IN VITRO ANTI-SICKLING EFFECT AND ACTIVITY GUIDED FRACTIONATION OF *AGERATUM CONYZOIDES* AND *MANGIFERA INDICA* METHANOLIC EXTRACTS ON HUMAN SICKLE CELLS

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MARCH, 2017

DECLARATION

I declare that the work in this dissertation titled "*In vitro* Anti-sickling effect and activity guided fractionation of *Ageratum conyzoides* and *Mangifera indica* methanolic extracts on human sickle cells" has been performed by me in the Department of Biochemistry. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree at this or any other institution.

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CERTIFICATION

This dissertation titled "*IN VITRO* ANTI-SICKLING EFFECT AND ACTIVITY GUIDED FRACTIONATION OF *AGERATUM CONYZOIDES* AND *MANGIFERA INDICA* METHANOLIC EXTRACTS ON HUMAN SICKLE CELLS" by Danja Murja Ibrahim meets the regulations governing the award of the degree of M.Sc. Biochemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literacy presentation.

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ABSTRACT

All over Africa, traditional healers use medicinal plants to prepare medicines to treat a wide range of illnesses. One of these illnesses is sickle cell anemia. Sickle-cell anemia is caused as a result of substitution of glutamic acid by valine at the sixth position of beta-globin chain of hemoglobin.The effect of *Ageratum conyzoides* and *Mangifera indica* on human sickle red blood cells *in vitro* were investigated. The effect of the crude methanolic extracts on sickle cell was determined by sodium metabisulphite induced sickling which is based on deoxygenation of the red blood cells. The ability of the plant extract to protect the red blood cells against induced hemolysis and methemoglobin concentration were also investigated. Fractionation of the most active extract (stem bark of *A. conyzoides*) using column chromatography revealed eight fractions while *M. indica* stem bark gave seven fractions. Preliminary phytochemical screening on crude extracts of the leaf, stem and root of *A. conyzoides* and *M. indica* and their fractions showed the presence of some phytochemicals such as alkaloids, flavonoids, tannins, cardiac glycosides, carbohydrate, saponins and steroids. The results of Anti-sickling test showed that the percentage of unsickled cells were 56.93%, 64.60% and 67.77% for *A. conyzoides* stem bark extract, 37.60%, 44.20% and 55.50% for leaf extract and 39.50%, 43.30% and 51.90% for root extract at concentration of 0.1, 0.2 and 0.3mg/ml respectively. While 55.20%, 63.20% and 70.60% was observed for *M. indica* stem bark, 48.40%, 50.50% and 56.60% for leaf and 32.43%, 48.50% and 52.60% for root at 0.1, 0.2 and 0.3mg/ml respectively. Analysis of the fractions obtained from column chromatography of crude methanolic extracts shows that 70% reversal of sickling was obtained in fraction F7 of *A.conyzoides,* while fraction M4 of *M.indica* gave 74% reversal of sickling. Evaluation of the extracts for membrane protection also showed that at 0.5% sodium chloride concentration *A. conyzoides* reduced % lysis from 81.13% of SS

control to 28.83%, 24.37% and 40.10% for stem, leaf and root extracts respectively, while *M. indica* gave 37.73%, 37.37% and 34.90% reduction by the stem, leaf and root respectively. The stem bark of *A. conyzoides* and *M.indica* significantly ($p<0.005$) decreased methemoglobinemia to values of 3.25% and 3.56% when compared with the control. Atomic absorption spectroscopy revealed the presence of magnesium, calcium, iron and zinc in both plants. The findings of this study suggest that different parts of *A.conyzoides* and *M. indica* possess promising anti-sickling activity and membrane protection capability to validate the use of the plants in the management of sickle-cell anemia by traditional practioners.

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LIST OFABBREVIATIONS

ACS	-	Acute chest syndrome
ANOVA	-	Analysis of Variance
CSSCD	-	Cooperative Study of Sickle Cell Disease
EAN	-	Epiphyseal Aseptic Necrosis
ETA	-	Ethyl acetate
HbF	-	Fetal hemoglobin
FDA	-	Food and Drug Administration
HSCT	-	Hematopoietic stem cell transplantion
Hb	-	Hemoglobin
HbA	-	Hemoglobin A
HbS	-	Hemoglobin S
HU	-	Hydroxyurea
ISCs	-	Irreversibly sickle cells
MEOH	-	Methanol
PABA	-	Para amino benzoic acid
ROS	-	Reactive oxygen species
RBC	-	Red blood cell
SNPs	-	Single Nucleotide Polymorphisms
SS	-	Homozygous Sickle cell
SCA	-	Sickle cell anaemia
TLC	-	Thin layer chromatography
VOC	-	Vaso-occlusive crisis

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Since the evolution of man, plants have played an indispensable role in the struggle to survive. Not only do plants represent the primary source of food for all animals, including humans but a large number of plant species have been shown over the ages to have extremely important medicinal uses. Over the years, traditional medicinal plants have been widely used to treat and/or to prevent diseases and even for daily consumption (Lam *et al*., 2016).Substances found in medicinal plants, containing the healing property of plants are known as the active principle.It differs from one plant to another and examples of active ingredients include: anthraquinones, flavonoids, glycosides, saponins, tannins etc. Plants also contain other compounds such as morphine, atropine, codeine, steroids, lactones and volatile oils, which possess medical values for the treatment of different diseases.Current assessments recommend that, in numerous developing nations, a substantial extent of the population depends on orthodox and traditionalmedicine to meet essential medicinal health care needs (Ajlan, 2016).

Sickle cell disease, is a genetic disease resulting from the inheritance of two variant allelomorphic genes that control the formation of the β chains of Hemoglobin (Hb) (Agbor*et al.,* 2015). Hemoglobin S (HbS) produced as a result of this defect, is a hemoglobin tetramer (α2/β2) which is poorly soluble and polymerizes when deoxygenated (Bunn,1997). The sickle hemoglobin (HbS) disorder is caused by a point mutation affecting the coding sequence ofthe β-globin gene, causing a substitution of glutamic acid by valine.This amino acid substitution leads to a drasticreduction in the solubility (gelation) of deoxy-HbS molecules (Chikeze, *et.,al* 2011).

Sickling of hemoglobin is the cause of precocious hemolysis of erythrocytes and various complications of HbSS subjects (Kambizi and Afolayan, 2001; Moody *et al*., 2003).The recurrent pain and complications caused by the disease can interfere with the quality of the patient's life, including education, employment and psychosocial development (Alabdallat and Adam, 2016). SCA is associated with high morbidity and mortality in developing countries (Ogamdi and Onwe.,2000).Life expectancy of people with SCA is 42 and 48 years for males and females, respectively (Emmanuel *et al*., 2015). SCA has been affecting people of African origin for centuries (Mehanna, 2001). Other races affected include Mediterranean, Carribean, South and Central American, Arab and East Indian origins (Tavs *et al.*,2014). In Nigeria 23,000 infant are born annually with sickle cell anemia (WHO, 2006).In 1910 sickle cell disease was first reported by Dr. James Herrick and subsequent characterization by Linus Pauling. He hypothesized on the nature of sickle cell hemoglobin (HbS) and its role in sickle cell anemia in 1952. Since then, cure and treatment for the disease has remained a challenge for the entire humanity (Kunle and Egharevba, 2013).

The Plant *Ageratum conyzoides*

Ageratum conyzoides is a common tropical annual herbaceous weed (Onuoha et al., 2013). It belongs to the family Asteraceae (Brojendro *et al.*, 2013). It is a branching herb which grows to approximately 1 m in height. The stems and leaves are covered with fine white hairs, the leaves are ovate and up to 7.5 cm long. The flowers are purple to white, less than 6 mm across and arranged in close terminal inflorescences (Chauhan and Rijhwani, 2015). The common name of the plant is billy goat weed while its hausa name is "*Iccen ciniki*".

Traditional uses of *Ageratum conyzoides*

The leaves are applied to cuts, burns and sores and externally for body rash. They are also used against sore throat, spasm, diarrhea, epilepsy stomach ailments, gynaecological diseases, leprosy and other skin diseases (Amadi *et al.,* 2012). It is the only plant used in the management of HIV/AIDS by Igede people in Nigeria (Amadi *et al.,* 2012).

The Plant *Mangifera indica*

Mangifera indica belongs to the family Anacardiaceaeand is commonly called mango (Ogbe *et al.,* 2012). It is found all over the tropical regions of the world where it is used as a horticultural and medicinal plant (Bbosa *et al.,* 2007).It consists of about sixty genera and six hundred species, which are mainly tropical trees shrubs. It is widely used as a source of food, medicines and timber.

Medicinal uses of *Mangifera indica*

Various parts of the tree have been reported to be used in traditional medicine. *Mangifera indica* (mango tree) are commonly used as herbal preparations in the treatment of tooth ache, gastrointestinal disorders, dysentery, respiratory and urinary tract infections (Mada *et al.,* 2016). The ashes of the leaves are used to treat burns, scalds, sores, cough and diarrhoea in South America and other parts of the world (Somkuwar and Kambale, 2013).

1.2 Statement of the ResearchProblem

Currently there is no widely available cure for sickle cell anemia and most available forms of treatment are limited in their actions without holistically addressing the basis of the disease.Bone marrow transplantation and gene therapy that offers a great promise for the treatment of sickle

cell disease are very expensive and inaccessible to the lower class individuals (Imaga *et al.*,2009).

1.3 Justification of Research

Sickle cell anemia is a chronic hemolytic disorder. Different approaches have been put in place to address the problem but none of the approaches gives promising result. The diagnosis of SCD has evolved over time. This has ranged from electrophoresis, HPLC, IEF to cutting edge molecular techniques. Earlier forms of diagnosis are limited by lack of application in pre-natal diagnonsis. Developmenthave added this limitation with PCR +RFLP utilized in prenatal diagnosis on chorionic villus sampling, amniotic fluid and fetal DNA in maternal circulation (Jain *et al*., 2015). However making diagnosis is only a part in the overall management of SCD. The most problematic of the presentation is related to VOC and the underlying sickling of RBCs.Currently, hydroxyurea is used to reduce the hemoglobin polymerization by increasing the concentration of hemoglobin F among other mechanisms (Archer *et al*., 2015). It has been reported to have a cytotoxic property in nature and also causes myelosuppression which leads to severe decrease in leucocyte and platelete count. Furthermore, it is also potentially carcinogenic andmutagenic. Bone marrow transplant that show great promise for the actual cure of sickle cell anemia is very expensive to the extent that it cannot be afforded by the lower class individuals.

Therefore, there is the need for an alternative approach which is less toxic and less expensive to address the problem of sickle cell anemia. *Ageratum conyzoides*and *Mangifera indica* have been used by traditional healers in the management of sickle cell anemia, though there are limitedscientific literatures that support the claim. Therefore, this research was designed to validate scientifically the use of these plants in the management of sickle cell anemia.

1.4. Null Hypothesis

Ageratum conyzoides and*Mangifera indica* do not have any anti-sickling effect.

1.5. Aim

This research aim to investigate the anti-sickling effect of different parts of *Ageratum conyzoides*and *Mangifera indica*

1.6 Specific Objectives

i. To determine the anti-sickling effect of different parts of the plant *Ageratum conyzoides,Mangifera indica*and their fractions.

ii. To determine the effect of *Ageratum conyzoides* and *Mangifera indica* on HbSS erythrocyte membrane protection and methemoglobin concentration

iii. To carry out qualitative phytochemical analysis of *Ageratum conyzoides, Mangifera indica*extracts and their fractions.

iv. To determine elemental composition of the different parts of the plants

1.7 Limitation

The main limitation of the study arises from the small quantity of fractions obtained from column chromatography which prevented the researcher to carry out other analysis such as quantitative phytochemistry, NMR spectroscopy and GC-MS.

CHAPTER TWO

2.0 Literature Review

2.1 Sickle cell anemia

Sickle cell anemia is an inherited blood disorder which is caused by inheritance of S hemoglobin (HbSS) from both parents. The variant hemoglobin S is less soluble than the normal hemoglobin A (HbA) and therefore tends to crystallize out resulting in deformation of cells from round shape to sickled shape. The abnormality also leads to red cell membrane damage and premature death of red blood cells. Therefore, the red blood cells are lysed causing anemia. Its tendencyto sickle causes adhesion and obstruction of the blood vessels causing recurrent manifestations of pain and multi-organ ischemic damage (Obeagu *et al*., 2015). It results from a point mutation in the genetic code such that glutamic acid is replaced by valine at 6^{th} position of β-globin chain of hemoglobin. (Mpiana *et al.*, 2014). It is known that amino acid variations have very large diverse effects affecting protein sequence, structure, stability, interactions, activity, abundance and other properties, such as shape, pKa and pH (Alli and Okoh, 2016). Hence, SCA demonstrates the importance of amino acid sequence variations in determining structure, functions and, also a good pointer to the fact that, most gene variations usually consist of differences in a single amino acid residue, with variants called alleles (Alli and Okoh, 2016).

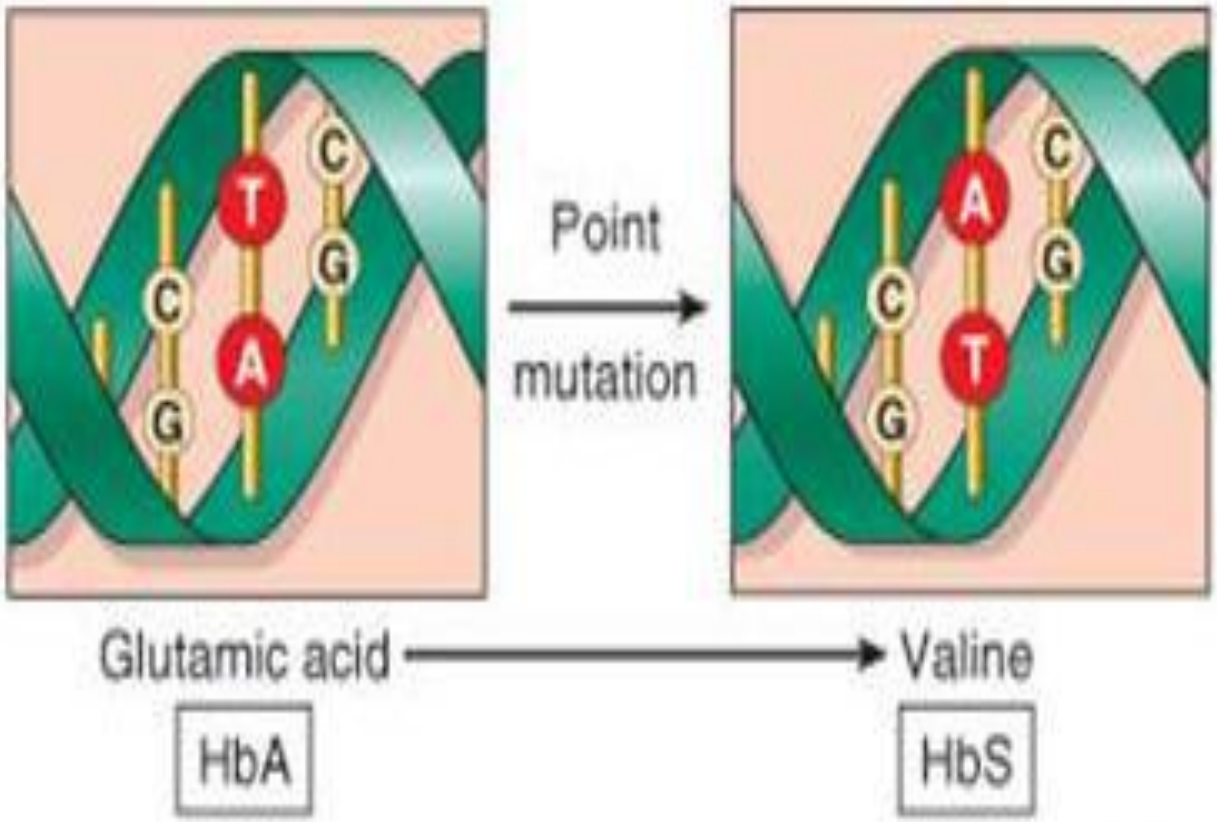


Figure 1.0:Representing substitution of adenine with thymine in Sickle Cell Anemia (Source;Kaur *et al.*,2013).

2.1.1. History of Sickle Cell Anaemia

Historians believed that DNA mutations, were responsible for the firstversions of the sickle cell gene, originated in various African regions including Central Africa Republic, Benin, Cameroon, and Senegal. Studies showed that the Trans-Atlantic slave trade introduced the sickle cell gene into the America and the Caribbean islands (Jones, 2008).

The clinical description of SCA were unknown until the explanation of the sickle RBCs in 1904 by the Chicago cardiologist and a Professor of Medicine James B. Herrick (1861 - 1954) whose intern Ernest Edward Irons (1877 -1959) found "peculiar elongated and sickle - shaped" cells in the blood of a young dental student of Granadian Nationality. He was admitted to the Chicago Presbyterian Hospital in December 1904 suffering from anemia (Emmanuel *et al*., 2015).

Linus Pauling and colleagues were the first, in 1949, to demonstrate that sickle cell disease occurs as a result of an abnormality in the hemoglobin molecule. This was the first time a genetic disease was linked to a mutation of a specific protein, a milestone in the history of molecular biology. This was published in their paper "Sickle cell Anemia, a Molecular Disease"(Emmanuel *et al*., 2015). The association between SCA and an abnormal hemoglobin was first discovered in 1954 in which the abnormality was found within the hemoglobin molecule and sickle cell hemoglobin could be separated from the normal hemoglobin using gel electrophoresis. The exact differences between normal and sickle cell hemoglobin was identified when Ingram in 1954 devised a new technique for detecting amino acid subsititution in proteins.The hemoglobin molecule was split into smaller units for analyses. Trypsin was used to specifically cleave hemoglobin on the carboxyl side of it's lysine and arginine residues. An αβ half of hemoglobin contains a total of 27 lysine and arginine residues, tryptic digestion produced 29 different peptides which was placed in a spot at one corner of a sheet of filter paper. Electrophoresis was

carried out in one direction, followed by chromatography in the perpendicular direction. The peptides were made visible by staining the filter paper with ninhydrin. This process is called finger printing.Studies later showed that SCA was due to substitution of glutamic amino acid by valine amino acid at position six of the beta globin chain(Clarke and Higgins, 2002).

2.1.2. Mode of Inheritance of Sickle Cell Anemia and its Traits

The transmission of the SCD follows the simple Mendelian principle and thus, expression of disease requires both aberrant copies of defective hemoglobin genes in most instances. The disease develops when a heterozygote (AS) (carrier) procreates with either a fellow carrier or a homozygote (SS) (Kaur *et.,al,* 2013). When both parents are carriers of sickle cell trait (AS), one out of four children will likely have SCD (25%). Procreation between a parent (AA) and a carrier (AS), the chances of having normal and carrier children is equal (50%)Marriage between normal parent (AA) and homozygote (SS), the chances of having all carrier children is 100%. The marriage between carrier (AS) and homozygote (SS) has been shown to have equal chances of having homozygote (SS)and carriers (50%), If marriage between homozygote (SS) occur which is very rare, then it has 100% chance of having homogygote (SS). Sickle cell hemoglobin has also been inherited in association with other hemoglobin variants such as Hb SC, Hb SD, Hb SE and Hb S alpha or beta thalassemia.These sickle cell traits occur when a gene for sickle cell hemoglobin is inherited from one parent and a gene for either hemoglobin A,C,D,E alpha or beta thalassemia is inherited from the other parent(Kaur *et al.,* 2013).

