family: Fabaceae

Biological Classification of *Detarium microcarpum*

- Kingdom: Plantae
- Phylum: Tracheophyta
- Class: Magnoliopsida

- Order: Fabales
- Family: Fabaceae
- Species: Detarium microcarpum

Main and unique feature of this family is its legumes which are the fruit of the plant. Species of this family ranges from dwarf herbs of arctic and alpine vegetation to massive tree of tropical forest. The family leguminosae is also divided into 3 subfamilies papilionodae, caesalpinioideae and mimosoideae. Sometimes these sub families are also recognizes as a separate and independent families. Identification of these subfamilies is done by their flowers (Ahmad *et al.,* 2016). Legumes of this family are also used for economically for nitrogen fixation.as legumes are able to convert the atmospheric nitrogen into useful nitrogenous compounds, which are used for the growth of plant. This is done by the bacteria of the genus Rhizobium present in the root nodules. There is development of symbiotic relationship among bacteria and legumes.so they able to fix free nitrogen for plants and in return legumes are able to provide fixed carbon produced by photosynthesis. The tendency of legumes for semi aired to aired habitat is related to nitrogen demanding metabolism and this is thought to be an adaption for unpredictable habitat (Patel and Shah, 2014).

2.4 Morphology of *Detarium Microcarpum*

Detarium microcarpum family Fabaceae is an African tree, with height of 15–25 m with distinguished green 8–12 cm leaves (FAO, 1995). As far as development rate, the shoots of the storage compartment can achieve a tallness of 1.5–2 m in 1–2 years and are considerably more overwhelming than seedlings which on proximate develop to 0.6 m following 3 years and may achieve 1.5 m in 4 years. It blossoms amid the rainy season (July to September/November), yet the principle blooming period just keeps going up to 8 days. It proves to be fruitful from September – January/May and in November; the tree sheds its leaves and delivers new leaves in March (Kouyaté and van Damme, 2006). A tree can give 675 fruits about 7 kg. The fruit is sweet and usually eaten

crisp, while the mash is utilized in the readiness of cakes and couscous. The fruit is about 4 cm long and 2.5 cm wide. The fruits can be stored for 1–3 years in jute bags (Mariod *et al.,* 2019).

It is also known as sweet detar and traditionally called ‘abu laila’ in western Sudan, ‘dank’ in Senegal, ‘tambadala’ in Mali, and ‘ofor’ in southeastern Nigeria (Aviara, 2015). D. microcarpum grows in dry regions of central and western Africa, where it can reach heights of up to 15 m. In high rainfall areas, it can grow up to 25 m (Hinawi *et al.,* 2018).

Biological Activities of Detarium Microcarpum

Detarium microcarpum (Fabaceae) is an African leguminous medicinal plant found in the tropical forests (Mabberley, 2017). Leaves, bark, roots, fruits and seeds of D. microcarpum are currently used in Cameroon for the treatment of stomachache, typhoid fever, dysentery, malaria, jaundice, digestive, nutritional and pregnancy disorders (Ebi and Afieroho, 2011).

Fruits are rich in vitamin C and are eaten raw or cooked, while leaves and flowers are used as spices and vegetables for the preparation of diets (Mbock *et al.,* 2020). Antimicrobial activities of some parts of D. microcarpum have been reported (Akah *et al.,* 2012; Ebi and Afieroho, 2011).

The bark, leaves and roots are widely used throughout the plant's native range because of their diuretic and astringent properties. Modern research has verified the presence of medicinally active compounds. The bark has been found to contain 2 tetranorditerpenes, the clerodane diterpenes catechine and cis-2-oxokolavenic acid (0.5%), the diterpene copalic acid (1.7%) and coumarin (1%).

An ethanol extract of the bark has demonstrated antimicrobial action against *Pseudomonas aeruginosa, Citrobacter freundii, Klebsiella pneumoniae, Staphylococcus aureus, Streptococcus pyogenes* and *Listeria monocytogenes* (Akah *et al.,* 2012; Ebi and Afieroho, 2011).

The extract also showed moderate antitumor activity against breast cancer cells. The flavones present in a methanol extract of the plant showed strong inhibitory effects on HIV-1 or HIV-2 infection. A bark extract showed significant molluscicidal activity against *Lymnaea natalensis*.

The bark, leaves and roots are prepared as infusions or decoctions to treat a wide range of ailments

including rheumatism; venereal diseases and urogenital infections; haemorrhoids; caries; problems of the digestive system such as biliousness, stomach-ache, intestinal worms, diarrhoea and dysentery. They are also used against malaria, leprosy and impotence (Mbock *et al.,* 2020).

Applied externally, the fresh bark or leaves are used to treat wounds, to prevent and cure infections. A decoction of the powdered bark is widely taken to alleviate pain such as in headache, sore throat, back pain and painful menstruation. The bark is also used to treat measles, nocturia, hypertension, itch and tiredness. A decoction of the leaves or roots is taken to treat paralysis, meningitis, tiredness, cramps and difficult delivery. A decoction of the leaves is taken to treat fainting and convulsions. The leaves, combined with the leaves of Sclerocarya birrea and Acacia macrostachya, and pounded in milk, are considered a very efficient treatment for snakebites. The powdered seeds are applied to skin infections and inflammations(Akah *et al.,* 2012; Ebi and Afieroho, 2011).

The fruit is eaten to cure meningitis and malaria. A preparation of the fruits is taken to treat dizziness. Externally, the fruit pulp is used for treating skin infections(Mbock, M.A.*et al.,* 2020).