2.2 Epidemiology

Sickle cell anemia is a common genetic condition due to a hemoglobin disorder i.e inheritance of mutant hemoglobin genes from both parents (WHO, 2006). Such hemoglobinopathies, mainly thalassaemias and SCA, are globally widespread. About 5–7% of the global population carries a variant hemoglobin gene and the most predominant form of hemoglobinopathy worldwide is sickle cell disease (Ademola,2015). About 300 000 infants are born yearly with major hemoglobin disorders worldwide and 70% global distribution of HbS is indicative of two factors: selection for carriers through their survival advantage are affected by SCD (Mpiana *et al.,* 2013). In 2013, more deaths, 176,000, were recorded compared to 113,000 deaths reported in 1990 (Pierre*et al.,* 2015). Globally, there are more carriers (i.e. healthy people who inherited only one mutant gene from one parent) of SCA, but the high frequency of the sickle cell gene in certain areas leads to a high rate of affected newborns (WHO, 2006). Sickle cell disease has become a challenge in the African continent with about 89% of global sufferers of SCD. About twenty-five (25%) of the patients with SCD in the world are in Nigeria. Hence, this confers on Nigeria the highest population of patients withSCD in the world (Olufunmilayo*et al.,* 2012).

2.2.1 Prevalence

The prevalence of SCD can be objectively determined by calculating the birth prevalence of affected children, which requires accurate diagnosis and registration at birth. Since this is not done in most African countries, an alternative method is to use the prevalence of the carrier or heterozygous states (HbAS) to calculate the expected birth rate of SCA based on the gene frequency and Hardy-Weinberg equation $(p^2) + 2(pq) + (q^2) = 1$(Makani *et al.,* 2013).

2.3 Pathophysiology of Sickle Cell Disease

Although the polymerization of sickle haemoglobin (HbS) is the primary event in the pathogenesis of SCD, the pathophysiology of SCD is far more complex and involves endogenous and exogenous dysfunctions (Darghouth *et al.*,2015). The major clinical features of SCD in all the four haplotypes are chronic hemolytic anaemia and obstruction of microvasculature resulting in acute and chronic ischemia, and subsequent organ damagethat results from infarction and fibrosis (Sergeant and Serjeant, 2001). The β-globin gene haplotype is believed to influence the severity of SCD in patients from different population (El-Hazmi *et al*., 2012). The haplotypes include Senegal (SEN),Benin (BEN), Bantu or Central African Republic (CAR), Cameroon (CAM) and Arab-Indian (ARAB) (Loggeto, 2013).SCD in North and South America, the Caribbean and much of Europe occurs in peopleof African origin. This is mostly theof the Benin haplotype and this form of the disease is relatively severe. The disease of the Asian haplotype is generally milder because mutation has occurred against a background of genetic features likelyto inhibit sickling (Sergent *et al*., 2016). The clinical symptoms of SCA originate from the unique properties of hemoglobin. Deoxygenation of HbS is the initiator event toPolymerisation which is the core pathophysiological event in SCD.The polymerization process results to sickling due to exhibition of low oxygen tension by the red blood cells. This results into the insolubilization of the hemoglobin in reduced or deoxygenated state.Polymerisation of of hemoglobin S is dependent on intra-erythrocytic HbS concentration, the degree of hemoglobin deoxygenation, pH, 2,3-diphosphoglycerate and intracellular concentration of HbF. Due to local microcirculatory conditions, the transit time of the erythrocyte in the microvessels may be prolonged, causing lower oxygen tensions(Darghouth *et al.*,2015). As a result polymerization of HbS and dehydration of the red blood cell, the shape of the erythrocyte changes from

biconcaveshapeto sickle shape. This may occur within 2-4 minutes after deoxygenation. Repeated cycles of HbS polymerisation and thereby sickling and unsickling can cause severe erythrocyte membrane injury (Sergeant and Serjeant, 2001).

An important pathophysiologic feature of SCD is episodic occurrence of vaso-occlusive events that precipitate acute painful episodes. Vaso-occlusion of small and sometimes large vessels is the hallmark of SCD, accounting for much of its morbidity and mortality. Numerous studies proved that a key contributor to vaso-occlusion may be the increased tendency of sickle red cells to adhere to vascular endothelium.The mechanisms by which abnormal hemoglobin produces the profound, systemic, and severe effects of SCD are complex some of which are increased adhesion molecules, inflammation and depletion of NO (Bunn,1997). NO is normally produced by the endothelium and regulates the basal vessel tone, inhibits platelet and hemostatic activation, inhibits adhesion molecules and reduces reactive oxygen species (ROS) levels. Serum levels of L-arginine, the precursor of NO, are decreased in patients with sickle cell anemia, particularly during vaso-occlusive and acute chest syndrome episodes; these levels are inversely proportional to painful crisis (Gonclaves, 2012).

Vaso-occlusion can occur whenyoung“sticky” erythrocytes containing hemoglobin S adhere to the postcapillary venule and transit time of red cells through the capillaries is longer than the delay time for deoxygenation- induced hemoglobin polymerization of sickle hemoglobin. As adherence of sickle red cells to vascular endothelium will impede blood flow due to narrowing of the capillary lumen and thereby increasingcapillary transit time (Wendell *et al.*, 2000). Deoxyhemoglobin S is much less soluble than the oxygenated variety, and a hydrophobic interaction between haemoglobin S chains precipitates a double stranded, helical polymer that causes erythrocyte sickling and rigidity. Distorted sickle cells become wedged and occlude the

small vessels, producing ischemia and eventually necrosis in the affected vasculature, initiating an inflammatory response and neuropeptide release.

2.3.1 Erythrocyte Dehydration

Patients with SCD have higher proportion of dense dehydrated erythrocytes than normal individuals. This dense cell fraction includes irreversible sickle cells (ISCs) and cells with more extensive membrane damage and thus plays important role in vaso-occlusion.HbS polymerization activates sickling induced pathway, permitting Ca^{2+}entry into the cell. Increased cytoplasmic Ca^{2+} activates Gardos pathway, which in turn mediates rapid KCl and water loss. This contributes to intracellular dehydration, worsen membrane damage and increases cellular adhesion to the endothelium. The permeability pathway generated by the interaction of deoxygenated hemoglobin S polymers with the red blood cell (RBC) membrane plays a major role in the mechanism of dehydration of sickle cell anaemia RBCs. (Wendell *et al.*, 2000).

2.3.2. Complications in Sickle Cell Disease

Despite the 'common' underlying genetic basis and a similar pathophysiology, patients with SCD present a highly different clinical phenotype due to Single Nucleotide Polymorphisms (SNPs) variability throughout the genome. Patients with SCD are at high risk of developing multi-system acute and chronic complications associatedwith significant morbidity and mortality (Ersi, 2014). The evolution of genome-wide association study(GWAS) has further shown that SCD although a monogenic disorder however multiple mutations in unrelated genes modify its severity. Sebastiani *et al* (2010) utilized a Bayesian network to develop a model of SCD severity and associated SNPs. Some of the genes they identified are the cytochrome P family: CYP2C18,

CYP2C9 and CYP4F8. SNPs have been identified which have association with subphenotypes of SCDs. Genes that regulate the TGF-β/SMAD/BMP pathway have been implicated(Steinberg, 2015).

2.3.2.1 Acute Complications of Sickle Cell Disease

Acute episode of severe pain also known as acute vaso-occlusive crisis (VOC) is the most common complication of SCD. Other common acute complications of SCD include fever which is usually due to infection, hepatobiliary complications, acute anemia, acute renal failure, splenic sequestration, acute chest syndrome (ACS) and acute stroke.

a. Vaso-occlusive crisis

Vaso-occlusion in SCD is a complex process not only caused by adhesion of sickled erythrocytes to the endothelium, but also by adhesion of leukocytes and platelets along with coagulative factors to the endothelial cells (Turhan *et al.*, 2002). The sickled erythrocytes block blood flow through small blood vessels resulting in ischemia. Sudden episodes of pain throughout the body are a common symptom of SCD due to this blockade. This pain can range from mild to severe form which usually lasts from hours to a few days. VOCs and the accompanying pain mostly occur in the extremities, chest, abdomen, back and head (Kaur *et al*., 2013). Well-known precipitants of VOC include cold weather, relative high hemoglobin concentration, dehydration, infection, exercise, dampness, poor diet, hypoxia, acidosis, emotional stress, and fatigue(Behrens et al., 2000).

b. Acute Chest Syndrome(ACS)

A major target organ for acute and chronic complications of SCD is the lung. Acute chest syndrome is considered as a frequent cause of death, hospitalization and common indication for transfusion and treatment with hydroxyurea in both children and adults with SCD (Kathleen

etal., 2011).Common causes of ACS include infection, vaso-occlusion, fat embolism, and thrombosis (Kaur *et al.,* 2013). Death may be due to sudden drop in oxygen supply following a loss of gas exchange interface.

c. Priapism

Priapism isanother common acute pain state in patients with SCD. Priapism is defined as a painfulprolonged, purposeless penile erection that persists beyond or is unrelated to sexual interest or stimulation (Andrea *et al*., 2013). Priapism lasting more than 3 hours is called prolonged priapism (PP), while the shorter episodes, which have a mean duration of less than one hour, are called stuttering. Nocturnal occurrence predominates and may seriously impair rest and quality of life. This happens because the sickled cells prevent blood to flow out of an erect penis. Repeated episodes of priapism lead to impotence, and subsequent episodes of priapism may be characterized by a painful, but not fully erect, penis(Robert*et al*., 2000)

d. Acute stroke

Central nervous system events can be particularly catastrophic in patients with SCD (Sergeant, 1997).Stroke is one of the most common and devastating complications (Ohene-Frempong *et al.*,1998). Sickle shaped red blood cells may stick to the walls of the tiny blood vessels in the brain. This type of stroke occurs mainly in children at a median age of 6 years, and affects 8% of patients with sickle cell anemia by the age of 14 years (Behrens*et al.,* 2000). This complication presents as sudden onset of weakness, aphasia, and sometimes seizures or coma and results in adverse motor and cognitive sequelae(Ersi, 2014). Patients with recurrent strokes may be candidates for high-risk treatments such as recurrent transfusions orbone marrow transplant(Behrens*et al.,* 2000).

e. Organ Damage

Organ damage is progressive throughout the course of the disease. It may be slow, steady and irreversibleor marked by acute deficits, some of which may be reversible. Spleen damage is universal in patients with SCD. Antibody formation, opsonization, and alternate complement pathway activation are all decreased by splenic hypofunction, which occurs early in childhood. Recurrent and irreversible small infarcts cause autosplenectomy and predispose the individual to infections with encapsulated organisms (pneumococci, meningococci, and salmonella).

2.3.2.2 Chronic Complications of Sickle Cell Disease

The incidence of chronic complications appears to increase with age and understanding of the pathophysiology and the involved factors is necessary to prevent or reduce long-term morbidity.

a. Bone and Joint Complications

Bone involvement in SCD may result from three mechanisms: osteopenia due to bone marrow expansion, bone infarction, and osteomyelitis (Diggs, 1967). The main consequence of osteopenia is vertebral instability with mechanically provoked pain and a greater risk of compression fractures. Osteonecrosis of epiphyseal segments of bones (EAN – Epiphyseal Aseptic Necrosis) is a leading cause of chronic pain in the adults, and has a devastating effect on quality of life. It may occur in all SCD genotypes and at least 40% of patients older than 30 years have one or more symptomatic EAN. In order of frequency, EAN affects the hips, the humeral heads and the knees. However bone infarction can be observed in any bone, or part of bones.

Humeral head osteonecrosis occurs frequently in all genotypes and particularly in individuals who already have femoral head involvement.Osteomyelitis frequency is linked to the environmental setting. It is a severe complication which most often originates from

bacteremia.The two main bacterial species involved are *Salmonella* and *Staphylococcus* (Mallouh and Talab, 1985).

b. Leg ulceration

Leg ulcers are more common in homozygous forms of HbSS commonly called SCA(Ladizinski*et al.,* 2012). (These occur mainly in the more severely anemic patients and are rare during early childhood (Jawad *et al.,* 1998). They have little or no spontaneous tendency to heal and even when well managed they may remain unhealed for years. The exact cause of sickle cell ulceration remains unclear, but theconsensus in the field is that the causation is multifactorial. Leg ulcers are produced by minor trauma, stasis, pressure, and sickling in the small skin vessels. Thechronic damage fromclotting and impeding themicro-circulationplays the key role in causing sickle cell–associated leg ulcers (Ladizinski *et al.,* 2012). Leg ulcers in patients with sickle cell are more common with increasing age (Jones *et al.,* 2013). Some heal rapidly, but others persist for years or recur(Ersi, 2014).Zinc deficiency in sickle cell anemia has been reported to retard wound healing and increase incidence of infection (Edamisan *et al*., 2011).

2.4. Generation of Reactive Oxygen Species (ROS)in SCD

Reactive species such superoxide (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO•), nitrogen oxide (NO•), peroxynitrite (ONOO-) and hypochlorous acid (HOCl), are all products of normal metabolic pathways of the human organs (You and Chan, 2015) but, when in excess, can exert harmful effects under certain conditions.Oxidative stress is defined as an imbalance between the production of ROS and the ability of the biological system to repair these damages. Such activated oxygen species are now increasingly recognized to be the mediator of the cell injury in diseases and have been implicated in the pathogenesis of certain human diseases such as, cancer, atherosclerosis, neurodegenerative disorders, digestive system disorders, autoimmune

pathologies and diabetes (Hermann *et al.*,2014).Hemoglobin S (HbS) auto-oxidation processes and recurrentHbS polymerization/depolymerization events that promoteintermittent vaso-occlusive episodes and reperfusion injury are a major source of enhanced reactive oxygen species(ROS) production in sickle cell disease (Hierso*et al.,* 2014). The increased pro-oxidant generation in SS patients results in excessive antioxidant consumption and thus decreased antioxidant capacity (Hierso*et al.,* 2014).

2.5 Management of SCD

In most countries where sickle-cell anaemia is a major public health concern, its management has remained inadequate, as national control programmes do not exist. The basic facilities to manage the patients are usually absent, systematic screening is not a common practice and the diagnosis is usually made when a patient presents in early childhood with dactilitis. In ABUTH Shika, diagnosis is done using alkaline electrophoresis in addition to clinical features.

2.5.1 Orthodox Treatment Line Approach

Various approaches have been adopted in an effort to find agents that inhibit the polymerization of hemoglobin and hence prevent or reduce the occurrence of crises in sickle cell disease (Iyamu *et al*., 2002). This is largely supportive in the form of good nutrition, infection prophylaxis, avoidance of extreme climatic conditions, maintenance of hydration, transfusion support, routine clinic visits and education of the family and patient onthe disease (Sergent,2001). Malaria prophylaxis, folic acid supplementation made remarkable improvements in SCA outlook.

2.5.1.1 Chemotherapeutic Approach

As a genetic disease, no specific drugs are yet available; however, management has been targeted at increasing the solubility and oxygen affinity of deoxygenated Hb S.Examples of such

treatments include, the use of Hydroxyurea, penicillin as prophylaxis to prevent infections which is common among sickle cell patients.Patients with SCD are hyposplenic and are at risk of infection, in particular from encapsulated bacteria such as *Neisseria meningitides, Streptococcus pneumonia and Haemophilus influenzae*.There is clear evidence that penicillin prophylaxis is of benefit to all patients with SCD as it decreases the rate of infection (Green-top Guideline, 2011). Unfortunately, all currently proposed therapies are quite expensive and have attendant risk factors in terms of clinical use (Vaishnava and Rangari, 2015).

2.5.1.2Hydroxyurea (HU)

Hydroxyurea (HU) is a chemotherapy drug used in myeloproliferative disorders and neoplastic diseases. This drug has the ability to prevent the formation of HbS polymers and increase levels of fetal hemoglobin. (Marilia *et al.,* 2014). Patients randomized on hydroxyurea (HU), had fewer pain episodes, less acute chest pain syndromes and less transfusion requirements (Nwaoguikpe *et al., 2*013). HU has been shown to reduce mortality in agroup of otherwise severely affected patients after 5 to 10 years follow-up (Steinberg *et al.,* 2003). However, it was found to be potentially mutagenic and carcinogenic (Nwaoguikpe *et al.,* 2013). The cancer risk of HU in person years is 1 per 1000 years (Steinberg *et al*., 2010).

Hydroxyurea (HU) was first approved by the Food and Drug Administration (FDA) for the treatment of sickle cell disease (SCD) in 1996 (Fathallah *et al.,* (2005). Its usefulness in SCD is related to its ability to induce increased levels of fetal hemoglobin production in sickle cells thus mitigating tendencies for red cell sickling (Ademola, 2015). HU is also known to increase steady state hemoglobin levels and reduce leucocyte and platelet counts. Also, as a rheological agent, HU improves cell hydration, limits interaction of the sickle cells with the vascular endothelium, and acts as a nitric oxide donor (Ademola, 2015). Chemotherapeutic agents such as HU, butyrate

and 5-azacytidine may have beneficial effect on activating γ-globin gene expression and HbF production in SCD; yet, the dose of inducing agents must be strictly monitored in order to ensure it is high enough to activate stress signaling pathway but not too high to trigger cell-cycle arrest or apoptosis in hematopoietic precursor cells that will result in dangerously low blood counts

2.5.1.3Bone Marrow Transplantation and Gene therapy

The only curative option currently available for SCD is hematopoietic stem cell transplant (HSCT) (Archer *et al.,* 2015).This therapeutic option is neither cheap nor available to most patients due to the lack of a Human Leukocyte Antigen (HLA)-matched bone marrow donor (Alli and Okoh, 2016). Transplantation procedure requires a highly compatible family member as a donor and is associated with potentially serious complications. Disease-free survival rate is about 85%, transplant related mortality is 7% while graft failure rate is 9% (Alli and Okoh, 2016).

2.5.2 Traditional Medicine Approach

The economic implications of the SCD is of public health importance in developing countries. This is why its treatment is beyond the reach of the populations of the sub-saharan Africa where the use of medicinal plants constitutes part of their culture and tradition (Kotue *et al.,* 2014). At least 60% of Nigerians use TAM as first port of care.There is a growing evidence that ethno-medicine is an efficient source of new pharmaceuticals (Ngbolua *et al.,* 2014). It is acknowledged world-wide that traditional medicine can be explored and exploited to be used along-side synthetic pharmaceutical products for enhanced health management.Due to the high mortality rate of SCD patients, especially in childhood, and since chemotherapy has its adverse effects, there is need for rational drug development that must embrace not only synthetic drugs, but also natural products (herbal drugs). Phytochemicals are chemical compounds formed during

the normal metabolic processes of a plant. These chemicals are also referred to as "secondary metabolities" which include different classes such as alkaloids, flavonoids, glycosides, gums, coumarins, phenols, tannins, terpenes and terpenoids (Okwu, 2004). Most plant parts (roots, stems, leaves, flowers) and others have been found to be good sources of anti-sickling agents and phytochemicals used in the management of sickle cell disease (Nwaoguikpe and Braide, 2011). Active constituents of medicinal plants and naturally occurring compounds (anti-sickling agents) that improve the health of sickle cell individuals are rich in aromatic amino acids, phenolic compounds and antioxidant nutrients (Abraham *et al.*, 1991) which are thought to be responsible for their observed anti-sickling action. Some of these active compouds with anti-sickling activity includes terpenoids, anthraquinones, phenolics, flavonoids and alkaloids (Vaishnava and Rangari, 2015).

2.5.2.1 *Ageratum conyzoides*

Ageratum conyzoides is among such medicinal plants that are effective against many diseases and may contain biologically active compounds, which are effective against ill health (Amadi *et al*., 2012). It belongs to the family of Asteraceae. It is an erect, annual, branched, slender, hairy and aromatic plant, which grows to approximately 1m in height. It is native to Central America, Southeast Asia, South China, India and West Africa (Prince and Prabakaram,2011).

The phytochemical screening of the ethanolic extracts of *Ageratum conyzoides* indicated the presence of tannins, steroids, saponins, alkaloids, phenols, flavonoids and carbohydrate (Odeleye*et al.*, 2014). In another study by (Amadi*et al.*, 2012), phytochemical screening revealed the presence of alkaloids, flavonoids, tannins, saponins, and cyanic acid (HCN) in the investigated parts of *Ageratum conyzoides.*

Ageratum conyzoides has antimicrobial,antimalarial, analgesic, antidiarrheal andanti-inflammatory(Brojendro *et al*., 2013). In some parts of Africa, the plant is used for headaches, dyspnoea, mental and infectious diseases (Singh *et al*.,2012). However despite the claims made by traditional healers on its role in management of sickle cell crisis, no literature supports their claims. Hence, the need to scientifically validate their claim.

2.5.2.2. *Mangifera indica*

Mangifera indica is a large evergreen tree, with a heavy, dome-shaped crown and it belongs to the family *Anacardiaceae*(Samkuwar and Kambale 2013) that consists of about sixty genera and six hundred species, which are mainly tropical shrubs. It is widely used as a source of food, medicines and timber (Mada*et al.,* 2012).The common name of the plant is Mango while its local names are mangwaro(Hausa), Mangoro(Yoruba) and mangolo (Igbo). Various parts of plant are used as a dentrifrice, antiseptic, astringent, diaphoretic, stomachic, vermifuge, tonic, laxative and diuretic and to treat diarrhea, dysentery, anemia, asthma, bronchitis, cough, hypertension, insomnia, rheumatism, toothache, leucorrhoea, hemorrhage and piles (Shah *et al*., 2015). Mango is also claimed to have anti malarial effect and has found to display *in vitro* activity against *Plasmodium falciparum*.

The ash of the leaves are used to treat burns, scalds, sores, cough and diarrhoea in South America and other parts of the world (Somkuwar and Kambale, 2013). Mango stem-bark aqueous extract has been reported to possess anti-inflammatory, analgesic and immunoprotective effects. A number of biological activities of mangiferin have been suggested, including anti-diabetic and anti-inflammatory abilities (Vyas *et al.,* 2012). Mango is also claimed to have anti malaria effect (Tsabang *et al*., 2012) and was found to display *in vitro* activity against *Plasmodium falciparum* (Rasoanaivo *et al.,* 2004). Stem bark of *M. indica* showed significant antibacterial and antifungal

activities against *Streptoccocus pneumonia,Enterobacter aerogenes*, *Klebsiella pneumonia*and *Candida albicans* with MIC of 0.08 mg/ml(El-Gied *et al*., 2012). The fruits have also been eaten with salt and honey as source of vitamin A and for the treatment of blood disorders. Sap from the leaves and unripe fruits have been eaten as colic and to treat irritations scorpion and bee stings (Doughari and Manzara, 2008). Mangifera indica is used in Ayuverdic system of medicines for a variety of medicinal purposes (Latha *et al.,* 2011). The local people are constantly using the root as part of Ayuverdic for Leucorrhoea (Shah, 2010). Aqueous decoction of the flower was also reported to have among other properties anti-ulcerative effect in piroxicam-induced gastric lesions (Severi *et al.,* 2009). Other reported activities include, anti-inflammatory and analgesic effects (Islam *et al*., 2010).

Phytochemical screening of *mangifera indica* stem bark showed the presence of active pharmacological components such as tannins, saponims, sterols, cardiac glycoside, flavonoid and alkaloids. These components are known to be biologically active because they protect the plant against infections and predations by animals (Mada *et al.,* 2012). Phytochemical screening of the leaf extract revealed the presence of high concentration of phenols and flavonoids.Its population and importance can easily be realized by the fact that it is often referred to as *King of Fruits in the Tropical World*(Mahato *et al*., 2016).

2.6 Prevention of Sickle Cell Disease

Sickle-cell anemia can be prevented. Couples at risk of having affected children can be identified by inexpensive and reliable blood tests; chorionic villus sampling from nine weeks of pregnancy can be performed for prenatal diagnosis targeting the sickle Hb gene

(Alli and Okoh, 2016). Adoption of such measures goes hand-in-hand with health education. However Experience has clearly shown that genetic counselling coupled with the offer of

prenatal diagnosis can lead to a large-scale reduction in births of affected children. The risk of having affected children can be detected before marriage or pregnancy. These approaches should be practiced in conformity with the three core principles of medical genetics: the autonomy of the individual or the couple; their right to adequate and complete information; and the highest standards of confidentiality.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All chemicals used were of analytical grade. These included; Sodium metabisulphite, paraffin, para hydroxy benzoic acid(Sigma Aldrich), methanol(Sigma Aldrich), petroleum ether(Sigma Aldrich), Ethyl acetate (Sigma Aldrich), Sodium monohydrogen phosphate, Sodium dihydrogen phosphate, Silica gel 60-120 mesh(Qualikems, United kingdom), Giemsa stain(BDH chemicals, United Kingdom), Immertion oil.

3.1.2 Reagents

Geimsa stain. 0.68% of geimsa in methanol/glycerol was diluted with distilled water in the ratio of 1:10.Mayer's reagent; 1.36g of mercuric chlorate and 5g of potassium iodide was dissolved in 100ml distilled water. Spraying reagent (p-anisaldehyde Sulphuric acid spray); 0.5ml of P-anisaldehyde, 5ml of Conc Sulpuric acid and 10ml of glacial acetic acid were dissolved in 85ml of methanol.Dragendroff's reagent; 1.27g of Iodine and 2g of Potassium iodide dissolved in 100ml of distiled water.

3.1.3 Equipment

Centrifuge machine(Labofuge 300, Heraeus), Microscope (Herius, United kingdom), Spectrophotomter (6305, Jenway), Soxhlet extractor(Pyrex United kingdom), Water bath(Grant jb series, B and T), Chromatograpic tank,Weighing balance(GF-2000), TLC plates(G60 F_{254}, EMerck), Cuvette, pH meter 3150,(Jenway), Heating oven (IGNS 5000),Rotory evaporator (B-490 Buchi).

3.1.4Plant Material

Fresh plant samples were collected from Dumbi, Zaria Local Government Area of Kaduna state, located at 11°4N and 7°42E.The samples were authenticated at Herbarium Unit of Biological Sciences Department, Ahmadu Bello University Zaria, with Voucher Numbers V3611 and V1312for *Ageratum conyzoides* and *Mangifera indica* respectively.

3.2 Sampling and sampling procedure

3.2.1 Area of Study

The study area, Zaria LGA is a major city in Kaduna State, Nigeria. It is located at 11°4N and 7°42E on the Nigerian map.

3.2.2Collection of Blood Sample

Blood sample used for the experiment were collected from patients confirmed to have sickle cell anemia as suggested by Alkaline Hemoglobin Electrophoretic pattern attending the day care unit of hematology department Ahmadu Bello University Teaching Hospital, Shika for medical care. Five(5)ml of blood was collected into anticoagulated tubes (Tripotassium EDTA bottles) under aseptic conditions.

3.2.3 Inclusion Criteria

- Patients with confirmed SCA by a combination of solubility test using sodium metabisulphite as well as Hb electrophoresis on cellulose acetate in an alkaline medium.
- Patients who signed an informed written consent
- Patients from 12 years and above
- HbAA subjects

3.2.4 Exclusion Criteria

- HbAS Subjects

- Patients with hemoglobin SC (HbSC)

3.2.5 Ethical Clearance

Ethical clearance was obtained from Health Research Ethics Committee Ahmadu Bello University Teaching Hospital Shika on 3rd October 2013 with assigned Number of ABUTH/HREC/GO7/2013. Informed consent form (page 106-108)was issued to patients to obtain their permission for blood collection.

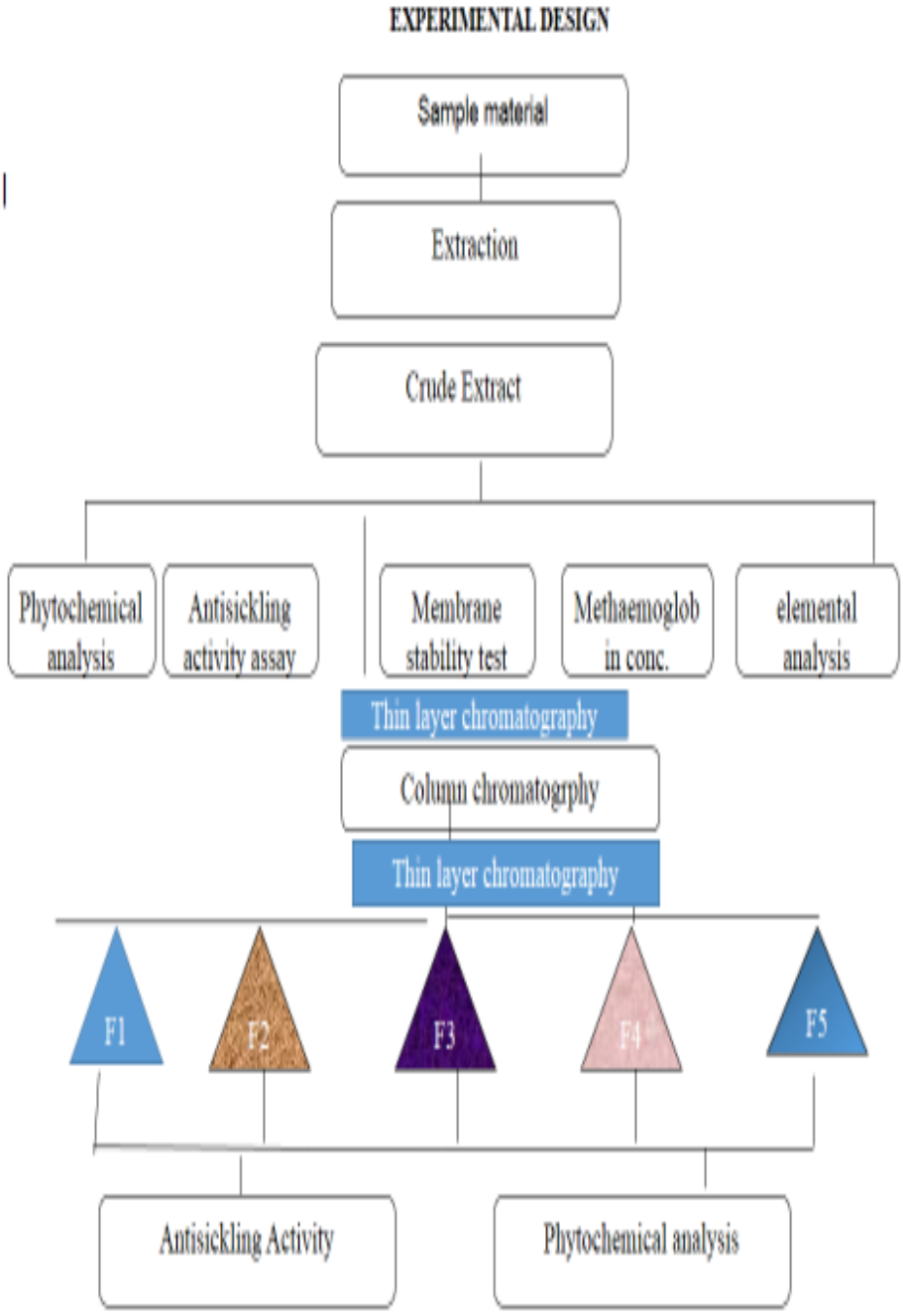


Fig 3.0: Experimental Design

3.3 METHODS

3.3.1 Exraction of the Plant Materials

Exactly 500g of each powdered plant material was weighed and then loaded into soxhlet extractor. Each Sample was then deffated with petroleumether for 8 hoursand then extracted with methanol for another 8 hours.The solvent was evaporated under reduced pressure using rotary evaporator, and thereafter kept in a dessicator to dry completely before being kept in a refrigerator (Sofowora, 1993).

3.3.2 Preparation of the Blood Sample

Blood sample collected from sickle cell patients was centrifuged at 1500g for 10 minutes. The supernatant plasma was decanted and the volume determined. Normal saline was then added to the packed erythrocytes and mixed gently. The amount of saline added was equal to the volume of plasma removed.This procedure was repeated three more times with normal saline. The washed erythrocytesto be used for the analyseswas stored in the refrigerator for18-20 hours.

3.4 Determination of Anti-Sickling Property of the Plant extract

Anti-sickling property of the plant extract was determined by the method of Imaga *et al.*(2010), which is based on removal of the oxygen content of the red cells by sodium metabisulphite. The ability of the plant extract to reverse sickling was measured under the microscope at x1000 magnification.

3.4.1 Procedure

The washed erythrocytes (0.5ml) were mixed with 0.2ml of 2% sodium metabisulphite in test tubes and sealed with parafin. The mixture was then incubated for 30 min at 37℃. Wet preparation was made and observed under the microscope at Magnification×1000 to confirm

wether sickling was induced.Various concentrations of the extract were then added to the test tubes and then incubated again for 120 min at 37 ^{o}C. Samples were then taken and then smeared on different microscopic slide after 0 min, 30 min, 60 min, 90 min and 120 min. Each sample smeared was dried and then fixed with 98% methanol, dried and stained with Giemsa stain, and each sample was examined under the oil immersion using light microscope before counting at least 500 red blood cells in each sample from five different fields of view across the slide. The number of sickled cells were counted and the percentage of unsickled cells was determined using the following formula;

$$\%\ \text{of unsickled cells} = \frac{\text{mean of sickled cells at 0 min} - \text{mean of sickled cell at time t}}{\text{Mean of sickled cell at 0 min}}$$

Apositivecontrol using p-hydroxy benzoic acid (5mg/ml) and a negative control involving the use of norrmal saline were employed in this analysis.

3.5 Osmotic Fragility Test

The ability of the plant extract to protect the cell against induced hemolysis was determined by the method of Umukoro and Ashorobi (2006). This is based on the ability of the extract to possess a compound(s) that facilitates the protection of biological membrane.

3.5.1 Procedure

The stock erythrocyte (RBC) suspension (0.50 ml) was mixed with various concentrations (0.1, 0.2, 0.3, 0.5 0.6 and 0.9%) of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the extract (0.5-2.5 mg/ml). The control samples consisted of 0.5 mlSS washed erythrocytes mixed with hypotonic-buffered saline solution aloneand 0.5mlAA erythrocytes mixed with only saline solution. The mixtures were incubated for 30 min at room temperature and centrifuged for 5 min at 3000 g and the absorbance of the supernatant was measured at 540 nm. The percentage lysis was calculated using the formular

$$\%\ lysis\ = \frac{Absorbance\ of\ Test}{Absorbance\ of\ 100\%\ lysis} \times 100$$

3.6 Determination of Methemoglobin Concentration

The effect of the plant extract on methemoglobin concentration was determined by themethod of Chikezie (2009). The Principle behind this test is that hemoglobin and methemoglobin absorbs light at diffrent wavelengths, at 540nm and 630nm as their respective peak absorbance, and hence increasein absorbance at 630nm signifies increase in methemoglobin concentration.

3.6.1 Procedure

In a test tube containing 5.0ml of distilled water, 0.02ml of whole blood was added. The mixture was allowed to stand for 1 hour at room tempreature (20-25℃), and absorbance was read at wavelength of maximum absorption of hemoglobin and methemoglobin which is 540 nm and 630 nm respectively. This serves as the control experiment.

The effect of each of the extracton plasma methemoglobin concentration was carried out by introducing 0.02ml of the specified concentrations (0.2-1.0g% w/v) of each extract solution into separate test tubes. This was followed by the addition of 5 ml of distilled water and 0.02ml of the blood sample. The mixture was allowed to stand for 60 minutes at room temperature, after which, the absorbance was read at 540 and 630 nm. The percentage plasma methemoglobin was calculated using the formula below;

$$\text{Percentage Methemoglobin}\ = \frac{(A630)^2}{(A540)^2+(A630)^2} \times \frac{100}{1}$$

1

Where A540 = absorbance at 540 nm andA630 = absorbance at 630 nm.

3.7Partial Purification of active extracts

Chromatography

The most active part was partially purified using column chromatography according to the method of Ejele *et al.,* (2012). Thin layer chromatography was carried out to ascertain the solvent system to be used for the column chromatography.

3.7.1 Procedure for Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out to determine the solvent system to be used for column chromatography. An aluminium chromatographic plate coated with silica gel was used. The crude extract was dissolved in methanol and then spotted ona strip of the coated plate using a micro hematocrit capillary tube. The plates were separately placed in a chromatographic tanks each containing the following solvent systems:

Hexane:ethylacetate 7:3

Chloroform : methanol 8 : 2

Ethylacetate : methanol 7 : 3

N-butanol : acetic acid : water 8 : 1: 1

Ethylacetate : methanol : water 10 : 1 : 1

Chloroform : ethylacetate : methanol : water 15 : 8 : 4 :1

The solvent that gave the best separation was Ethyl acetate –Methanol 7:3 and was therefore used for the elusion in column chromatography.

3.7.2. Bioassay Activity Guided Fractionation

The highest anti-sickling activity was obtained with stem barks of both *A. conyzoides* and*M.indica*. Therefore, the stem bark extracts of the two plants were further subjected to fractionation.

3.7.3. Procedure for Colomn Chromatography

The crude extracts of *A. conyzoides and M. indica*stem barks were subjected to column chromatography in order to separate the extracts into their component fractions. Silica gel 60-120 mesh was used as the stationary phase while solvent combinations of increasing polarity were used as the mobile phase. In the setting up of column chromatography, the lower part of the glass column(62×2.5cm) was stocked with glass wool with the aid of glass rod. The slurry was prepared by mixing 100g of silica gel and 250 ml of ethylacetate and then poured down carefully into the column (wet packing method). The tap of the glass column was left open to allow free flow of solvent into a conical flask below. The set-up was seen to be in order when the solvent drained freely without carrying either the silica gel or glass wool into the tap. At the end of the packing process, the tap was locked. The column was allowed to stabilize, after which, the clear solvent on top of the silica gel was allowed to drain down to the silica gel meniscus. The crude extract to be loaded was properly mixed with activated sand to loosen the sample. Spatula was used to stir the sample and then gently layered on top of the column and the tap was opened to allow the eluent to flow. Elusion of the extract was done with solvent system of gradually increasing polarity using ethylacetate and methanol. The following ratios of solvent combinations were sequentially used in the elution process, ethylacetate: methanol 100:0, 90:10, 80:20, 70: 30 and the eluted fractions were collected in aliquots of 20 ml in beakers.

3.7.4 Thin layer chromatography (TLC) and pooling of fractions

The fractions were concentrated by air drying and then the content of each beaker was spotted on a strip of aluminium coated TLC plate using micro hematocrit capillary tube and placed in a small chromatographic tank to separate the different fractions based on their relative mobilities in solvent system. The strip was lowered into a small chromatographic jar containing the solvent system. The jar was covered with a glass lid. The solvent was allowed to ascend until the solvent front was about ¾ of the length of the strip. The strip was removed and air dried.

Thereafter, the plates were removed and air dried.The spots were developed by spraying the plates with P-anisaldehyde spraying reagent and heated in an oven for 1 min. The spots were then compared and all the fractions that travelled same distance were pooled together (i.e they have same $R_f$value) and then subjected to phytochemical screening and anti-sickling activity (Ejele *et al*.,2012).

3.8 Preliminary Phytochemical Screening.

Phytochemical screening was carried out on all the extracts according to the method of sofowara, (1993).

3.8.1 Test for Carbohydrates

3.8.1.1 Molisch's test

Few drops of molisch's reagent was added to 2ml of the extract and then concentrated sulphuric acid was added down the side of the test tube to form a lower layer with reddish colored ring at the interphase which indicates presence of carbohydrates (Evans, 1989).

3.8.2 Test for free Anthracene Derivatives

3.8.2.1 Bontrager's Test

To a portion of the extract in a dry test tube, 5ml of chloroform was added and shaken for at least 5 minutes. This was filtered and the filtrate shaken with equal volume of 10% ammonia solution,

bright pink color in the aqueous (upper) layer indicates the presence of free anthraquinones (Evans, 1989).