Chemical Constituents isolated from Detarium Microcarpum

The Phyto-constituents identified from the ethyl Acetate extract of the stem bark of Detarium Microcarpum were:

- Anthocyanidin
- 3,4-epoxyclerodan-13E-en-15-oic acid
- 5α, 8α (2-oxokolavenic acid)
- 2-Oxokolavenic acid
- 3,4-dihydroxyclerodan-13E-en-15-oic acid
- 3,4-dihydroclerodan-13z-en-15-oic acid
- Copalic
- Hydroxyurea

Figure 2.2: Detarium Microcarpum in fresh habitat (Mbock *et al.,* 2020)

Figure 2.3: Detarium Microcarpum Stem Bark (Mbock *et al.,* 2020)

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Laboratory Materials

3.1.1 Equipment/Apparatus

- Drying Oven
- Refrigerator
- Centrifuge
- Thermostat Water Bath (TT42D Multi-purpose use, Techmel and Techmel, USA)
- Beakers (Pyrex, England)
- Micropipette (Microlux)
- Measuring Cylinder (Pyrex, England)
- UV/visible Spectrophotometer (21D Milton Ray Unleam SP 1800)
- Rotary evaporator (RE 300, Bibby Scientific, UK)
- Autoclave
- Incubator
- Digital weighing balance
- Syringe
- Hand Gloves
- Spatula
- EDTA bottles
- Universal Bottles
- Retort Stand
- Round bottom flask (Pyrex, England)
- Pipettes (pyrex, England)
- Conical flask (pyrex, England)

- Cuvette
- Separating Funnels
- Test tubes (pyrex, England)
- Test tubes rack
- Centrifuges tubes (pyrex, England)
- Wire loop
- Petri dish (pyrex, England)
- Electric stirrer
- Water bath
- Cotton wool and Masking tape
- Filter paper
- Foil
- Dry Chemicals
    1. P-hydrobenzoic acid
    2. Sodium metebisulphite
- Wet Chemicals
    1. Ethyl Acetate
    2. Distilled Water
    3. Normal Saline

3.2 Methods

3.2.1 Collection and identification of plant extracts

The fresh *Detarium Microcarpum* leaves and stem bark were ordered from Lagos State, Nigeria. The plant material was identified and authenticated by the Department of plant Biology and Biotechnology university of Benin and was given a reference voucher number. The Stem Bark of D.

Microcarpum was air-dried and grinded into powder using the Thomas Viking Milling Machine at the Faculty of Pharmacy, Department of Pharmacognosy, University of Benin. The crude powdered sample was stored in an air-tight container until ready for use.

3.2.2 Plant Extraction

Preparation of Crude Plant Extract

Precisely 980g of the pulverized Detarium Microcarpum were soaked with 750mL methanol, for 72 hrs. The extract was freeze-dried using a freeze dryer. The powdered extract was weighed and stored in an air-tight container.

3.3 Chemicals and Reagents

Methanol, Ethyl Acetate, Dichloromethane, N-hexane, Phosphate Buffer Saline (pH 7.4), Disodium Hydrogen Phosphate, Potassium dihydrogen Phosphate, distilled water, sodium metabisulphite, phosphate buffer saline, potassium phosphate buffer pH 7.4), EDTA.

3.4 Study Area and Subject Recruitment

3.4.1 Inclusion Criteria

This research was conducted in Benin City, Edo State, Nigeria. A total of forty (40) subjects was recruited for this study. The subjects included forty sickle cell patients of ages between 5-40years of age. Informed consent was obtained from each participant after proper notification and information on the nature of the research, risk involved, benefits as well as confidentiality.

3.4.2 Exclusion Criteria

Those excluded from this study were patients who have received blood transfusion for the last six months and patients on any kind of medication.

Sodium metabisulphite solution served as the negative control. 5mg/mL solution of p-

hydroxybenzoic acid (PABA) in normal saline was used as the positive control.

3.5 Collection of HbSS blood sample

Samples of HbSS blood were collected by venipuncture from the sickle cell patients no in crisis at the Sickle Cell Anaemia Centre GRA, Benin City, Nigeria. A total number of 40 samples were collected into EDTA bottle to prevent coagulation and used within 24hours. Ethical approval was obtained from the Edo State Ministry of Health, with reference number ES/HM.1208/269

Normal cell morphology of collected blood samples

A sample of the HbSS blood (5ml) was poured into a sample tube, made up to mark with phosphate buffer saline, mixed gentle and centrifuged at 2000 – 3000rpm to remove the serum. The resulting packed erythrocytes were washed three times with sterile phosphate buffer saline and centrifuged each time to remove the supernatant.

3.6 Haemoglobin Polymerization Assay

Sodium metabisulfite {Na2S2O5; (BDH, UK)}-induced polymerization of HbS molecules was ascertained as described previously with minor modification according to;

Principle:

The underlying principle is that HbS molecules undergo gelation when deprived of oxygen, transiting to deoxyHbS molecules; Na2S2O5was used as the reductant. The level of polymerization was measured by recording increasing absorbance of the assay mixture with progression of time.

Preparation of RBC Suspension:

Red blood cell (RBC) suspension was prepared. Blood sample is collected from confirmed sickle cell disease patients in an EDTA tube. The sample is washed in in an ice-cold PBS thrice to remove plasma and the packed erythrocyte is re-suspended in PBS and stored at 4^{o}C in a freezer to form ice. The washed erythrocyte is lysed by freeze-thawing which is freezing a cell suspension in dry ice or

freezer and gradually warming at 37^{o}C. This will cause the cell to break and ultimately break as ice crystals form during freezing process and contract during thawing.

The resulting erythrocyte hemolysate was used for polymerization analysis.

The hemolysate was centrifuged at 1000rpm for 9minutes. The supernatant obtained is used for the analysis.

Standard: 50µl of washed erythrocyte hemolysate was put in a plain tube and 250ml of Phosphate Buffer Saline (PBS), p-hydroxybenzoic acid was added,

1.7ml of Sodium metabisulphite was added and read immediately at 0min, 20mins, 60mins and 90mins respectively.

Control: 50µl of washed erythrocyte hemolysate was put in a plain tube and 250ml of Phosphate Buffer Saline (PBS), 500µl of distilled water (H_20) was added, 1.7ml of Sodium Metabisulphite was added and read immediately at 0mins, 20mins, 40mins, 60mins and 90 minutes respectively.

Ethyl Acetate Extract: 50µl of washed erythrocyte hemolysate was put in a plain tube and 250ml of Phosphate Buffer Saline (PBS), concentrated Ethyl Acetate plant extract was added, 1.7ml of Sodium metabisulphite was added and read immediately at 0min, 20mins, 60mins and 90mins respectively.