3.8.3 Test for Steroids and Triterpenes

3.8.3.1 Lieberman- Burchard's Test

To a portion of the extract, equal volume of acetic anhydride was added and mixed gently. One (1ml) of concentrated sulphuric acid was added down the side of thetest tube to form a lower layer. Color changes were observed immediately and over a period of one hour. Blue to blue-green color in the upper layer and reddish, pink or purple color indicates the presence of triterpenes (Evans, 1989).

3.8.4 Test for Cardiac glycosides

3.8.4.1 Keller-Killani Test

A portion of the extract was dissolved in 1ml of glacial acetic acid containing traces of ferric chloride solution. This was then transferred into a dry test tube and 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer at the bottom. It was observed carefully at the interface for purple-brown ring. This indicates the presence of deoxy sugars and a pale green color in the upper acetic acid layer indicates the presence of cardiac glycosides (Evans, 1989).

3.8.5 Test for Saponin Glycoside

3.8.5.1 Frothing Test

About 10ml of distilled water was added to a portion of the extract and was shaken vigourosly for 30 seconds. The tube was allowed to stand in a vertical position and was observed for 30

minutes. A honeycomb froth that persist for 10-15 minutes indicates presence of saponins (Evans, 1989).

3.8.6 Test for Tannins

3.8.6.1 Ferric chloride Test**;**

To a portion of the extract, 3-5 drops of ferric chloride solution was added. A greenish-black precipitate indicates presence of condensed tannins while hydrolysable tannins giveto a blue or brownish blue precipitate (Evans, 1989).

3.8.7 Test for Flavonoids

3.8.7.1 Sodium hyroxide test

Few drops of 10% sodium hydroxide were added to the extract. Yellow coloration indicates presence of flavonoids (Evans, 1989).

3.8.8 Test for Alkaloids

3.8.8.1 Dragendoff's Test

To a portion ofthe extract, few drops of Dragendroff's reagent were added. A reddish brown precipitate indicates the presence of alkaloids (Evans, 1989).The phytochemical screening of the fractions was carried out using TLC because the quantity of the fractions were small.

3.8.9 Test for phenolic compounds

3.8.9.1 Ferric chloride test

Respective fractions were dissolved in methanol and then spotted on the TLC plates using a micro hematocrit capillary tube. The plates were placed in a chromatographic tank containing Ethyl acetate –Methanol 7:3 which gave the best separation in column

chromatography.Thereafter, the plates were removed and air dried.The spots were developed by spraying the plates with Ferric chloride spray.

3.8.10 Test for Alkaloids of fractions

3.8.10.1 Dragendroff test

Respective fractions were dissolved in methanol and then spotted on the TLC plates using a micro hematocrit capillary tube. The plates were placed in a chromatank containing Ethyl acetate –Methanol 7:3 which gave the best separation in column chromatography.There after the plates were removed and air dried.The spots were developed by spraying the plates with Dragendroff spray.

3.8.11 Test for triterpenes and steroids of fractions

3.8.11.1 Libermann Buchard test

Respective fractions were dissolved in methanol and then spotted on the TLC plates using a micro hematocrit capillary tube. The plates were placed in a chromatank containing Ethyl acetate –Methanol 7:3 which gave the best separation in column chromatography.There after the plates were removed and air dried.The spots were developed by spraying the plates with Liberman Bucchard spray.

3.8.12 Test for flavonoids of fractions

3.8.12.1 Aluminum chloride test

Respective fractions were dissolved in methanol and then spotted on the TLC plates using a micro hematocrit capillary tube. The plates were placed in a chromatank containing Ethyl acetate –Methanol 7:3 which gave the best separation in column chromatography.Thereafter, the plates

were removed and air dried.The spots were developed by spraying the plates with Aluminum chloride spray. The TLC plates were allowed to dry and viewed under Uv light.

3.8.13 Test for Anthraquinone

3.8.13.1 Anthraquinone test

Respective fractions were dissolved in methanol and then spotted on the TLC plates using a micro hematocrit capillary tube. The plates were placed in a chromatank containing Ethyl acetate –Methanol 7:3 which gave the best separation in column chromatography.Thereafter, the plates were removed and air dried.The spots were developed by spraying the plates with Anthraquinone spray.

3.9 Atomic Absorption Spectroscopy

HCL nitrite ratio 1:3 was added to 0.5g of the extract and then heated at 100^{o}C for two hours to almost dryness. The mixture was allowed to cool and then transferred to 50ml volumetric flask. Deionised water was added up to the 50ml mark. The mixture was then subjected to analysis using AA240 FS (Atomic Absorption Spectroscopy).

3.10Statistical analysis of data

Data generated during the course of the study were analyzed using Analysis of Variance (ANOVA) whilestatistical significance wastested at 95% confidence limit (i.e $P \leq 0.05$) was considered significant.

CHAPTERFOUR

4.0 RESULT

4.1 Anti-sickling Activity of extracts

Table 4.1shows the percentage of unisickled cells treated with methanolic extract of *Ageratum conyzoides*stem bark at various concentration (0.1-0.3mg/ml). The Table clearly shows that the extract exhibited significant ($p<0.05$) increase in number of unsickle cells,when compared with normal saline (negative control) with peak value of 67.77% at 0.3mg/ml concentration even though its activity was lower than that of p-hydroxy benzoic acid (positive control) that gave 84.60 ± 0.64^{a}. This indicates that the reversal of sickling was time and concentration-dependent, because there were significant ($p<0.05$) differences in activity between the concentrations used at 30, 60, 90 and 120 min

Table 4.2 shows the percentage of unsickle cells treated with methanolic extract of *Ageratum conyzoides*leaf at different concentrations. There was a significant ($p<0.05$) increase in the percentage of unsickle cells as the concentration of the extract increased, with a peak value of 55.50% at 0.3mg/ml. Also there weresignificant ($p<0.05$) differences in the percentage of

unsickled cells treated with various concentrations of the leaf extract, suggesting that, the anti sickling effect was also time-and-concentration-dependent.

Table 4.3 shows the effect of methanolic extract of *Ageratum conyzoides*root on human sickle red blood cells. Incubation of the sickled cells with the extract at various concentrations for 120 min showedthat there was a significant ($p<0.05$) increase in the percentage of unsickle cells when compared with normal saline.This was however lower than the Anti-sickling activityrecorded for the stem bark and leaf extracts.

Table 4.1: Percentage of cells unsickled after treatment withmethanolic extract of *A. conyzoides*stem barkat various concentrations.

Time(min)	Normal saline	0.1mg/ml	0.2mg/ml	0.3mg/ml	PABA(5mg/ml)
30	3.10±0.29^{d}	29.90±1.62^{c}	37.40±1.44^{b}	44.30±2.48^{a}	30.90±0.23^{c}
60	3.90±0.23^{d}	36.30±0.69^{c}	42.70±1.33^{b}	54.50±1.04^{a}	53.60±0.35^{a}
90	4.70±0.35^{d}	41.20±0.92^{c}	47.90±0.46^{b}	51.10±2.37^{b}	65.00±2.37^{a}
120	6.20±0.46^{d}	56.93±2.37^{c}	64.60±2.42^{b}	67.77±1.24^{b}	84.60±0.64^{a}

The values in the table are the Mean±SD from triplicate experiment

Table 4.2: Percentage of cells unsickled after treatment withmethanolic extract of *A. conyzoides*leafat different concentrations.

Time(min)	Normal saline	0.1mg/ml	0.2mg/ml	0.3mg/ml	PABA(5mg/ml)
30	3.10±0.29^{d}	25.80±0.29^{c}	28.90±1.10^{b}	36.80±1.33^{a}	30.90±0.23^{b}
60	3.90±0.23^{d}	29.40±2.60^{c}	29.43±0.32^{c}	42.30±1.50^{b}	53.60±0.35^{a}
90	4.70±0.35^{e}	25.90±0.69^{d}	39.20±1.50^{c}	48.90±2.60^{b}	65.00±2.37^{a}
120	6.20±0.46^{e}	37.60±0.64^{d}	44.20±1.21^{c}	55.50±1.50^{b}	84.60±0.64^{a}

The values in the table are the Mean±SD from triplicate experiment

Table 4.3: Percentage of cells unsickled after treatment withmethanolic extract of *A. conyzoides*rootat different concentrations.

Time(min)	Normal saline	0.1mg/ml	0.2mg/ml	0.3mg/ml	PABA(5mg/ml)
30	3.10 ± 0.29^{dd}	20.80 ± 0.64^{c}	22.20 ± 0.98^{c}	27.40 ± 0.29^{b}	30.90 ± 0.23^{a}
60	3.90 ± 0.23^{d}	20.90 ± 0.98^{c}	32.20 ± 1.96^{b}	31.20 ± 2.54^{b}	53.60 ± 0.35^{a}
90	4.70 ± 0.35^{d}	27.10 ± 1.56^{c}	39.00 ± 2.83^{b}	41.20 ± 1.10^{b}	65.00 ± 2.37^{a}
120	6.20 ± 0.46^{d}	39.50 ± 2.66^{c}	43.30 ± 1.91^{c}	51.90 ± 0.99^{b}	84.60 ± 0.64^{a}

The values in the table are the Mean±SD from triplicate experiment

Table 4.4 shows the percentage of unsickleds cell after treatment with various concentrations of *Mangifera indica* stem for 120 min. It shows that there was significant increase in percentage of unsickled cells when compared to the normal saline control. The highest antisickling percentage was observed with a concentration of 0.3mg/ml at 120min indicating that the activity is time and concentration dependent.

The results presented in Table 4.5shows the percentage of unsickled cells after treatment with various concentrations of *Mangifera indica* leaf methanolic extract. A significant increase of percentage unsickled cells was observed after incubation at different time with a peak value of 56.60% at.

While Table 4.6 shows the percentage of unsickled cells after treatment with various concentrations of *Mangifera indica* root methanolic extract at different times of incubation. At 120min of incubation, 0.3mg/ml of the extract gave the highest percentage of unsickled cells (52.60%). Comparing the three parts of the plant (stem bark, leaf and root), the stem bark exhibited the highest reversal of sickled cells and the least was observed with the root extract.

Table 4.4: Percentage of cells unsickled after treatment withmethanolic extract of *M. indica*stem bark at different concentrations.

Time(min)	Normal saline	0.1mg/ml	0.2mg/ml	0.3mg/ml	PABA(5mg/ml)
30	3.10 ± 0.29^{dd}	26.10 ± 1.10^{c}	30.97 ± 1.21^{b}	37.23 ± 0.38^{a}	30.90 ± 0.23^{b}
60	3.90 ± 0.90^{e}	36.40 ± 0.64^{d}	40.10 ± 0.38^{c}	49.00 ± 1.79^{b}	53.60 ± 0.35^{a}
90	4.70 ± 0.29^{d}	46.70 ± 2.25^{c}	56.93 ± 0.92^{b}	59.60 ± 0.29^{b}	65.00 ± 2.36^{a}
120	6.20 ± 0.46^{e}	55.20 ± 2.77^{d}	63.20 ± 0.36^{c}	70.60 ± 1.27^{b}	84.60 ± 0.64^{a}

The values in the table are the Mean±SD from triplicate experiment

Table 4.5: Percentage of cells unsickled after treatment withmethanolic extract of *M. indica*leaf at different concentrations.

Time(min)	Normal saline	0.1mg/ml	0.2mg/ml	0.3mg/ml	PABA(5mg/ml)
30	3.10±0.29^{d}	30.90±0.18^{a}	31.50±1.35^{a}	31.57±0.83^{a}	30.90±0.23^{a}
60	3.90±0.90^{e}	29.30±0.56^{d}	32.90±0.23^{c}	38.80±0.52^{b}	53.60±0.35^{a}
90	4.70±0.29^{c}	40.73±0.46^{b}	42.00±0.38^{b}	44.00±0.12^{b}	65.00±2.36^{a}
120	6.20±0.46^{d}	48.40±0.69^{c}	50.50±0.92^{c}	56.60±0.69^{b}	84.60±0.64^{a}

The values in the table are the Mean±SD from triplicate experiment

Table 4.6: Percentage of cells unsickled after treatment withmethanolic extract of *M. indica*rootat different concentrations.

Time(min)	Normal saline	0.1mg/ml	0.2mg/ml	0.3mg/ml	PABA(5mg/ml)
30	3.10±0.29^{d}	18.30±0.35^{c}	22.00±0.81^{b}	22.20±1.50^{b}	30.90±0.23^{a}
60	3.90±0.90^{d}	24.90±0.98^{c}	27.20±1.21^{c}	36.40±1.10^{b}	53.60±0.35^{a}
90	4.70±0.29^{e}	35.80±0.23^{d}	41.60±0.75^{c}	51.40±0.44^{b}	65.00±2.36^{a}
120	6.20±0.46^{e}	32.43±1.36^{d}	48.50±0.52^{c}	52.60±0.61^{b}	84.60±0.64^{a}

The values in the table are the Mean±SD from triplicate experiment

Table 4.7 depicts the anti-sickling activitiesof partially purified fractions of *Ageratum conyzoides* stem bark methanol extract obtained from column chromatography. From the Table, it can be seen that, fraction F4 gave 40% of unsickled cells after 120 min of incubation. And the highest was seen in fraction F7 (70%) The positive control (PABA) gave 87.3% reversal of sickle cells while only 12.4% of unsickled cell on Normal saline (negative control) treated sickle cells.

Table 4.12 shows the antisickling activity of partially purified *Mangifera indica* stem bark extracts at 0.3mg/ml. The highest anti-sickling activity was observed in fraction M4which gave 72%, while the fraction with the least antisickling activity was fraction M1with 27% reversal rate.

Table 4.7: Anti-sickling Activity of Fractions Obtained from *A.conyzoides*Stem bark

Fraction	Anti-sickling Effect(%)
F1	40±0.47
F2	38±0.82
F3	33±2.50
F4	40±0.47
F5	48±1.25
F6	61±1.25
F7	70±1.63
F8	42±0.94
PABA	87.3±1.21
Normal saline	6.9±0.23

Key (*Ageratun conyzoides*)

Fraction 1(F1) → Ethyl acetate 100%

Fraction 2 (F2) → Ethylacetate:Methanol 90:10a

Fraction 3 (F3) →Ethylacetate:Methanol 90:10b

Fraction 4 (F4) → Ethylacetate:Methanol 80:20a

Fraction 5 (F5) → Ethylacetate:Methanol 80:20b

Fraction 6 (F6) → Ethylacetate:Methanol 70:30a

Fraction 7 (F7) → Ethylacetate:Methanol 70:30b

Fraction 8 (F8) → Ethylacetate:Methanol 70:30c

Table 4.8: Anti-sickling Activity of Fractions Obtained from *M.indica*Stem bark

Fraction	Peak Antisickling Value(%)
M1	27±0.47
M2	37±1.25
M3	46±1.63
M4	72±1.25
M5	57±0.82
M6	53±1.41
M7	39±1.41
PABA	87.3±1.21
Normal saline	6.9±0.23

Key (*Mangifera indica)*

Fraction 1 (M1) → Ethyl acetate 100%

Fraction2 (M2) → Ethylacetate:Methanol 90:10a

Fraction 3 (M3) → Ethylacetate:Methanol 90:10b

Fraction 4 (M4) → Ethylacetate:Methanol 80:20a

Fraction 5 (M5) → Ethylacetate:Methanol 80:20b

Fraction 6 (M6) → Ethylacetate:Methanol 70:30a

Fraction 7 (M7) → Ethylacetate:Methanol 70:30b

4.2 Erythrocyte Membrane Protection

Table 4.9 Shows the effect of stem methanolic extract at various concentrations on membrane protection of human sickle erythrocyte. From the result, the stem methanolic extract of *A.*

conyzoides exhibited a membrane protective effect in a dose-dependent manner. The highest protection was observed at a concentration of 2.5mg/ml which was 29.13±0.47% protection the while lowest protection was observed at 0.5mg/ml which was 36.27±1.51%. while the SS control (negativecontrol)was 81.30±0.3%.

Table 4.10shows the effect of leaf methanolic extract of *A. conyzoides* which demonstrated a significant ($p < 0.05$) membrane protection ability at various concentration of the extract. The highest protection was observed at 2.5mg/ml with 24.13±0.47% and 34.90±1.10% as the lowest activity observed at 0.5mg/ml.

Table 4.11 presents the effect of root methanolic extract of *A.conyzoides* on human sickle cell. The result showes that, the extract exhibited the highest membrane protection activity of 40.73±1.04% at 2.5mg/ml concentration. While it shows a minimum protection of 50.80±1.27% at 0.5mg/ml

Table 4.12 shows the membrane protective effect of *M.indica* stem bark extract on human sickle cell. It shows that the extract has the highest membrane protective effect. The highest protection was observed at 2.0mg/ml of the extract which gave 36.63±1.13% and the lowest was seen at 0.5mg/ml of the extract.

On the other hand, the result presented in Table 4.13demonstrates the effect of leaves methanolic extract of *M. indica* on human sickle cell. The result showed that, the extract exhibited membrane protection effect by reducing percentage lysis to 37.37±0.47d at 2.5mg/ml concentration. While it shows a minimum reduction of percentage lysis of 52.17±1.31% at 0.5mg/ml.

Table 4.14 shows that the root methanolic extract of *M.indica*reduces percentage of lysis from 81.30±0.36% of SS control to55.73±2.56% and 34.87±1.39% at 0.5mg/ml and 2.0mg/ml respectively.

Table 4.9: Effect of methanolic extract of *A. conyzoides* stem bark on red blood cell membrane fragility

	Mean % RBC lysis					
NaCl %	AA Blood	SS Blood	SS blood+0.5mg/ml	SS blood+1.0mg/ml	SS blood+2.0mg/ml	SS blood +2.5mg/ml
0.1	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]
0.2	76.20±0.92[b]	98.63±0.39[b]	91.13±0.24[b]	77.60±0.42[b]	76.30±0.76[b]	73.80±0.49[b]
0.3	69.17±1.54[c]	95.90±0.20[c]	61.00±1.11[c]	61.70±2.01[c]	60.13±2.49[c]	53.37±1.17[c]
0.5	30.80±0.40[d]	81.30±0.36[d]	36.27±1.51[d]	30.93±1.26[d]	30.93±1.26[d]	29.13±0.47[d]
0.6	4.87±0.57[e]	40.57±0.28[e]	16.20±1.01[e]	12.23±0.84[e]	12.23±0.84[e]	12.60±0.35[e]
0.9	0.40±0.12[f]	12.97±0.34[f]	5.00±0.31[f]	3.27±0.64[f]	3.27±1.11[f]	2.93±0.03[f]

Values are mean ± SD of the mean of triplicate experiments.

Table 4.10: Effect of methanolic extract of *A. conyzoides* leaf on red blood cell membrane fragility

	Mean % RBC lysis					
NaCl %	AA Control	SS Control	SS blood+0.5mg/ml	SS blood+1.0mg/ml	SS blood+2.0mg/ml	SS blood +2.5mg/ml
0.1	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]
0.2	76.20±0.92[b]	76.20±0.92[b]	88.27±0.75[b]	79.33±0.29[b]	75.30±0.70[b]	75.23±1.94[b]
0.3	69.17±1.54[c]	69.17±1.54[c]	61.57±1.04[c]	60.30±0.79[c]	58.27±2.26[c]	61.80±1.22[c]
0.5	30.80±0.40[d]	30.80±0.40[d]	34.90±1.10[d]	32.27±1.05[d]	31.53±1.90[d]	24.13±0.47[d]
0.6	4.87±0.57[e]	4.87±0.57[e]	16.33±1.73[e]	13.53±0.87[e]	17.00±1.73[e]	11.70±0.57[e]
0.9	0.40±0.12[f]	0.40±0.12[f]	4.27±1.18[f]	2.90±0.52[f]	7.53±0.90[f]	7.40±0.74[f]

Values are mean ± SD of the mean of triplicate experiments

Table 4.11: Effect of methanolic extract of *A. conyzoides* root on red blood cell membrane fragility

Mean % RBC lysis

NaCl %	AA Control	SS Control	SS blood+0.5mg/ml	SS blood+1.0mg/ml	SS blood+2.0mg/ml	SS blood +2.5mg/ml
0.1	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]
0.2	76.20±0.92[b]	98.63±0.39[b]	95.73±0.52[b]	89.57±0.68[b]	79.40±5.46[b]	83.17±0.23[b]
0.3	69.17±1.54[c]	95.90±0.20[c]	84.30±0.71[c]	72.33±1.49[c]	66.10±0.64c	54.03±5.30[c]
0.5	30.80±0.40[d]	81.30±0.36[d]	50.80±1.27[d]	45.23±1.91[d]	44.13±2.62[d]	40.73±1.04[d]
0.6	4.87±0.57[e]	40.57±0.28[e]	9.27±1.36[e]	8.27±1.77[e]	10.83±1.30[e]	8.60±1.78[e]
0.9	0.40±0.12[f]	12.97±0.34[f]	5.43±1.45[f]	4.13±1.33[e]	5.67±1.98[e]	4.30±1.36[e]

Values are mean ± SD of the mean of triplicate experiments

Table 4.12: Effect of methanolic extract of *M. indica* stem bark on red blood cell membrane fragility

	Mean % RBC lysis					
NaCl %	AA Control	SS Control	SS blood+0.5mg/ml	SS blood+1.0mg/ml	SS blood+2.0mg/ml	SS blood +2.5mg/ml
0.1	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]
0.2	76.20±0.92[b]	98.63±0.39[b]	90.10±0.66[b]	87.83±0.37[b]	83.70±0.51[b]	81.70±0.76[b]
0.3	69.17±1.54[c]	95.90±0.20[c]	78.40±0.82[c]	66.57±1.13[c]	62.97±1.30[c]	70.73±0.52[c]
0.5	30.80±0.40[d]	81.30±0.36[d]	50.33±1.77[d]	40.70±1.49[d]	36.63±1.13[d]	37.73±0.97[d]
0.6	4.87±0.57[e]	40.57±0.28[e]	6.27±1.03[e]	6.73±0.90[e]	2.80±0.52[e]	3.33±0.75[e]
0.9	0.40±0.12[f]	12.97±0.34[f]	3.10±0.85[f]	5.30±0.68[e]	2.20±0.30[e]	2.73±0.46[e]

Values are mean ± SD of the mean of triplicate experiments

Table 4.13: Effect of methanolic extract *M. indica* leaf on red blood cell membrane fragility

	Mean % RBC lysis					
NaCl %	AA Control	SS Control	SS blood+0.5mg/ml	SS blood+1.0mg/ml	SS blood+2.0mg/ml	SS blood +2.5mg/ml
0.1	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]
0.2	76.20±0.92[b]	98.63±0.39[b]	91.73±0.97[b]	91.50±0.82[b]	81.97±3.59[b]	83.13±0.27[b]
0.3	69.17±1.54[c]	95.90±0.20[c]	73.83±3.51[c]	68.47±0.70[c]	64.43±0.12[c]	68.13±1.74[c]
0.5	30.80±0.40$_d$	81.30±0.36[d]	52.17±1.31[d]	42.70±1.08[d]	38.83±1.27[d]	37.37±0.47[d]
0.6	4.87±0.57[e]	40.57±0.28[e]	10.43±1.84[e]	10.37±1.75[e]	6.33±1.33[e]	4.43±0.43[e]
0.9	0.40±0.12[f]	12.97±0.34[f]	6.53±1.39[e]	6.80±0.92[f]	5.43±1.33[e]	3.27±0.23[e]

Values are mean ± SD of the mean of triplicate experiments

Table 4.14: Effect of methanolic extract *M. indica* root on red blood cell membrane fragility

	Mean % RBC lysis					
NaCl %	AA Control	SS Control	SS blood+0.5mg/ml	SS blood+1.0mg/ml	SS blood+2.0mg/ml	SS blood +2.5mg/ml
0.1	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]	100.00±0.00[a]
0.2	76.20±0.92[b]	98.63±0.39[b]	93.13±0.52[b]	84.40±0.10[b]	82.23±0.56[b]	76.70±0.29[b]
0.3	69.17±1.54[c]	95.90±0.20[c]	79.80±1.37[c]	67.73±1.52[c]	68.13±2.12[c]	65.13±2.57[c]
0.5	30.80±0.40[d]	81.30±0.36[d]	55.73±2.56[d]	41.70±2.31[d]	34.87±1.39[d]	34.90±1.88[d]
0.6	4.87±0.57[e]	40.57±0.28[e]	12.00±0.36[e]	7.87±2.21[e]	8.20±1.80[e]	9.33±2.16[e]
0.9	0.40±0.12[f]	12.97±0.34[f]	6.00±0.35[f]	4.47±1.66[e]	5.67±2.06[e]	5.47±1.87[e]

Values are mean ± SD of the mean of triplicate experiments

4.3 Methemoglobin Concentration

The percentage of plasma methemoglobin following the addition of various concentrations of the methanolic extract of *Ageratum conyzoides* parts was presented in Table 4.15.The stem, leaf and root methanolic extracts of *A. conyzoides* produced significant ($p<0.05$) decrease in the percentage of methemoglobin concentration in a dose-dependent pattern. The stem methanolic extract of *A. conyzoides* also produced a significant ($p<0.05$) decrease in the percentage of methemoglobin concentration with increase in concentration. The percentage of plasma methemoglobin concentration following the addition of various concentrations of the methanolic extract of *Mangifera indica* parts was presented in Table 4.16.

Table 4.15: Effect of stem, leaf and root extracts of *A. conyzoides* on methemoglobin concentration

Concentration (mg/ml)	Stem *A.conyzoides*	leaf *A.conyzoides*	root *A.conyzoides*
Control	12.82±0.06[a]	12.82±0.06[a]	12.82±0.06[a]
0.2	10.05±0.16[b]	10.77±0.64[b]	10.65±0.53[b]
0.4	8.21±0.64[c]	9.07±0.38[c]	8.54±0.32[c]
0.6	6.55±0.74[d]	7.25±0.19[d]	6.44±0.21[d]
0.8	5.20±0.14[e]	6.76±0.23[d]	5.09±0.17[e]
1.0	3.25±0.14[f]	4.77±0.44[e]	4.70±0.09[e]

Values are Mean±SD triplicate analysis.Values withdifferentsuperscripts in the same row are significantly different ($p<0.05$).

Table 4.16 Effect of stem, leaf and root extracts of *M. indica* on methemoglobin concentration

Concentration (mg/ml)	Stem *M. indica*	leaf *M.indica*	root *M.indica*
Control	12.82±0.06[a]	12.82±0.06[a]	12.82±0.06[a]
0.2	9.16±0.13[b]	12.66±0.05[a]	11.28±0.08[b]
0.4	7.67±0.17[c]	10.30±0.21[b]	8.53±0.12[c]
0.6	6.64±0.13[d]	7.76±0.16[c]	6.83±0.12[d]
0.8	4.57±0.11[e]	6.00±0.04[d]	5.40±0.18[e]
1.0	3.56±0.32[f]	3.96±0.37[e]	4.66±0.24[f]

Values are Mean±SD triplicate analysis.Values withdifferentsuperscripts in the same row are significantly different ($p<0.05$).

4.4 Phytochemical Composition of crude extracts

Table 4.17 shows the results of qualitative screening of crude methanolic extracts of *Ageratum conyzoides*.The result in Table 4.17 indicates that methanolic extract of the leaf of *A.conyzoides* was found to contain carbohydrates, triterpenes, cardiac glycosides, saponins, flavonoids and alkaloids. But anthraquinones and tannins were absent. The stem bark methanolic extract was found to contain all the phytochemicals present in the leaf extract, while the methanolic extract of the root revealed the presence of carbohydrates, triterpenes,cardiac glycosides and flavonoids. Table 4.18 shows phytochemical composition of the leaf, stem and root methanolic extracts of *Mangifera indica.* The leaf extract revealed the presence ofcarbohydrate, steroides and triterpens, cardiac glycosides, saponins, tannins, flavonoids and alkaloids. The stem bark extract showed the presence of all the phytochemicals present in the leaf extract with addition of anthraquinone. The root extract tested positive for all the phytochemicals present in the leaf extract.

Table 4.17:Phytochemical Composition of the Different Parts of*A. conyzoides*.

Phytochemical	leaves	Stem bark	Root
Carbohydrates Molisch Test	+	+	+
Anthraquinone Bontrager's Test	-	-	-
Steroid and triterpens Liberman Bucchard Test	+	+	+
Cardiac Glycosides Keller Killiani Test	+	+	+
Saponin glycoside Froth Test	+	+	-
Tannins Ferric chloride Test	-	-	-
Flavonoids NaOH Test	+	+	+
Alkaloids Dragendroff's test	+	+	-

KEY:

- = Absent
+ = Present

Table 4.18: Phytochemical Composition of the Different Parts of *M. indica*.

Phytochemical	leaves	Stem	Root
Carbohydrates Molisch Test	+	+	+
Anthraquinone Bontrager's Test	-	+	-
Steroid and triterpens Liberman Bucchard Test	+	+	+
Cardiac Glycosides Keller Killiani Test	+	+	+
Saponin glycoside Froth Test	+	+	-
Tannins Ferric chloride Test	+	+	+
Flavonoids NaOH Test	+	+	+
Alkaloids Dragendroff's test	+	+	+

KEY:

- = Absent
+ = Present

4.5. Phytochemical Composition of Column Chromatographic Fractions.

The fractions were subjected to six phytochemical tests i.e test for phenolic compounds, anthraquinone, steroids, triterpenes, flavonoids and alkaloids. The results of screening of *A. Conyzoides* fractionswere presented in Table 4.19.F1 showed the presence phenolic compounds, F2 showed the presence of phenolic compounds and flavonoids, while F3 showed the absence of all the tested phytochemicals. F4 tested positive for phenolic compounds, flavonoids and alkaloids, F5 tested positive for all except anthraquinone, while F6 showed the presence of all except flavonoids. F7 showed positive to all the tests and F8 tested negative to flavonoids and alkaloids only.

On the other hand, the results of phytochemical screening of *M. indica*fractions were presented in Table 4.20. All fractions showed positive for phenolic compounds except M1, while all showed positive for anthraquinone except M1 and M3 fraction. Only fractions M4, M5 and M6 showed positive for steroid and triterpenes but for flavonoids all tested positive with the exception of M1 fraction. From M3–M7 all tested positive for alkaloids while M1 and M2 showed negative.

Table 4.19: Phytochemical Screening of *A. Conyzoides*fractions.

Phytochemical	F1	F2	F3	F4	F5	F6	F7	F8
Phenolic compounds	+	+	_	+	+	+	+	+
Anthraquinone	_	_	_	_	_	+	+	+
Steroid and triterpens	_	_	_	_	+	+	+	+
Flavonoids	_	+	_	+	+	_	+	_
Alkaloids	_	_	_	+	+	+	+	_

KEY; + = present, - =Absent

Table 4.20: Phytochemical Screening of *M. Indica*Fractions

Phytochemical	M1	M2	M3	M4	M5	M6	M7
Phenolic compounds	_	+	+	+	+	+	+
Anthraquinone	_	+	_	+	+	+	+
Steroid and triterpens	_	_	_	+	+	+	_
Flavonoids	_	+	+	+	+	+	+
Alkaloids	_	_	+	+	+	+	+

KEY; + = present, - =Absent

4.5 Elemental composition of extracts and fractions

The results obtained from the elemental analysis of the plants parts are presented in Table 4.21. All the elements were detected in the different plant parts with the exception of Zinc in *Ageratum* stem bark, Zinc and Calcium in *Mangifera* root which were below detection limit. *Ageratum* stem bark has the highest Magnesium content of 176.48mg/Kg and the least in *Mangifera* stem bark with 3.10mg/Kg. The highest zinc content was found in *Ageratum* leaves with 2.48mg/Kg and the least was obtained in *Ageratum* stem and *Mangifera* root. Calcium content is highest in *Ageratum* stem bark 143.66mg/Kg and the lowest was *Mangifera* root below detection limit. Iron was found to be highest in *Mangifera* leaves 7.40mg/Kg and lowest in *Mangifera* stem 2.92mg/Kg. Elements of interest detected were, Zn, Ca, Fe, and Mg.

The anti-sickling activity of the plants studied may be attributed to the presence of Mg, Ca, Fe and Zn in these plants. The elemental contents were determined to verify the biological role of the selected trace elements in the selected anti-sickling medicinal plants. As shown in table 4.22, Mg was appreciably present in all the plants except in the root of *Mangifera indica.*. The results indicate that *Ageratum conyzoides* stem bark contain high Mg (176.48 mg/kg) and low in *Mangifera indica*, root bark (3.10mg/kg). Zn was present in all the studied plants in significant quantities (Table 4.21).

Table 4.22 shows the results obtained from the elemental analysis of the partially purified fractions of *Ageratum conyzoides* and *Mangifera indica.* Most of the fractions had higher concentration than the crude extracts.Fraction M6 of *Mangifera indica* has the highest concentration of Mg (81.58mg/ml) while Zn is highest M7 fraction of *M. indica* (19.88mg/ml). Ca and Fe has the highest concentration in F5of *Ageratum conyzoides*.

Table 4.21: Compositionof elements in methanolic extracts of different parts of *A.conyzoides* and *M.indica*(mg/kg)

Plant name	Plant part	Mg	Zn	Ca	Fe
Ageratum conyzoides	Stem bark	176.48	-	143.66	4.00
	Leaves	19.80	2.48	27.60	4.30
	Root	17.52	0.66	21.52	3.00
Mangifera indica	Stem bark	15.28	1.78	3.78	2.92
	Leaves	27.16	1.32	10.98	7.40
	Root	3.10	-	-	2.96

Key:
- = below detection limit

Table 4.22: Composition of elements in most active fractions of *A.conyzoides*and *M.indica*stem bark(mg/kg)

Plant name	Plant part	Mg	Zn	Ca	Fe
A. conyzoides	F7	16.21	4.66	35.54	86.66
M. indica	M4	29.48	11.50	71.20	25.10

CHAPTER FIVE

5.0 DISCUSSION

Research into phytotherapy of diseases is the current trend in the management of diseases such as typhoid fever, cancer, hypertension and also genetic disorders like SCA, with a view to finding cheaper, alternative medicines that the lower class individuals which are majority of the wide populace can have immediate access to (Imaga *et al.,* 2009).For the pharmacological discovery of novel drugs, the primary essential information regarding the chemical constituents are generally provided by the qualitative phytochemical screening of plant extracts (Nanfack *et al.,* 2013).

The anti-sickling results revealed that the extracts significantly ($P<0.05$) reverted sickled cells *in vitro* when compared with the control group for *A.conyzoides* and *M. indica* respectively. The highest percentage of reversion was 67.8 % obtained at the 120th Minute of incubation for*A.conyzoides* stem and 70.60% for *M.indica* stem. This compared favourably with PABA, a standard anti-sickling agent. These findings are in line with previous studies of Mpiana *et al.(*2014). The contributions of phytochemicals to the anti-sickling activity have been reported byseveral researchers (Adejumo *et al.*,2012). Most of these studies are *in vitro* and their modes of actions are not properly understood (Dash *et al,* 2013). The ability of any substance to elicit anti-sickling potential implies that such material wouldinterfere in different stages of sickling process. Some medicinal plants have been shown to have different activities in SCD condition, ranging from anti-sickling, anti-aggregating, anti-polymerization, radical scavenging or antioxidant, anti-inflammatory, analgesic, anti-pyretic, anti-dehydrating to anti-osmotic effect, etc. All these effects add up to give more stable and tolerable patient condition (Chikezie *et al*., (2011); Afolabi *et al.*,(2012); Ameh *et al*., (2012); Sulaiman and Gopalakrishnan,(2013).

A good number of studies have been carried out to identify and characterize some anti-sickling compounds from different plant sources. The most promising were found to be anthocyanins, anthraquinones, steriodal gylcosides, cardiac glycosides, alkaloids, flavonoids, saponins, tannins, phenols, hydroxybenzoic acids, liminoids, 5-hydroxymethyl-2-furfurals (5HMF), isomeric divanilloylquinic acid and certain amino acids such as arginine, tyrosine, aspartic acid and phenylalanine (Ameh *et al*., 2012; Dash *et al*., 2013). Even though, *in vitro* anti-sickling activity was observed with these compounds, their mode of action and the mechanism through which these actions are exerted is yet to be properly elucidated. The extract was found to reverse sickling in a dose dependent manner.

In the present study, phytochemical qualitative screening of different plant showed the presence of several groups of bioactive molecules such as alkaloids, glycosides, saponins, flavonoids, tannins, carbohydrates, anthraquinones, steroids and triterpenes. This result is similar to that reported by (Egunyomi *et al.,* 2009). The presence of alkaloids in the stem, leaf and root of both plants is an indication that they may be useful in alleviating pains associated with SCA (Ejele and Aneke, 2011). Anthraquinones act on the gastro-intestinal tract to increase peristalsis (Evans, 1989). The presence of anthraquinones in both plants is an indication that it may be useful as a mild laxative especially in cases where SC patients complain of constipation. Tannins are phenolic derivatives and are non-nitrogenous plant constituents with astringent properties on mucous membranes (Egunyomil *et al*., 2009). Thetannins that are present in the stem,leaf and root of *M.indica* may be useful in bathing or cleansing the surface of the skin ulcers that develop as a result of SCD. The presence of cardiac glycosides indicates that they may be potent in curing cardiac insufficiency, coughs and circulatory problems. Also, they may act as good sedatives and have antispasmodic properties (Egunyomil*et al*., 2009).

Osmotic fragility of erythrocytes demonstrates the integrity of red cell membranes in pathological and normal states (Salami, *et al.,* 2014). The findings obtained fromosmotic fragility tests of sickle cell blood showed appreciable membrane protective effects of the plant extracts and their inhibitory action on the hemolysis of red blood cells. The percentage of hemolysis of the control and test samples showed a sigmoidal relationship with increasing concentrations of saline solution. There was significant decrease ($p<0.005$) of percentage lysis from 95.70% of the negative control at 0.3% of NaCl solution to 52.60% at 2.5mg/ml *A.conyzoides* stem methanolic extract and 62.90% at 2.0mg/ml of *M.indica* stem methanolic extractThis result is similar to that reported by (Imaga *et al.,* 2009 and Arokoyo *et al.,* 2015).Tamer *et al*., 2000 reported that higher levels of free radicals in human sickle erythrocytes was associated with increased tendency of diminished mechanical and osmotic stability. Therefore, accumulation of oxidant contributes to accelerated damage of sickle cell erythrocyte membrane and senescence of these cells (Rice-Evans *et al*.,1986). The erythrocyte membrane protective ability of these extract may be due to the presence of flavonoids and flavonoids have been reported to have antioxidant property (Ahur *et al.*,2014). The effect of varied concentrations of *A.conyzoides* and *M.indica*plant extracts on the red blood cell membrane,analyzed using the Osmotic fragility Test, revealedappreciable membrane protective effects of the extracts andtheirinhibitory action on hemolysis of red blood cells. All extracts of the plants' stem,leaf and root were found to have anappreciable antihemolitic activity for *A. conyzoides* and *M.indica* respectively, suggesting that theextract would be effective in prolonging the delay time ofpolymerization of sickle hemoglobin *in vivo* and thusaffect the time course for sickling.