3.7 Statistical Analysis

Data were presented as Means ± SEM (standard error of means); n represents the number of subjects from which 5mls of blood will be collected. Comparison of the means will be effected using the Student's t-test, ANOVA and Graph pad prism version 7.03 statistical package. p – Values less than 0.05 ($p < 0.05$) were considered statistically significant for two independent variables (test and control).

CHAPTER FOUR

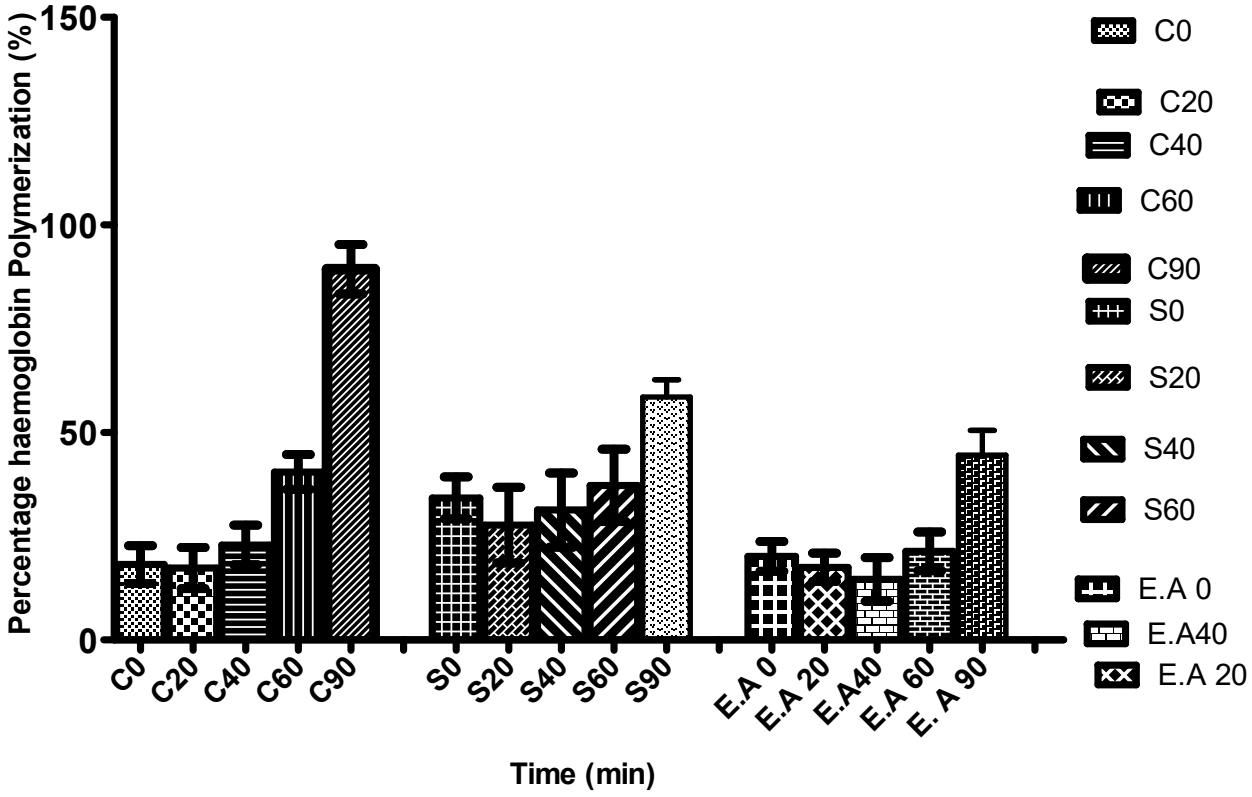
4.0 RESULTS

4.1 Percentage haemoglobin polymerization of Ethyl Acetate fraction of Detarium microcarpum (stem bark)

The effect of Ethyl Acetate (EA) fraction of D. microcarpum stem bark was evaluated using blood samples collected from sickle cell patients.

The result from this study showed that;

Ethyl Acetate (EA) fraction of D. microcarpum extract showed a significant ($p<0.05$) reduction in haemoglobin polymerization @90min when compared to the control. This was also significant ($p<0.05$) @ 90min with a reduction of about 46.05% against the control which was 90%. This was also observed when compared to the standard with a percentage of 70% in haemoglobin polymerization when compared to the control. Fig 4.1 below is a graphical representation of the results gotten from samples of sickle cell patients, with the C column serving as control, S column serving as standard and EA column administered Ethyl Acetate fraction of D. microcarpum stem bark. C0 and C90 are seen to be significantly different ($p<0.05$), C0 and S90 is also seen to be statistically different, C20 and C90, C20 and S90, C40 and C90, C40 and S90, C60 and C90, C90 and S0, C90 and S20, C90 and S40, C90 and S60, C90 and S90, C90 and EA0, C90 and EA20, C90 and EA40, C90 and EA60, C90 and EA90, S20 and S90, S90 and EA0, S90 and EA20, S90 and EA40, S90 and EA60, EA40 and EA90, are all seen to have statistical difference ($p<0.05$).



4.1 Percentage haemoglobin polymerization of Ethyl Acetate fraction of Detarium microcarpum (stem bark)

The result showed a reduction ($p < 0.05$) in percentage sickling or hemoglobin polymerization of red blood cells when 5 mg/ml of D. microcarpum stem bark ethyl acetate extract (20% sickling at 40 min and 46.05% sickling at 90 min) was compared to the control, phosphate buffer saline (20% sickling at 40 min and 90% sickling at 90 min). At 5 mg/ml concentration, D. microcarpum ethyl acetate showed a percentage sickling of 22% at 20 min and 25% at 60min.

This also showed a reduction ($p < 0.05$) in percentage sickling or hemoglobin polymerization of red blood cells when 5 mg/ml of D. microcarpum stem bark standard; p-hydroxybenzoic acid (35% sickling at 20 min and 40% sickling at 40 min) was compared to the control, phosphate buffer saline (18% sickling at 20 min and 20% sickling at 40 min). At 5 mg/ml concentration, D. microcarpum standard; p-hydroxybenzoic acid showed a percentage sickling of 45% at 60 min

and 60% at 90min.

The result showed a reduction ($p < 0.05$) in percentage sickling or hemoglobin polymerization of red blood cells when 5 mg/ml of D. microcarpum stem bark ethyl acetate extract (15% sickling at 40 min and 46.05% sickling at 90 min) was compared to the standard, p-hydroxybenzoic acid (40% sickling at 40 min and 70% sickling at 90 min). At 5 mg/ml concentration, D. microcarpum ethyl acetate showed a percentage sickling of 22% at 20 min and 25% at 60min. Detarium microcarpum stem bark extract gave a higher percentage inhibition of sickling at 45 min and 90 min by a reduction of hemoglobin polymerization when compared to the standard, p-hydroxybenzoic acid and the control which was just phosphate buffer saline.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATONS

5.1 Discussion

Sickle cell disease (SCD) is a hereditary chronic hemolytic anemia with numerous clinical consequences (Janet *et al.,* 2012). Sickle cell disease (SCD) is an umbrella term that defines a group of inherited diseases (including sickle cell anaemia (SCA), HbSC and HbSβ-thalassaemia, characterized by mutations in the gene encoding the haemoglobin subunit β (HBB) (Gregory *et al.,* 2018).

Humans have relied on nature throughout their ages to cater for their basic needs including medicines to cure a wide spectrum of diseases. Plants have formed the basis for sophisticated systems of traditional medicines. For therapeutic agents many of the presently known lead compounds are natural products or their derivatives. Ethnomedicinal studies play a vital role to pdiscover new drugs from indigenous medicinal plants. Green pharmaceuticals are getting popularity and extraordinary importance because vast opportunities for new drug discoveries are provided by the unmatched availability of chemical diversity and natural products either as pure compounds or as homogenous plant extracts (Ghulam *et al.,* 2017).

Plants had been used for medicinal purposes long before recorded history. Ancient Chinese and Egyptian papyrus writings describe medicinal uses for plants as early as 3,000 BC. Indigenous cultures (such as African and Native American) used herbs in their healing rituals, while others developed traditional medical systems (such as Ayurveda and Traditional Chinese Medicine) in which herbal therapies were used (Odoh *et al.,* 2020). Ethnomedicinal studies play a vital role to discover new drugs from indigenous medicinal plants and green pharmaceuticals are getting popularity and extraordinary importance (Ghulam *et al.,* 2017), because vast chances for new

drug discoveries are provided by the unrivaled availability of chemical diversity and natural products either as pure compounds or as homogenous plant extracts (Jigna *et al.,* 2007).

There has been a recent explosion in the interest among investigators in both the hematology academic community and in the pharmaceutical industry to develop new treatments for sickle cell disease, as indicated by the large number of active clinical protocols (William and Franklin, 2017).

For sickle cell disorder, the study of haemoglobin polymerization especially in various anti-sickling agents has a great importance because different antisickling agents have different degrees of effect. Although a number of chemical components described in Detarium Microcarpum are also found in other species, the secondary metabolites produced by this species are an important source of potential phytotherapeutic and medicinal agents.

The treatment of the sickled erythrocytes with sodium metabisulphite (2%w/v) showed a significant augmentation of sickling; this helped to suck up oxygen from the red blood cell thus creating a state of reduced oxygen tension which mimics the event that occurs during sicle cell crisis. Thus sodium metabisulphite created hypoxic conditions for red blood cells leading to loss of the morphology and sickled erythrocytes. In vitro deoxygenation of RBC by sodium metabisulphite caused progressive aggregation and polymerization of the individual haemoglobin molecules. The process of polymerization of haemoglobin molecules increases the formation of sickle cells. This causes the cells to assume the characteristic sickle cell shapes such as the crescent, holly leaf and spindle shapes. The sickle cell haemoglobin (HbS) is a product of a defective genetic code of haemoglobin molecule (Chikezie, 2011).

The Ethyl Acetate Leaf extract of Detarium Microcarpum showed antisickling activity of the sodium metabisulphite induced sickled cells. It had 60% inhibition at 90minutes incubation time

which was comparable to p-hydrozybenzoic acid (reference antisickling drug) which is a pure compound ($p< 0.05$).

p-hydrozybenzoic acid is freely soluble in Ethyl Acetate, it is a monohydroxybenzoic acid, a phenolic derivative of benzoic acid, a white crystalline solid that has been reported to be slightly soluble in water and chloroform but more soluble in polar organic solvents such as alcohols and acetone. It is a popular antioxidant because of its low toxicity.

The Ethyl acetate reduced haemoglobin polymerization in sickle cell by 60% compared to p-hydrozybenzoic acid.

This clearly shows the possible mechanism of action of D. microcarpum and further substantiates the antisickling potential of these compounds derived from D. microcarpum leaves and fruits (Ayevbuomwan *et al.,* 2020).

5.2 Conclusion and Recommendation

Haemoglobin polymerization and sickling of erythrocytes is favored when HbS molecules are deoxygenated (Chikezie *et al.,* 2013).

There is no doubt that there are many ways to inhibit haemoglobin polymerization. From the data and results obtained after experiment, it is evident that this is one of the ways and that is a cause for optimism but there is a need to know the standards for which toxicity information already exists. Also it has shown that most well-know anticancer, antitumor, anti-inflammatory, antibacterial, antimalarial, antifungal, antiviral, antidiabetic, immunomodulatory and antioxidant phytochemicals play significant role in ameliorating the SCD crisis. The plant that contains some chemical constituents or phytochemicals that might be responsible in the decrease or reduction of hemoglobin polymerization which are:

- Anthocyanidin
- 3,4-epoxyclerodan-13E-en-15-oic acid
- 5α, 8α (2-oxokolavenic acid)
- 2-Oxokolavenic acid
- 3,4-dihydroxyclerodan-13E-en-15-oic acid
- 3,4-dihydroclerodan-13z-en-15-oic acid
- Copalic
- Hydroxyurea

Detarium microcarpum stem bark extract gave a higher percentage inhibition of sickling at 40 min and 90 min when compared to the control, phosphate buffer saline and the standard, p-hydroxybenzoic acid.

From the results of this study, it can be concluded that;

- The ethyl acetate extract of Detarium Microcarpum demonstrated significant antisickling and antioxidant properties.
- Thus, further studies are therefore recommended to establish these data in human sickle cell disease (SCD). In addition, the toxicity profiles and lead compounds for drug formation can be isolated from this plant.