Previous reports have associated the HbSS erythrocytes with raised level of oxidized Hb (Chikezie *et al.,* 2009). They noted that the primary reason for the relatively raised concentration

of oxidized Hb (MethHb) in HbSS erythrocytes was the higher production of super oxide ion by these erythrocytes compared to those of HbAS and HbAA (Chikezie, 2011). This study showed a significant reduction in methemoglobin concentration after treatment with *A.conyzoides* and *M. indica* extracts, thus indicating the possible antioxidant effect of the extracts. This finding is in line with the work of Mpiana *et.,al* 2014. This may be due to the presence of bioactive agents such flavonoids and saponins present in the stem, leaf and roots of the plants. These phytochemicals have been reported to scavenge peroxyl radicals, alkyl peroxyl radicals, superoxide hydroxyl radicals, and peroxynitrite in aqueous and organic environments, which mitigate against lipid peroxidation, change in cellular osmotic pressure and subsequent inflammation and cell death (Mira *et al*., 2002).This indicates that the methanolic extracts of *A.conyzoides* and *M.idica* prevent the oxidation of Fe^{2+} into Fe^{3+} or the transformation of hemoglobin into methemoglobin. It therefore, decreases Fe^{3+}/ Fe^{2+} ratio in sickle cell blood., This would be a gain for patients with SCD since the increase of methemoglobin (Fe^{3+}) decreases the capacity of hemoglobin to bind to oxygen so increases the deoxyhemoglobin form and therefore, the polymerization conducive to the sickling process (Mpiana, 2010).

Some minerals and vitamins have been found to bebeneficial in the control of anaemia in sickle cell disease. These include iron (Fe), copper (Cu), zinc (Zn) and folate (Prasad, 1999).Zinc was present in all the studied plant parts in small quantites with the exception of *A.conyzoides* stem and *M.indica* root which was below detection limit,while Magnesium was found in significant quantities in all parts.(Table 4.21). The nutritional approach to the management of SCD has been the most modern and most effective protocol adopted in the management of the disease (Imaga, 2013. Many studies have provided humanity with reliable data on the deficiencies of various nutrients some of which are exacerbated by the sickling episode.Zinc has been found to inhibit

polymerization by 89.69 % and Magnesium 48.40%(Nwaoguikpe and Braide, 2012). Zinc deficiency has been associated with a myriad of disease states (Hambidge and Krebs, 2007). The main mechanism of the action of zinc in blood and in general metabolism has been to antagonize calcium retention and moreover, stabilizing the erythrocyte, thereby preventing hemolysis. (Solomon, 1998). Zinc has equally been found to bea strong antioxidant, since it is a component of many antioxidant enzymes like catalase, Glutathionine -S-transferase and Alkaline phosphatase and over 200 other enzymes that are zinc dependent (Sandstead,1994; Maret and Sandstead, 2006).This action of Zinc and magnesium in improving the Fe^{2+}/Fe^{3+} ratio is likened to reversing the functional state of sickle erythrocytes to their original biconcave structure, thereby increasing the oxygen affinity of the sickled cells. (Nwaoguikpe and Braide, 2012).Low levels of total magnesium in sickle cell erythrocytes have been associated with increased sickling due to propensity for red cell dehydration and hence, increased HbS polymerization (Franceschi, Bachir and Galacteros. 2000). Iron deficiency in patients with sickle cell may result in lowering the intracellular hemoglobin concentration and this may ameliorate sickling (Mohanty*et al*., 2008).

Iron deficiency is also very common in subjects with SCD (Davies*et al.,* 1983) especially among children living in developing countries where iron deficiency anemia is widespread (Jeyakumar *et al.,* 1987).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

The result obtained for Anti-sickling activity of extracts/fractions showed statistically significant ($p>0.05$) increase in the percentage of unsickle cells in the order of increasing percentage from 51.90±0.99, 55.50±0.99 and 67.77±1.24% for root, leaves and stem bark of the *Ageratum conyzoides* respectively with the stem bark showing the highest percentage of unsickled cells. While for *Mangifera indica*, it shows 52.60± 0.61%, 56.60±0.69% and 70.60±1.27% for root, leaves and stem respectively.

Partialy purified stem bark extract of *Ageratum conyzoides* gave eight fractions, and fraction F7 producing the highest percentage of unsickled cells of 70.1%%. While that of *Mangifera indica* gave seven fractions with fraction M4 producing the highest percentage of unsickle cells with the value of 72%.

Both plants extracts demonstrated antioxidant capacity by lowering the percentage of plasma methemoglobin with the stem of both*Ageratum conyzoides* and *Mangifera indica*demonstrating highactivity by decreasing the percentage to 3.25± 0.14% and 3.56± 0.32% respectively.

The stem bark extract of *Ageratum conyzoides*gave the highest protection power against hypotonic induced lysis followed by the leaves and root extracts. *Mangifera indica* also gave a significant protection to the cells against hypotonic induced lysis but there was no significant difference between root, leaf and stem extracts.

6.2 Conclusion

The results obtained revealed that the extracts possess significant sickling reversion potentials.

6.3 Recommendation

Further work on the screening of the secondary metabolites of these plants for biological and pharmacological studies will be necessary as well as the isolation of active compounds and their structural elucidation using spectroscopic techniques and subsequent *invivo* trials for maximal exploitation of the medicinal properties of these medicinal plants.

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APPENDIX

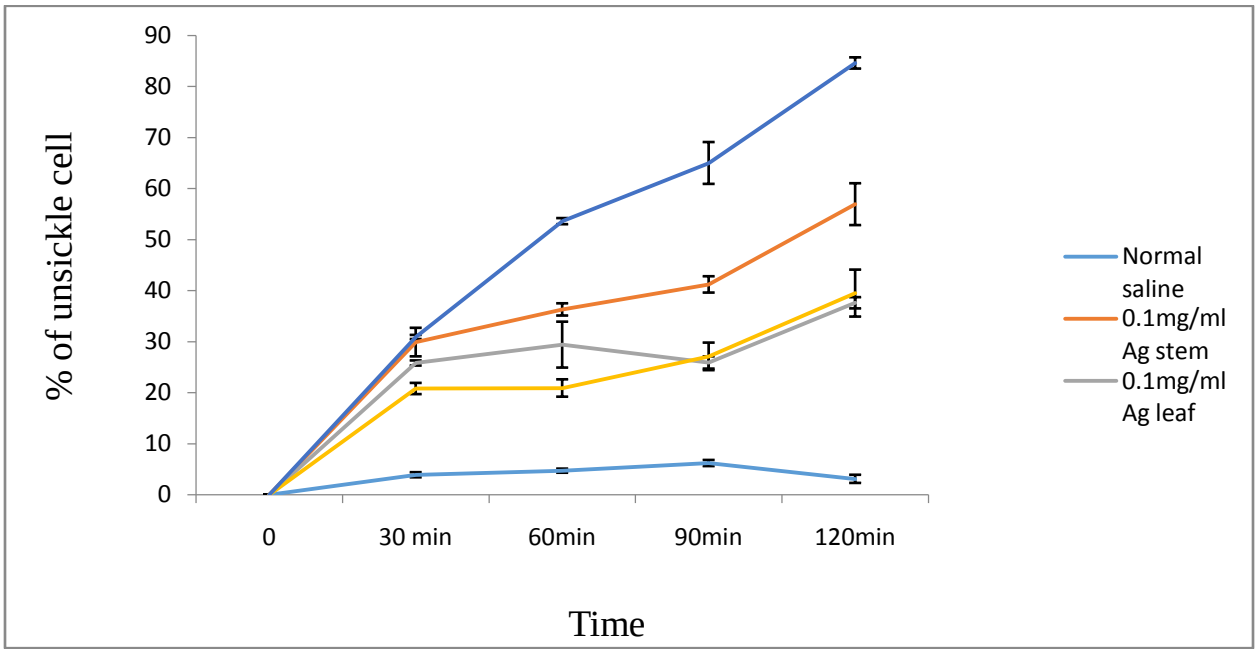


Figure 1: Effect of 0.1mg/ml methanolic extract of *A. conyzoides (Ag)* on human sickle cell *in vitro* after different period of incubation

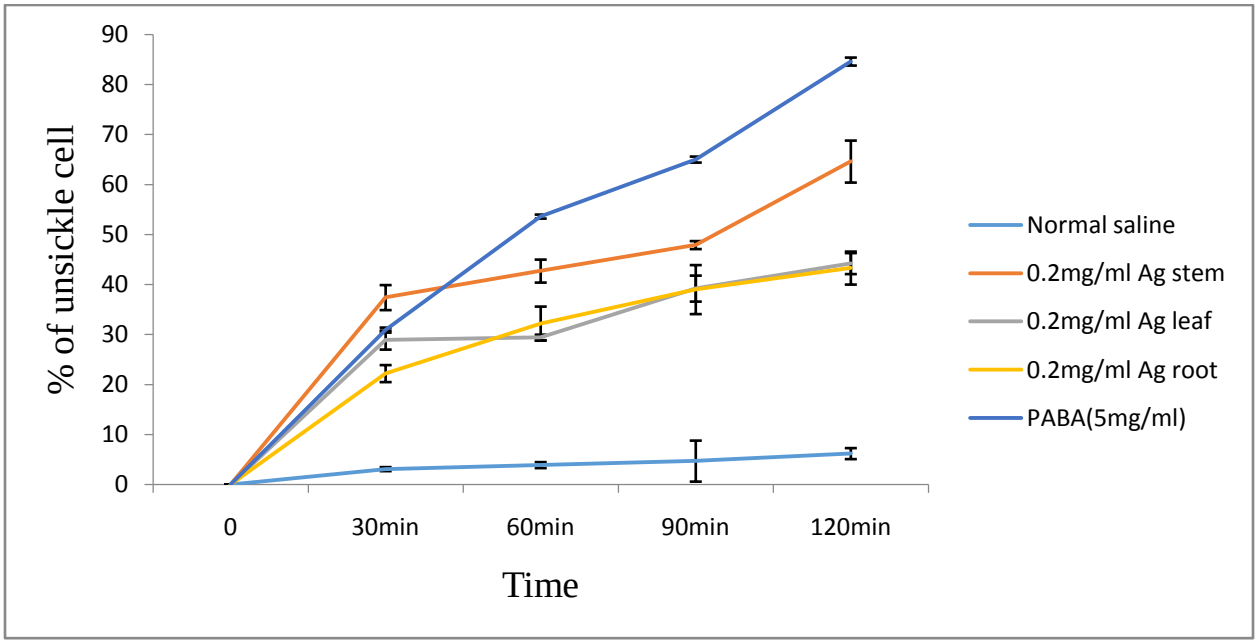


Figure 2: Effect of 0.2mg/ml methanolic extract of *A. conyzoides (Ag)* on human sickle cell *in vitro* after different period of incubation

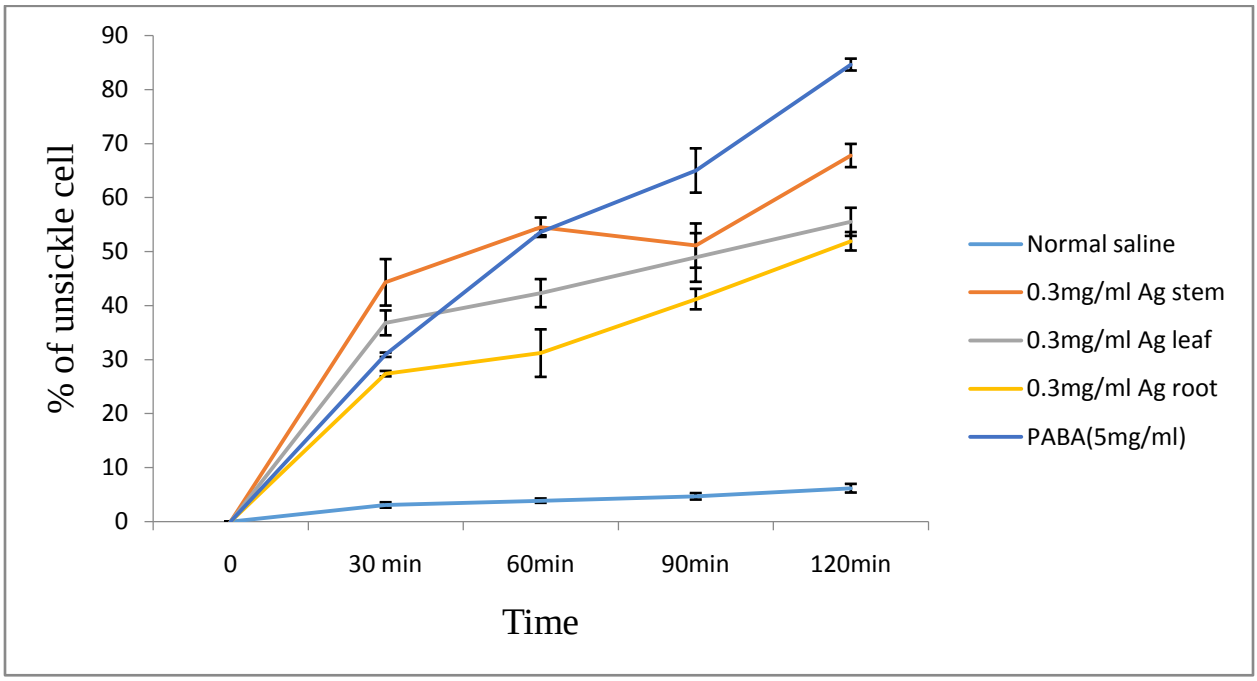


Figure 3: Effect of 0.3mg/ml methanolic extract of *A. conyzoides (Ag)* on human sickle cell *in vitro* after different period of incubation.

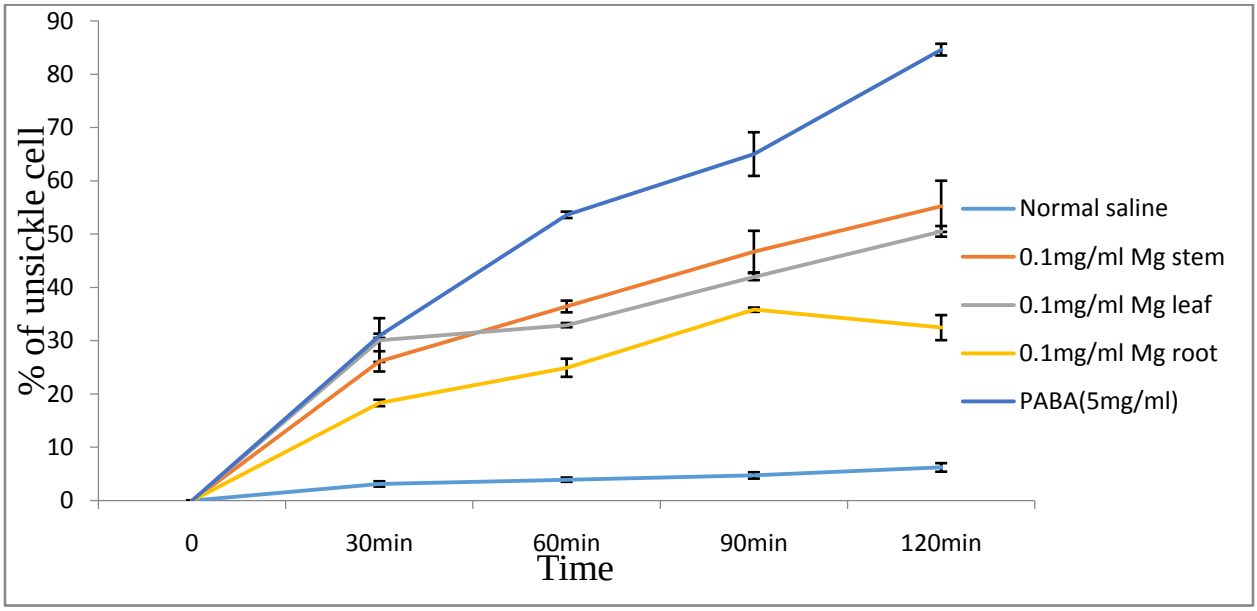


Figure 4: Effect of 0.1mg/ml methanolic extract of *M. indica (Mg)* on human sickle cell *in vitro* after different period of incubation.

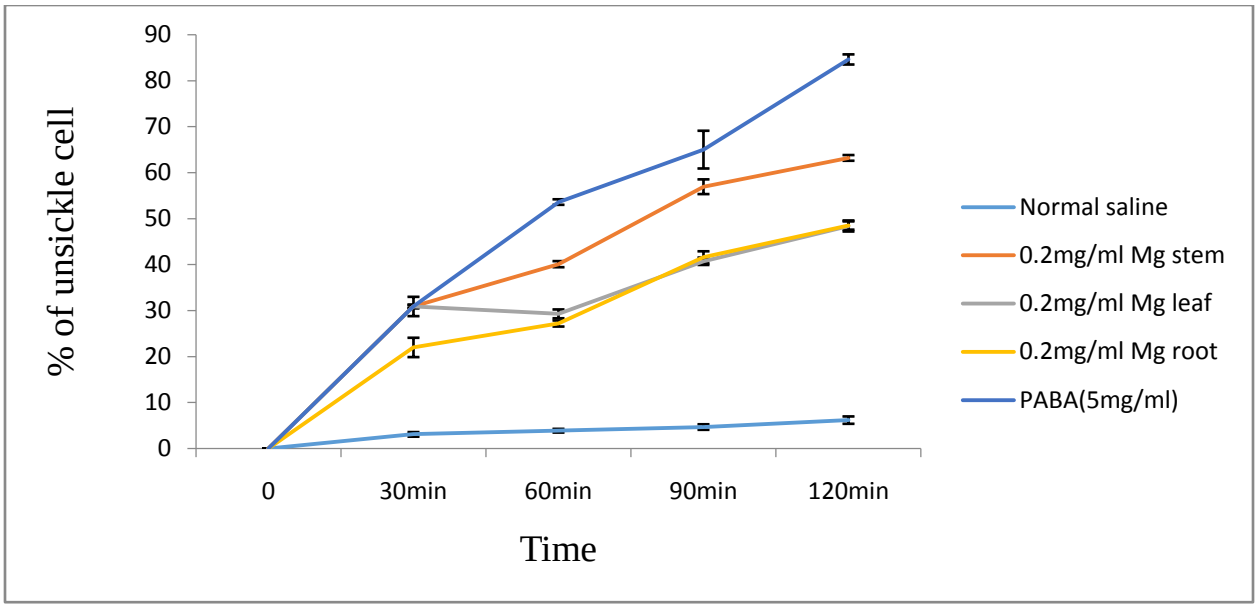


Figure 5: Effect of 0.2mg/ml methanolic extract of *M.indica (Mg)* on human sickle cell *in vitro* after different period of incubation.

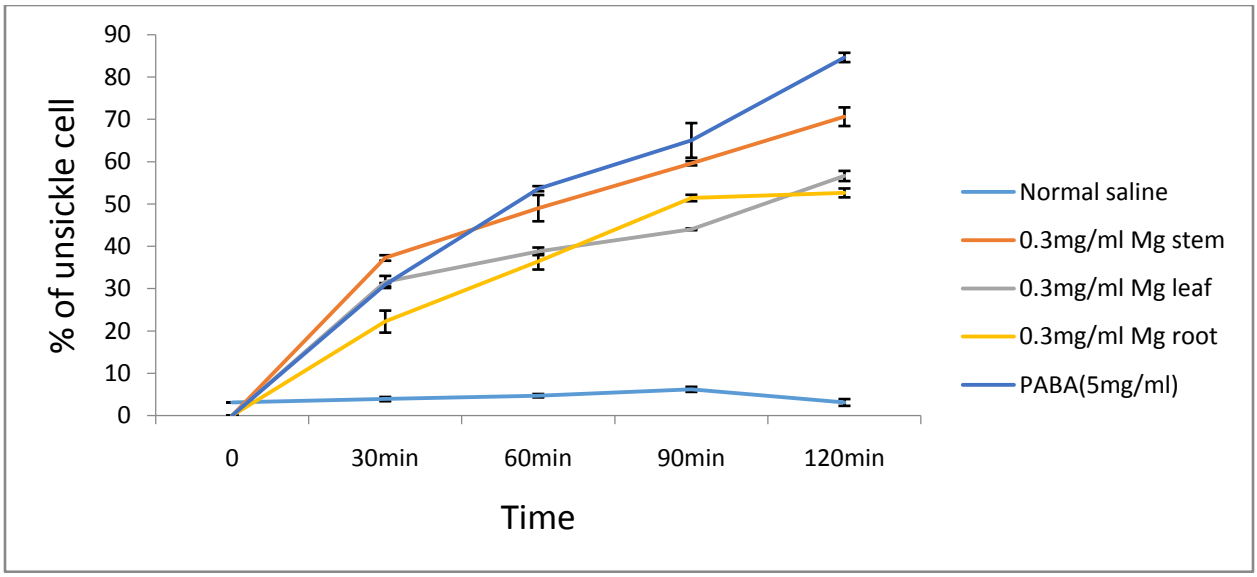


Figure 6: Effect of 0.3mg/ml methanolic extract of *M. indica (Mg)* on human sickle cell *in vitro* after different period of incubation.