REFERENCES

Adejumo, O.E., Adelodun, L.K., Oladimeji, P.R and Lateef, S.K (2010). Studies on phytochemical screening and antimicrobial potential of Phyllantus amarus against multiple antibiotic resistant bacteria. *International Journal of Applied Research in Natural Products*. 3(3): 6-12.

Ahmad, F., Anwar, F. and Hira, S. (2016). Review on medicinal importance of Fabaceae family. *Pharmacologyonline*. 3: 151-157.

Akah, P.A., Nworu, C.S., Mbaoji, F.N., Nwabunike, I.A. and Onyeto, C.A. (2012). Genus Detarium: Ethnomedicinal, phytochemical and pharmacological profile. *Phytopharmacology*. 3(2), 367-375.

Alabdallat, N.G. and Adam, I.A. (2016). In vitro antisickling activity of Zingiber officinale Roscoe (Ginger) Methanolic Extract on Sickle cell disease. *British Journal of Medicine and Medical Rvjesearch.* 12(12): 1-7

Aliyu, Z. Y., Kato, G. J., Taylor IV, J., Babadoko, A., Mamman, A. I., Gordeuk, V. R. and Gladwin, M. T. (2008).Sickle cell disease and pulmonary hypertension in Africa: a global perspective and review of epidemiology, pathophysiology, and management. *American Journal of Hematology*. 83(1): 63 - 70.

American Academy of Pediatrics, (2000). Committee on Infectious Diseases. Policy statement:recommendations for the prevention of pneumococcal infections, including the use of pneumococcal conjugate vaccine (Prevnar), pneumococcal polysaccharide vaccine, and antibiotic prophylaxis. *Pediatrics*. 106:362–6

Amit, K.M., Amrita, M. and Rajdeep, D. (2020). Sickle Cell Hemoglobin. *Cell.* 8: 298 – 308.

Andrea, P., Ciaran, M., Elva, E., Maria, B.R., Massimo, D.C.,Vecchiato, D.W., Corrina, M.M. and Owen, P.S. (2019). Insight into the complex pathophysiology of sickle cell anaemia and possible treatment. *European Journal of Haematology*. 102: 319 – 330.

Aviara, N.A. (2015). Moisture-dependent Physical Properties of Detarium microcarpum Seeds. *Journal of Biosystems Engineering*. 40(3): 212-223.

Ballas, S.K., Lieff, S. and Benjamin, L.J. (2010). Defifinitions of the phenotypic manifestations of sickle cell disease. *American Journal of Hematology.* 85: 6 – 13.

Banerjee, S. and Chopra, S. (2010). Hepatic manifestations of sickle cell disease. *Up To Date*. 8: 20 - 30.

Bauer, T.W., Moore, G.W. and Hutchins, G.M. (1980). The liver in sickle cell disease. A clinicopathologic study of 70 patients. *American Journal of Medicine.* 69: 833 – 837.

Bosman, G.J. (2004). Erythrocyte aging in sickle cell disease. *Cell Molecular Biology.* 50(1): 81-86.

Chikezie, P.C., Akuwudike, A.R. and Chikezie, C.M. (2013); Polymerization Studies of Sickle Cell Hemoglobin Incubated in Aqueous Leaf Extract of Nicotianatabacum Product. *Bulletin Environmental Pharmacology of Life Science.* 2(2): 47 - 51.

Chikezie, P.C. (2011). Sodium metabisulphite induced polymerization of sickle cell hemoglobinincubated in the extracts of three medicinal plants (*Anacardium occidentale, Psidium guajave and Terminalia catappa*). *African Journal of Biotechnology*. 10(61): 54 – 61.

Danielson, C.F. (2002). The role of red blood cell exchange transfusion in the treatment and prevention of complications of sickle cell disease. *Therapeutic Apherasial.* 6: 24 – 31.

Dayamba, S.D., Savadogo, P., Sawadogo, L., Zida, D., Tiveau, D. and Oden, P.C. (2011). Dominant species' resprout biomass dynamics after cutting in the Sudanian savannawoodlands of West Africa: long term effects of annual early fire and grazing. *Annals of Forest Science*. 68(3): 555 – 564.

Dillip, F.A., Mani, A. and Thawani, V. (2017). An invitro study on antisickling activity of *Terminalia arjuna* and *Terminalia belirica*. *Journal of Herbal Drugs (An international journal on medicinal herbs)*. 8(1): 1 – 7.

Ebi, G. and Afieroho, O. (2011). Phytochemical and antimicrobial studies on Detarium microcarpum Guilland Sperr (Caesalpinioceae) seeds coat. *African Journal of Biotechnology*. 10(3): 457 – 462.

FAO, (1995). Fruit and vegetable processing. *FAO Agricultural Services Bulletin*. 119: 4 – 10.

Fitzhugh, C.D., Lauder, N. and Jonassaint, J.C. (2010). Cardiopulmonary complications leading to premature deaths in adult patients with sickle cell disease. *American Journal of Hematology.* 85: 36 – 40.

Ghulam, M., Rawaba, A., Asia, A., Sumaira, S. and Amer, J. (2017). Bioactive Compounds from Medicinal Plants and Their Importance in Drug Discovery in Pakistan / `Mat. Sc. *Pharmaceutical Science.* 1(1): 17 - 26.

Gladwin, M.T., Crawford, J.H. and Patel, R.P. (204).The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation. *Free Radical Biological Medicine*. 36(6): 707 – 717.

Hassanin, H.A.M., Koko, M., Abdalla, M., Mu, W. and Jiang, B. (2018). Detarium microcarpum: a novel source of nutrition and medicine: a review. *Food Chemistry*. 12: 6 – 9.

Hebbel, R.P., Solovey, A.N., Soltero, E.G., Palzer, E.F., Ryder, J.R., Shaibi, G.Q. and Kelly, A.S. (2021). Relationship of circulating endothelial cells with obesity and cardiometabolic risk factors in children and adolescents. *Journal of the American Heart Association*, 10(1): e018092.

Jigna, P. and C.V. Sumitra. 2007. In vitro antimicrobial activity and phytochemical analysis of some Indian Medicinal plants. *Turkish Journal of Biology*. 31: 53 - 58.

Kapoor, S., Little, J.A. and Pecker, L.H. (2018). Advances in the treatment of sickle cell disease. In Mayo Clinic Proceedings . *Sickle Cell.* 93(12): 1810 – 1824.

Kouyaté, A.M. and van Damme, P. (2006). Medicinal plants/plantes médicinales: Detarium microcarpum Guill. & Perr. Prota. pp.11- 19.

Lamien-Meda, A., Lamien, C.E., Compaore, M.M.Y., Meda, R.N.T., Kiendrebeogo, M., Zeba, B., Millogo, J.F. and Nacoulma, O.G. (2008). Polyphenol content and antioxidant activity of fourteen wild edible fruits from Burkina Faso. *Molecules*. 13(3): 581 – 594.

Mabberley. D.J. (2017). Mabberley's plant-book: a portable dictionary of plants, their classification and uses. Cambridge University Press. http://database.prota.org/PROTAhtml/Detarium%20microcarpum_En.html.

Malowany, J. I. and Butany, J. (2012, February). Pathology of sickle cell disease. In Seminars in diagnostic pathology. WB Saunders. pp. 49 – 55.

Mbock, M.A., Fouatio, W.F., Kamkumo, R.G., Tsouh Fokou, Patrick.Valè., Tsofack, F.N., Lunga, P.-K., Essia Ngang, J.J., Boyomo, O., Nkengfack, A.E., Ndjakou, B.L., Sewald,

N., Boyom, F.F. and Dimo, T. (2020). In vitro and in vivo anti-salmonella properties of hydroethanolic extract of Detarium microcarpum Guill. & Perr. (Leguminosae) root bark and LC-MS-based phytochemical analysis, *Journal of Ethnopharmacology*. 8: 7 – 9.

Mariod A.A., Tahir H.E. and Komla M.G. (2019). Detarium microcarpum: Chemical Composition, Bioactivities and Uses. In: Mariod A. (eds) Wild Fruits: Composition, Nutritional Value and Products. Springer, Cham. pp. 5 – 10.

Martin, H.S. (2008). Sickle Cell Anemia, the First Molecular Disease: Overview of Molecular Etiology, Pathophysiology, and Therapeutic Approaches. *The Scientific World Journal*. 8: 1295 – 1324.

Meda, N.R., Fraisse, D., Gnoula, C., Vivier, M., Felgines, C. and Senejoux, F. (2017). Of antioxidants from Detarium microcarpum Guill. et Perr. leaves using HPLC-DAD coupled with pre-column DPPH assay. *European Food Research and Technology.* 243(9): 1659 - 1666.

Merit, E.A., Olusola, O.E., Francis, A.O. and Ehimwenma, S.O. (2020). Antisickling potential of compounds derived from *Detarium microcarpum* (Fabaceae): in vitro and in silico studies Mu, Wanmeng, Hinawi AM Hassanin, Leon Zhou, and Bo Jiang. *Chemistry behind rare sugars and bioprocessing*. 13(6): 13343 – 13345.\

Nessim, Y. and Gehr, R. (2006). Fouling Mechanisms in a Laboratory-Scale UV Disinfection System. *Water Environment Research*. 78(12): 2311 – 2323.

Odoh, U.E. and Ene, C. (2020): Phtyochemical Studies And Investigation on the Antiinflammatory Activity Of *Detarium Microcarpum Guill (Fabaceae).* 9(7): 38 - 51.

Oke, D. (2014). Proximate and phytochemical analysis of Cajanus cajan(Pigeon pea) leaves. *Chemistry Science Trans*. 3(3): 1172 – 1178.

Patel, S. and Shah, D.B. (2014). Phylogeny in Few Species of Leguminosae Family Based on matK Sequence. *Computational Molecular Biology*. 4(4): 2 – 7.

Platt, O.S. (2000). Sickle cell anemia as an inflammatory disease. *The Journal of clinical investigation*. 106(3): 337 – 338.

Reiter, C.D., Wang, X.T. and Santos, J.E. (2002). Cellfree hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *National Medicine*. 8(12):1383 – 1389.

Rother, R.P., Bell, L., Hillmen, P. and Gladwin, M.T. (2005). The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. *Journal of American Medical Associaton.* 293: 1653 – 1662.

Sani, A., Agunu, A., Danmalam, U.H. and Hajara, I. (2014) Pharmacognostic studies of the stem bark of *Detarium Microcarpum* – Guill. and Perr. (Fabaceae). *National Produce of Chemistry Research.* 1(4): 1 – 8.

William, A.E. and Franklin, H.B. (2017). Targeting *HbS Polymerization Blood.* 17(02): 765 – 891.

World Health Organization, (2015). Sickle cell disease prevention and control. Retrieved from : http://www.Afro.who.int/en/clusters-a-programmes/dpc/non-communicable-diseases-managementndm/programme-components/programe-components/sickle-cell-disease.html.

Appendix

Table Analyzed	Data 1
One-way analysis of variance	
P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	15
F	11.56
R squared	0.5189
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	28.60
P value	0.0118
P value summary	*
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	60630	14	4331
Residual (within columns)	56210	150	374.8
Total	116800	164	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
C0 vs C20	0.8425	0.1443	No	ns	-28.59 to 30.28
C0 vs C40	-4.687	0.8031	No	ns	-34.12 to 24.75
C0 vs C60	-22.29	3.818	No	ns	-51.72 to 7.147
C0 vs C90	-71.15	12.19	Yes	***	-100.6 to -41.72
C0 vs Column G	18.18	0.0000	No	ns	0.0000 to 0.0000
C0 vs S0	-16.05	2.750	No	ns	-45.49 to 13.38