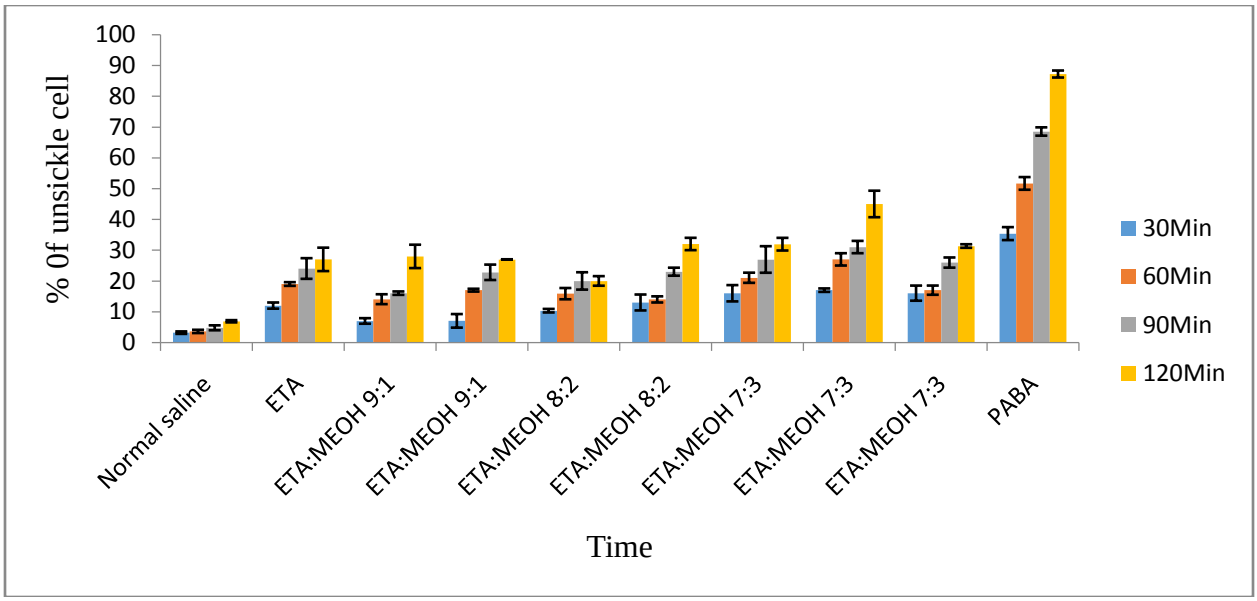


Figure 7: Effect of 0.1mg/ml methanolic extract of *A. conyzoides* fractions on human sickle cell *in vitro* after different period of incubation.

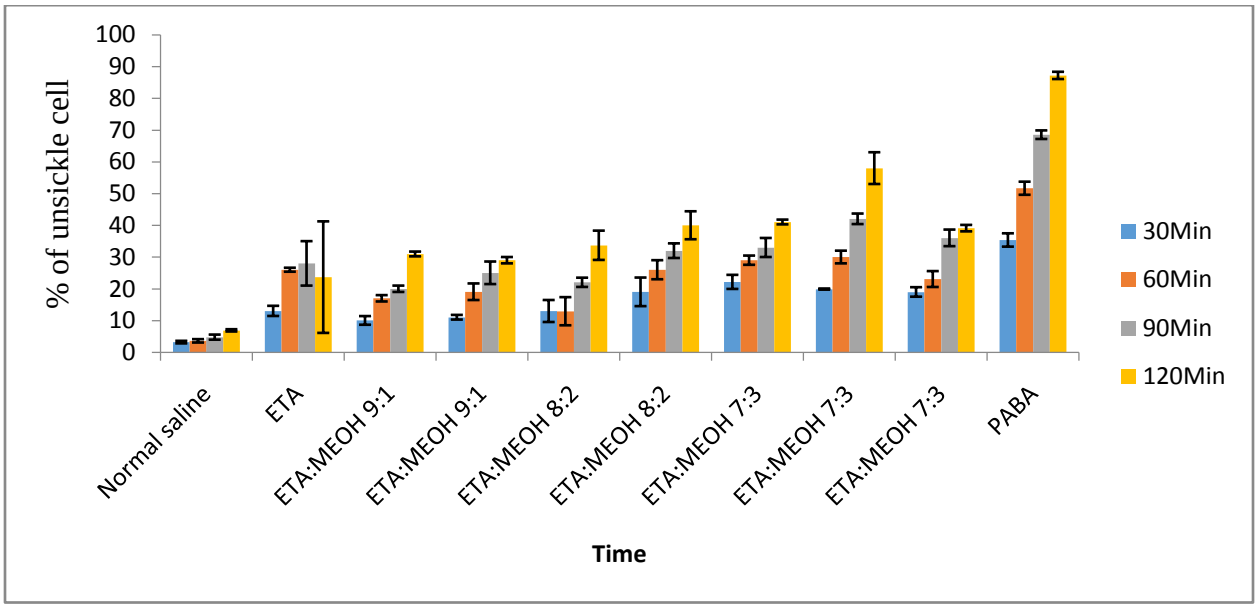


Figure 8: Effect of 0.2mg/ml methanolic extract of *A. conyzoides* fractions on human sickle cell *in vitro* after different period of incubation

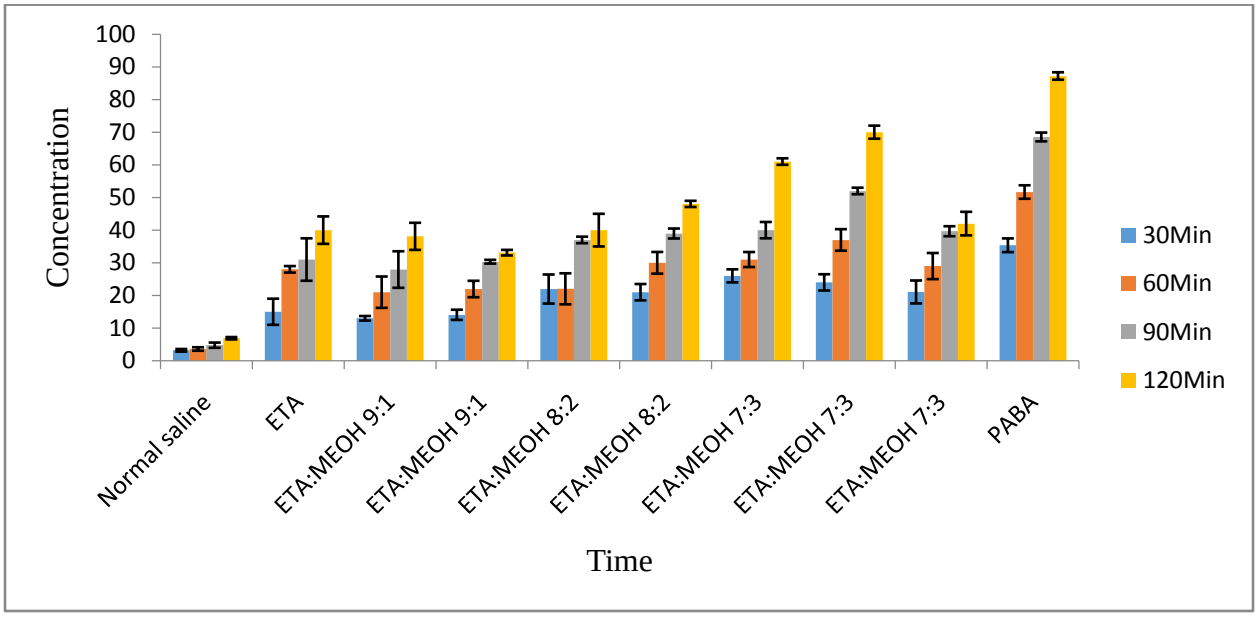


Figure 9: Effect of 0.3mg/ml methanolic extract of *A. conyzoides* fractions on human sickle cell in vitro after different period of incubation.

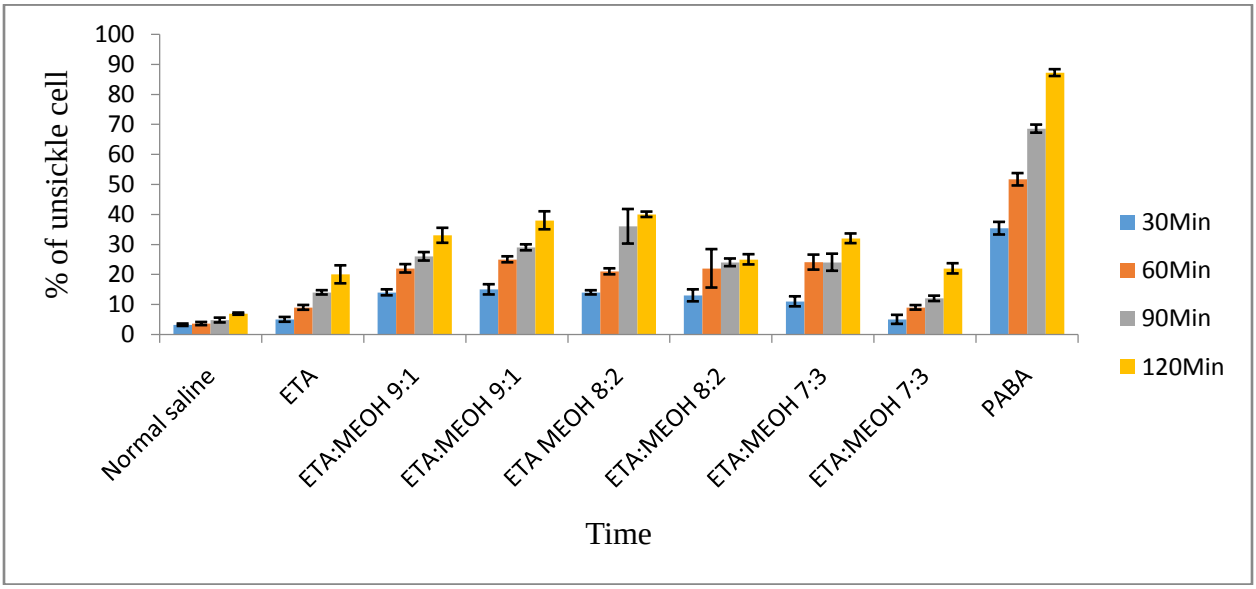


Figure 10: Effect of 0.1mg/ml methanolic extract of *M. indica* fractions on human sickle cell *in vitro* after different period of incubation.

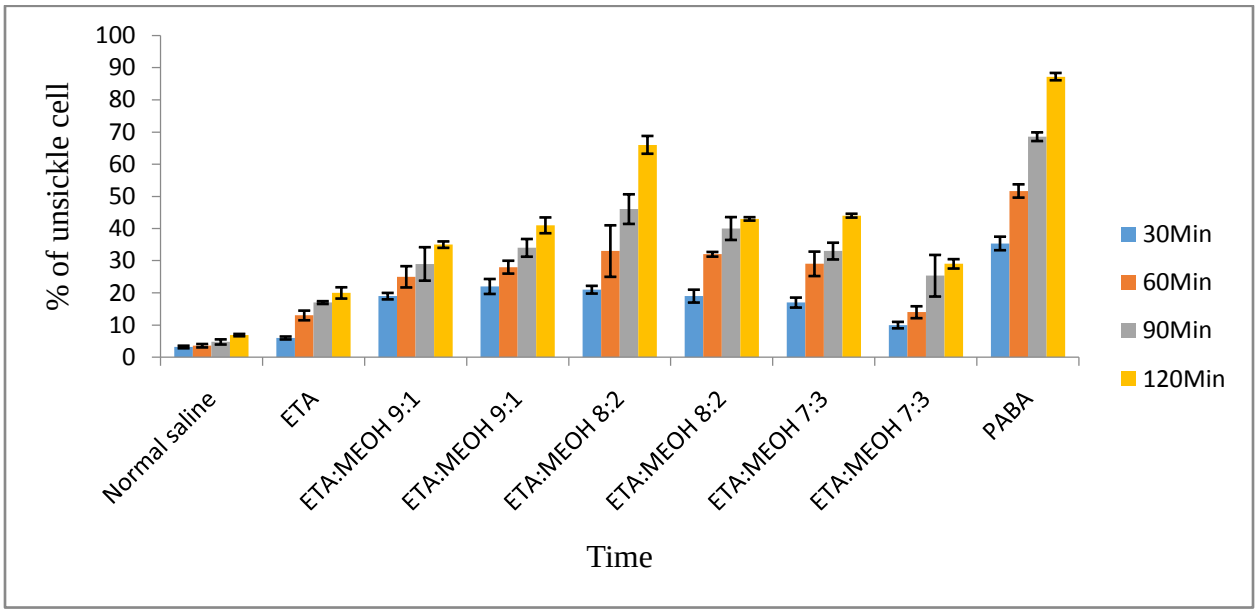


Figure 11: Effect of 0.2mg/ml methanolic extract of *M. indica* fractions on human sickle cell *in vitro* after different period of incubation.

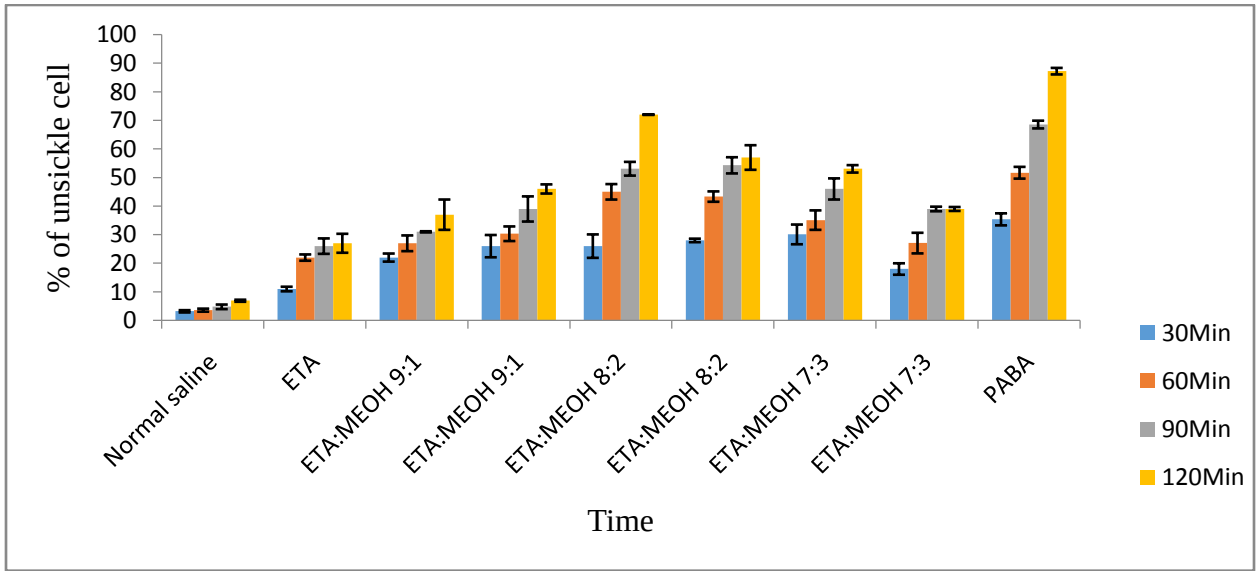


Figure 12: Effect of 0.3mg/ml methanolic extract of *M. indica* fractions on human sickle cell *in vitro* after different period of incubation.

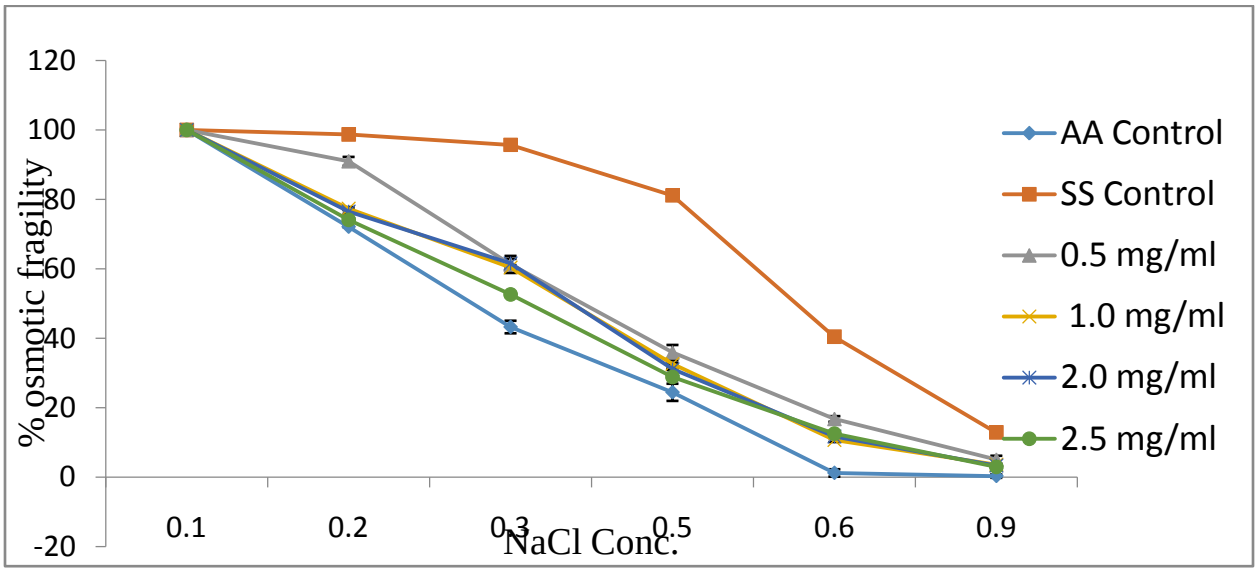


Figure 13: Effect of methanolic extract of *A. conyzoides*stem on human sickle cell *in vitro* at various NaCl concentrations

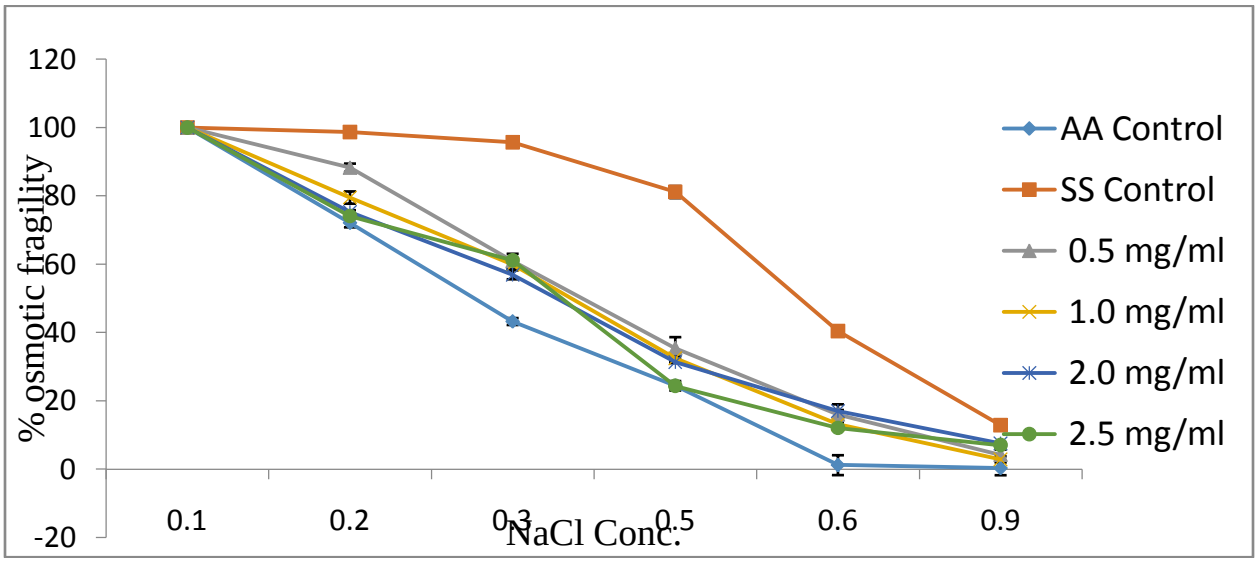


Figure 14: Effect of methanolic extract of *A. conyzoides* leaf on human sickle cell *in vitro* at various NaCl concentration.