C0 vs S20	-9.485	1.625	No	ns	-38.92 to 19.95
C0 vs S40	-13.13	2.249	No	ns	-42.56 to 16.31
C0 vs S60	-19.06	3.266	No	ns	-48.50 to 10.37
C0 vs S90	-40.32	6.908	Yes	***	-69.76 to -10.89
C0 vs Column M	18.18	0.0000	No	ns	0.0000 to 0.0000
C0 vs Column N	18.18	0.0000	No	ns	0.0000 to 0.0000
C0 vs E.A 0	-1.956	0.3351	No	ns	-31.39 to 27.48
C0 vs E.A 20	0.6797	0.1164	No	ns	-28.75 to 30.11
C0 vs E.A40	3.582	0.6137	No	ns	-25.85 to 33.02
C0 vs E.A 60	-3.167	0.5426	No	ns	-32.60 to 26.27
C0 vs E. A 90	-26.38	4.520	No	ns	-55.82 to 3.050
C20 vs C40	-5.530	0.9474	No	ns	-34.96 to 23.90
C20 vs C60	-23.13	3.963	No	ns	-52.56 to 6.304
C20 vs C90	-72.00	12.33	Yes	***	-101.4 to -42.56
C20 vs Column G	17.34	0.0000	No	ns	0.0000 to 0.0000
C20 vs S0	-16.89	2.894	No	ns	-46.33

					to 12.54
C20 vs S20	-10.33	1.769	No	ns	-39.76 to 19.11
C20 vs S40	-13.97	2.393	No	ns	-43.40 to 15.47
C20 vs S60	-19.90	3.410	No	ns	-49.34 to 9.531
C20 vs S90	-41.16	7.052	Yes	***	-70.60 to -11.73
C20 vs Column M	17.34	0.0000	No	ns	0.0000 to 0.0000
C20 vs Column N	17.34	0.0000	No	ns	0.0000 to 0.0000
C20 vs E.A 0	-2.799	0.4795	No	ns	-32.23 to 26.64
C20 vs E.A 20	-0.1628	0.02789	No	ns	-29.60 to 29.27
C20 vs E.A40	2.740	0.4694	No	ns	-26.69 to 32.17
C20 vs E.A 60	-4.009	0.6869	No	ns	-33.44 to 25.43
C20 vs E. A 90	-27.23	4.665	No	ns	-56.66 to 2.207
C40 vs C60	-17.60	3.015	No	ns	-47.03 to 11.83
C40 vs C90	-66.47	11.39	Yes	***	-95.90 to -37.03
C40 vs Column G	22.87	0.0000	No	ns	0.0000 to 0.0000
C40 vs S0	-11.36	1.947	No	ns	-40.80 to

C40 vs S20	-4.797	0.8219	No	ns	18.07 to -34.23
C40 vs S40	-8.439	1.446	No	ns	21.00 to -37.87
C40 vs S60	-14.37	2.462	No	ns	15.06 to -43.81
C40 vs S90	-35.63	6.105	Yes	**	-6.199 to -65.07
C40 vs Column M	22.87	0.0000	No	ns	0.0000 to 0.0000
C40 vs Column N	22.87	0.0000	No	ns	0.0000 to 0.0000
C40 vs E.A 0	2.731	0.4679	No	ns	32.17 to -26.70
C40 vs E.A 20	5.367	0.9195	No	ns	34.80 to -24.07
C40 vs E.A40	8.270	1.417	No	ns	37.70 to -21.16
C40 vs E.A 60	1.521	0.2605	No	ns	30.96 to -27.91
C40 vs E. A 90	-21.70	3.717	No	ns	7.737 to -51.13
C60 vs C90	-48.87	8.372	Yes	***	-19.43 to -78.30
C60 vs Column G	40.47	0.0000	No	ns	0.0000 to 0.0000
C60 vs S0	6.237	1.069	No	ns	35.67 to -23.20
C60 vs S20	12.80	2.193	No	ns	42.24 to -16.63

C60 vs S40	9.161	1.569	No	ns	-20.27 to 38.60
C60 vs S60	3.227	0.5529	No	ns	-26.21 to 32.66
C60 vs S90	-18.03	3.090	No	ns	-47.47 to 11.40
C60 vs Column M	40.47	0.0000	No	ns	0.0000 to 0.0000
C60 vs Column N	40.47	0.0000	No	ns	0.0000 to 0.0000
C60 vs E.A 0	20.33	3.483	No	ns	-9.103 to 49.77
C60 vs E.A 20	22.97	3.935	No	ns	-6.467 to 52.40
C60 vs E.A40	25.87	4.432	No	ns	-3.565 to 55.30
C60 vs E.A 60	19.12	3.276	No	ns	-10.31 to 48.56
C60 vs E. A 90	-4.097	0.7020	No	ns	-33.53 to 25.34
C90 vs Column G	89.33	0.0000	No	ns	0.0000 to 0.0000
C90 vs S0	55.10	9.440	Yes	***	25.67 to 84.54
C90 vs S20	61.67	10.57	Yes	***	32.23 to 91.10
C90 vs S40	58.03	9.941	Yes	***	28.59 to 87.46
C90 vs S60	52.09	8.925	Yes	***	22.66 to 81.53
C90 vs S90	30.83	5.282	Yes	*	1.397

					to 60.27
C90 vs Column M	89.33	0.0000	No	ns	0.0000 to 0.0000
C90 vs Column N	89.33	0.0000	No	ns	0.0000 to 0.0000
C90 vs E.A 0	69.20	11.86	Yes	***	39.76 to 98.63
C90 vs E.A 20	71.83	12.31	Yes	***	42.40 to 101.3
C90 vs E.A40	74.73	12.80	Yes	***	45.30 to 104.2
C90 vs E.A 60	67.99	11.65	Yes	***	38.55 to 97.42
C90 vs E. A 90	44.77	7.670	Yes	***	15.33 to 74.20
Column G vs S0	-34.23	0.0000	No	ns	0.0000 to 0.0000
Column G vs S20	-27.66	0.0000	No	ns	0.0000 to 0.0000
Column G vs S40	-31.31	0.0000	No	ns	0.0000 to 0.0000
Column G vs S60	-37.24	0.0000	No	ns	0.0000 to 0.0000
Column G vs S90	-58.50	0.0000	No	ns	0.0000 to 0.0000
Column G vs Column M	0.0000	0.0000	No	ns	0.0000 to 0.0000
Column G vs Column N	0.0000	0.0000	No	ns	0.0000 to 0.0000
Column G vs E.A 0	-20.14	0.0000	No	ns	0.0000 to

Column G vs E.A 20	-17.50	0.0000	No	ns	0.0000 to 0.0000
Column G vs E.A40	-14.60	0.0000	No	ns	0.0000 to 0.0000
Column G vs E.A 60	-21.35	0.0000	No	ns	0.0000 to 0.0000
Column G vs E. A 90	-44.57	0.0000	No	ns	0.0000 to -22.87
S0 vs S20	6.566	1.125	No	ns	-26.51 to 36.00
S0 vs S40	2.924	0.5010	No	ns	-32.44 to 32.36
S0 vs S60	-3.010	0.5157	No	ns	-53.70 to 26.42
S0 vs S90	-24.27	4.158	No	ns	0.0000 to 5.164
S0 vs Column M	34.23	0.0000	No	ns	0.0000 to 0.0000
S0 vs Column N	34.23	0.0000	No	ns	-15.34 to 0.0000
S0 vs E.A 0	14.09	2.415	No	ns	-12.70 to 43.53
S0 vs E.A 20	16.73	2.866	No	ns	-9.802 to 46.16
S0 vs E.A40	19.63	3.364	No	ns	-16.55 to 49.07
S0 vs E.A 60	12.88	2.207	No	ns	-39.77 to 42.32
S0 vs E. A 90	-10.33	1.771	No	ns	19.10

S20 vs S40	-3.642	0.6240	No	ns	-33.08 to 25.79
S20 vs S60	-9.576	1.641	No	ns	-39.01 to 19.86
S20 vs S90	-30.84	5.283	Yes	*	-60.27 to -1.402
S20 vs Column M	27.66	0.0000	No	ns	0.0000 to 0.0000
S20 vs Column N	27.66	0.0000	No	ns	0.0000 to 0.0000
S20 vs E.A 0	7.528	1.290	No	ns	-21.91 to 36.96
S20 vs E.A 20	10.16	1.741	No	ns	-19.27 to 39.60
S20 vs E.A40	13.07	2.239	No	ns	-16.37 to 42.50
S20 vs E.A 60	6.318	1.082	No	ns	-23.12 to 35.75
S20 vs E. A 90	-16.90	2.895	No	ns	-46.33 to 12.53
S40 vs S60	-5.934	1.017	No	ns	-35.37 to 23.50
S40 vs S90	-27.19	4.659	No	ns	-56.63 to 2.240
S40 vs Column M	31.31	0.0000	No	ns	0.0000 to 0.0000
S40 vs Column N	31.31	0.0000	No	ns	0.0000 to 0.0000
S40 vs E.A 0	11.17	1.914	No	ns	-18.26 to 40.61
S40 vs E.A 20	13.81	2.365	No	ns	-15.63

					to 43.24
S40 vs E.A40	16.71	2.863	No	ns	-12.73 to 46.14
S40 vs E.A 60	9.960	1.706	No	ns	-19.47 to 39.39
S40 vs E. A 90	-13.26	2.271	No	ns	-42.69 to 16.18
S60 vs S90	-21.26	3.642	No	ns	-50.69 to 8.174
S60 vs Column M	37.24	0.0000	No	ns	0.0000 to 0.0000
S60 vs Column N	37.24	0.0000	No	ns	0.0000 to 0.0000
S60 vs E.A 0	17.10	2.930	No	ns	-12.33 to 46.54
S60 vs E.A 20	19.74	3.382	No	ns	-9.694 to 49.17
S60 vs E.A40	22.64	3.879	No	ns	-6.792 to 52.08
S60 vs E.A 60	15.89	2.723	No	ns	-13.54 to 45.33
S60 vs E. A 90	-7.324	1.255	No	ns	-36.76 to 22.11
S90 vs Column M	58.50	0.0000	No	ns	0.0000 to 0.0000
S90 vs Column N	58.50	0.0000	No	ns	0.0000 to 0.0000
S90 vs E.A 0	38.37	6.573	Yes	**	8.931 to 67.80
S90 vs E.A 20	41.00	7.024	Yes	***	11.57 to

S90 vs E.A40	43.90	7.522	Yes	***	70.44 to 14.47
S90 vs E.A 60	37.15	6.365	Yes	**	66.59 to 7.720
S90 vs E. A 90	13.94	2.388	No	ns	43.37 to -15.50
Column M vs Column N	0.0000	0.0000	No	ns	0.0000 to 0.0000
Column M vs E.A 0	-20.14	0.0000	No	ns	0.0000 to 0.0000
Column M vs E.A 20	-17.50	0.0000	No	ns	0.0000 to 0.0000
Column M vs E.A40	-14.60	0.0000	No	ns	0.0000 to 0.0000
Column M vs E.A 60	-21.35	0.0000	No	ns	0.0000 to 0.0000
Column M vs E. A 90	-44.57	0.0000	No	ns	0.0000 to 0.0000
Column N vs E.A 0	-20.14	0.0000	No	ns	0.0000 to 0.0000
Column N vs E.A 20	-17.50	0.0000	No	ns	0.0000 to 0.0000
Column N vs E.A40	-14.60	0.0000	No	ns	0.0000 to 0.0000
Column N vs E.A 60	-21.35	0.0000	No	ns	0.0000 to 0.0000
Column N vs E. A 90	-44.57	0.0000	No	ns	0.0000 to -26.80
E.A 0 vs E.A 20	2.636	0.4516	No	ns	32.07

E.A 0 vs E.A40	5.538	0.9488	No	ns	-23.90 to 34.97
E.A 0 vs E.A 60	-1.211	0.2074	No	ns	-30.65 to 28.22
E.A 0 vs E. A 90	-24.43	4.185	No	ns	-53.86 to 5.006
E.A 20 vs E.A40	2.902	0.4973	No	ns	-26.53 to 32.34
E.A 20 vs E.A 60	-3.847	0.6590	No	ns	-33.28 to 25.59
E.A 20 vs E. A 90	-27.06	4.637	No	ns	-56.50 to 2.370
E.A40 vs E.A 60	-6.749	1.156	No	ns	-36.18 to 22.69
E.A40 vs E. A 90	-29.97	5.134	Yes	*	-59.40 to -0.5326
E.A 60 vs E. A 90	-23.22	3.978	No	ns	-52.65 to 6.216