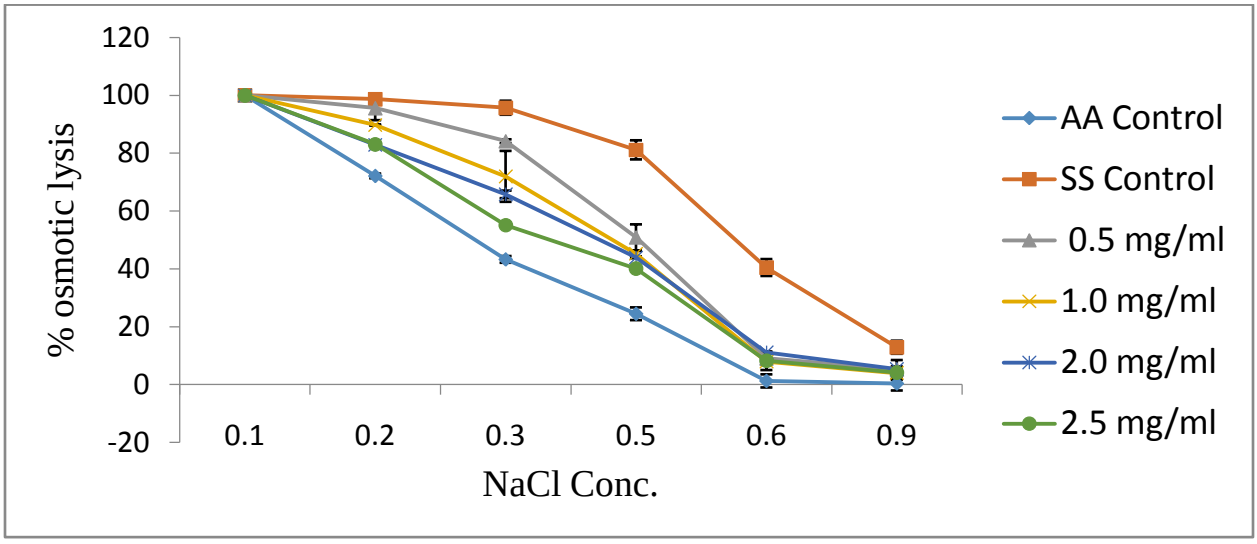


Figure 15: Effect of methanolic extract of *Ageratum conyzoides* root on human sickle cell *in vitro* at various NaCl concentrations

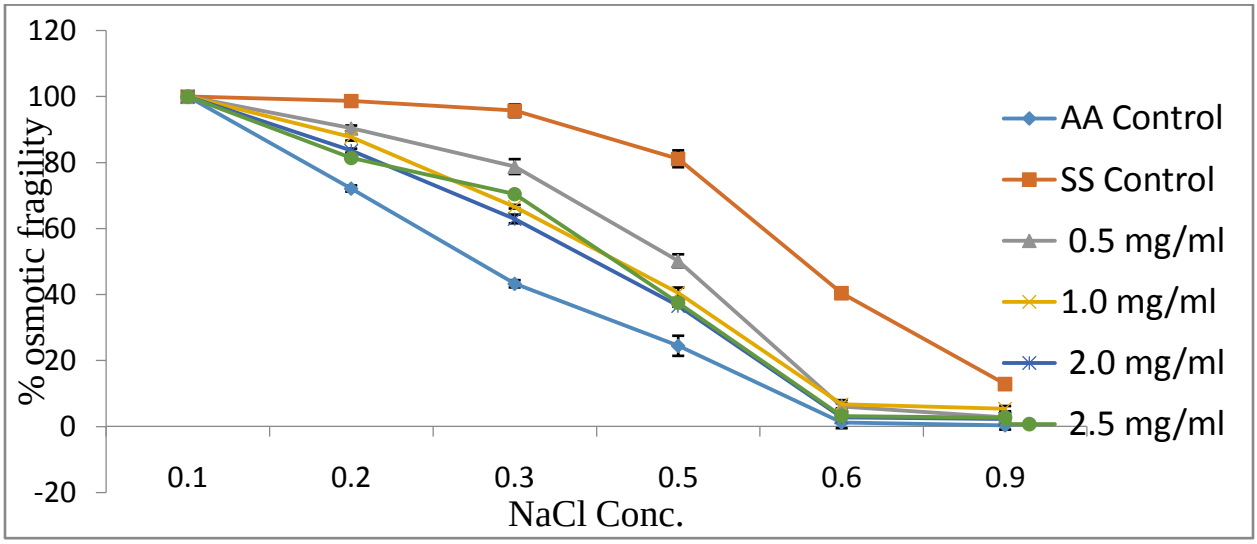


Figure 16: Effect of methanolic extract of *M. indica* stem on human sickle cell *in vitro* at various NaCl concentrations

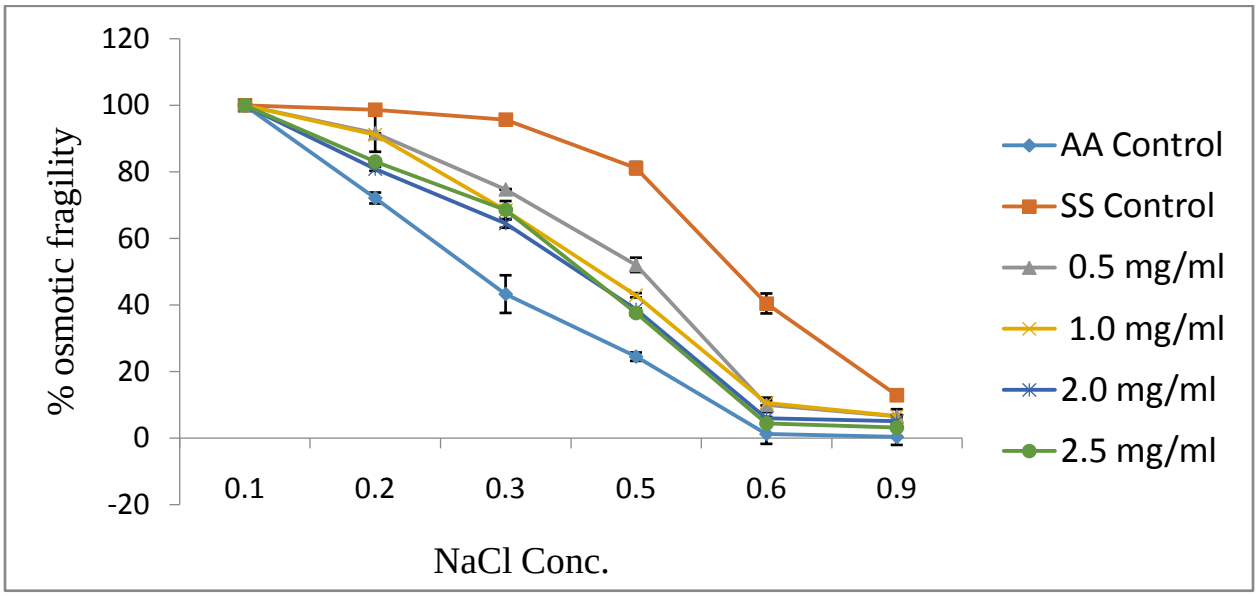


Figure 17: The effect of methanolic extract of *M. indica* leaf on human sickle cell *in vitro* at various NaCl concentrations

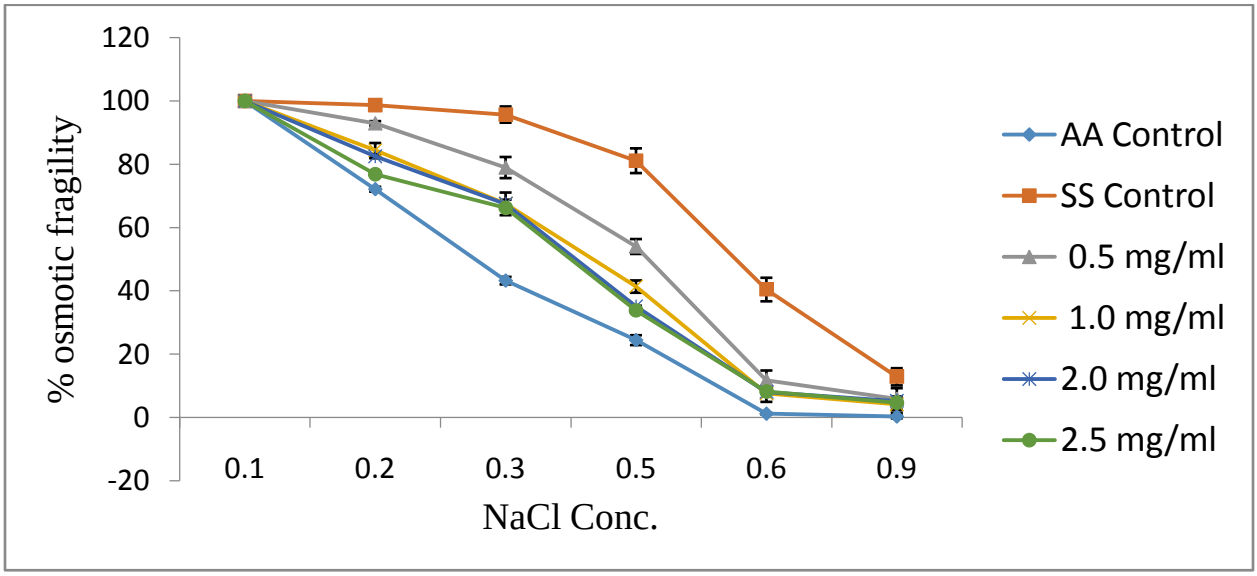


Figure 18: Effect of methanolic extract of *M. indica* root on human sickle cell *in vitro* at various NaCl concentrations

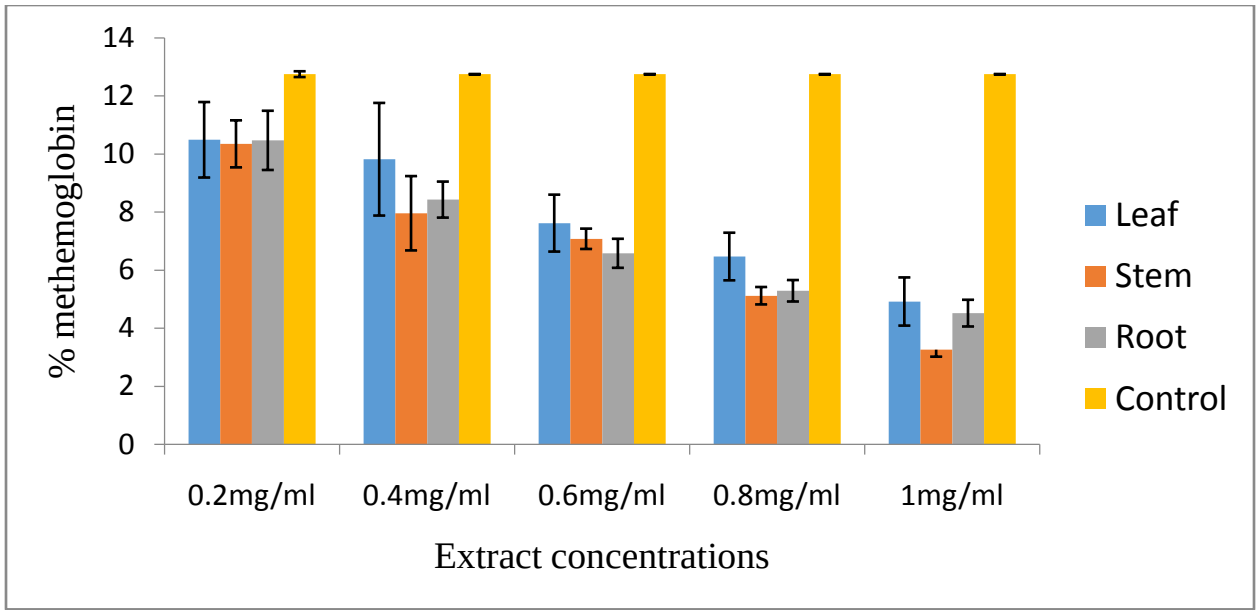


Figure 19: Comparism of % methemoglobin after treatment with different parts of *A. conyzoides* at various concentration of the plant extracts

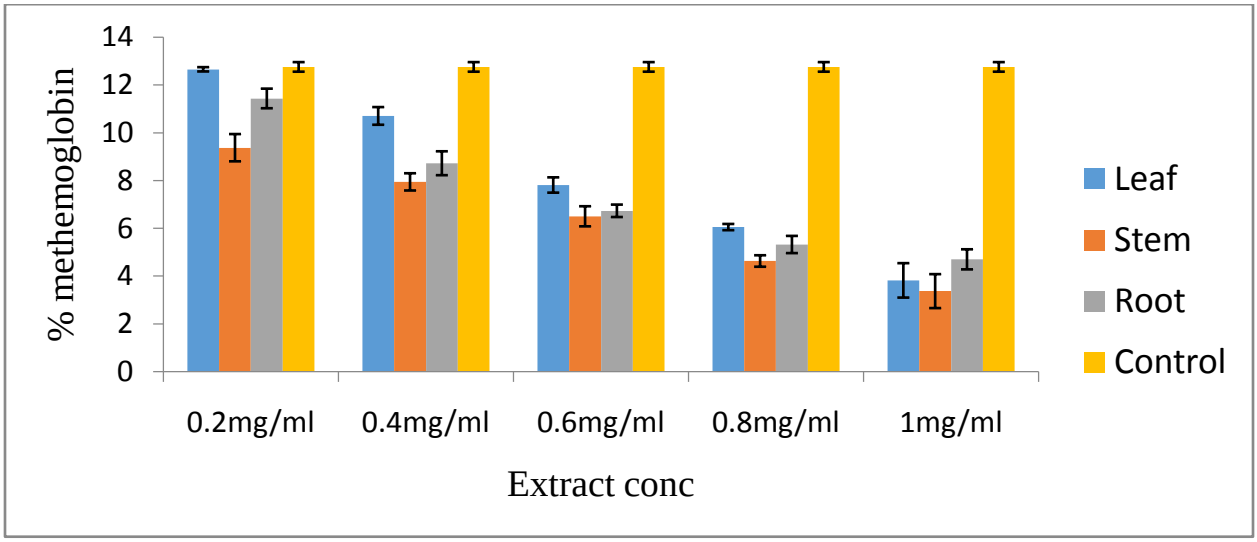


Figure 20: Comparism of % methemoglobin after treatment with different parts of *M. indica* at various concentration of the plant extracts

Figure 21: Distribution of Zinc, Magnesium, Calcium and Iron in leaves, stem bark and root of *A. conyzoides*

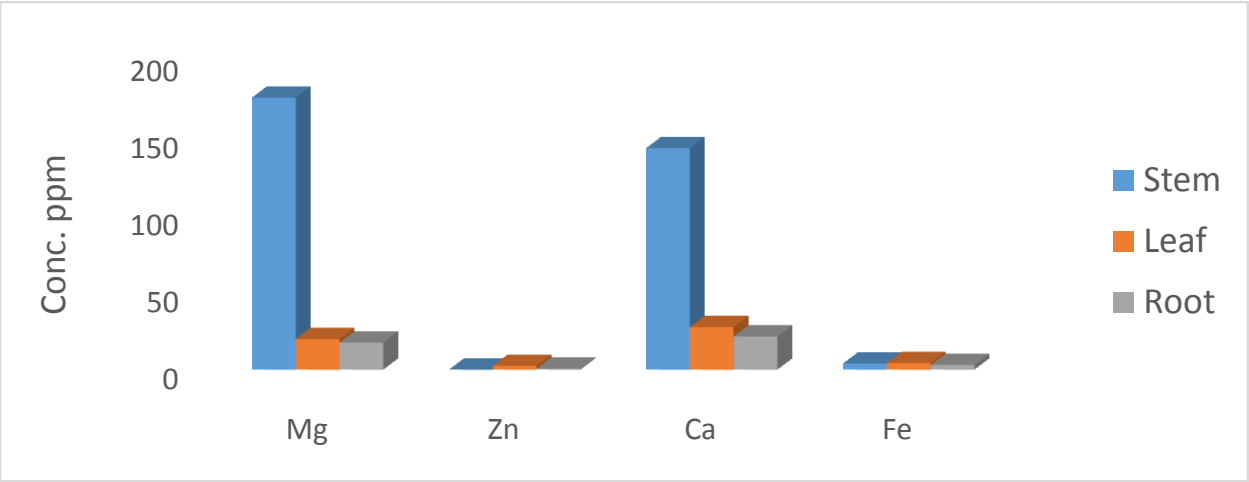


Figure 22: Distribution of Zinc, Magnesium, Calcium and Iron in leaves, stem bark and root of *M.indica*

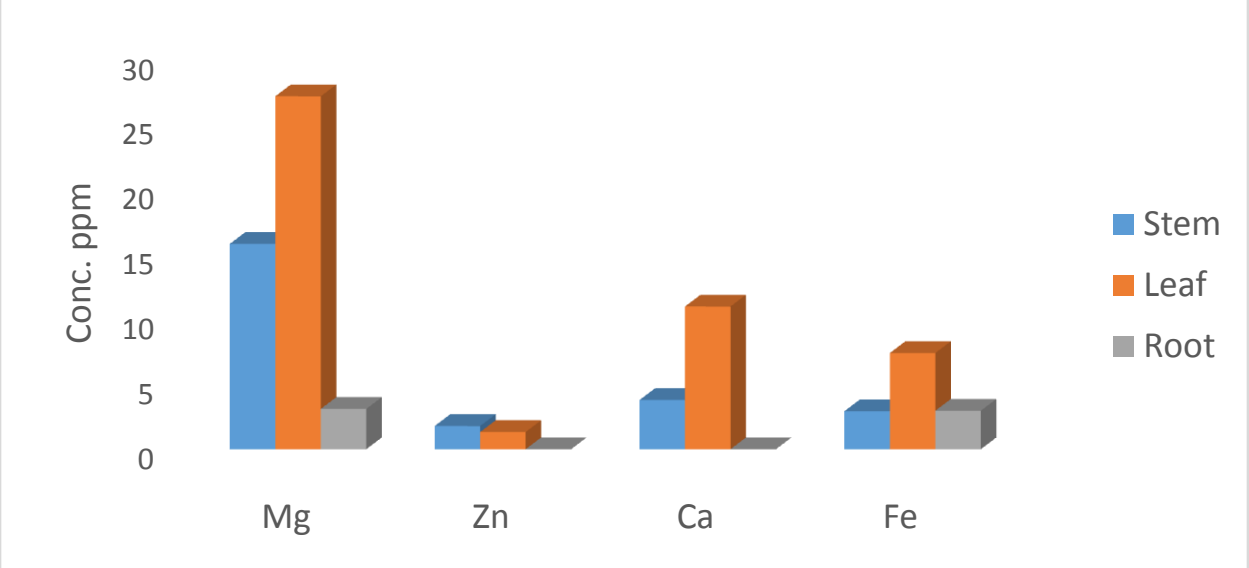
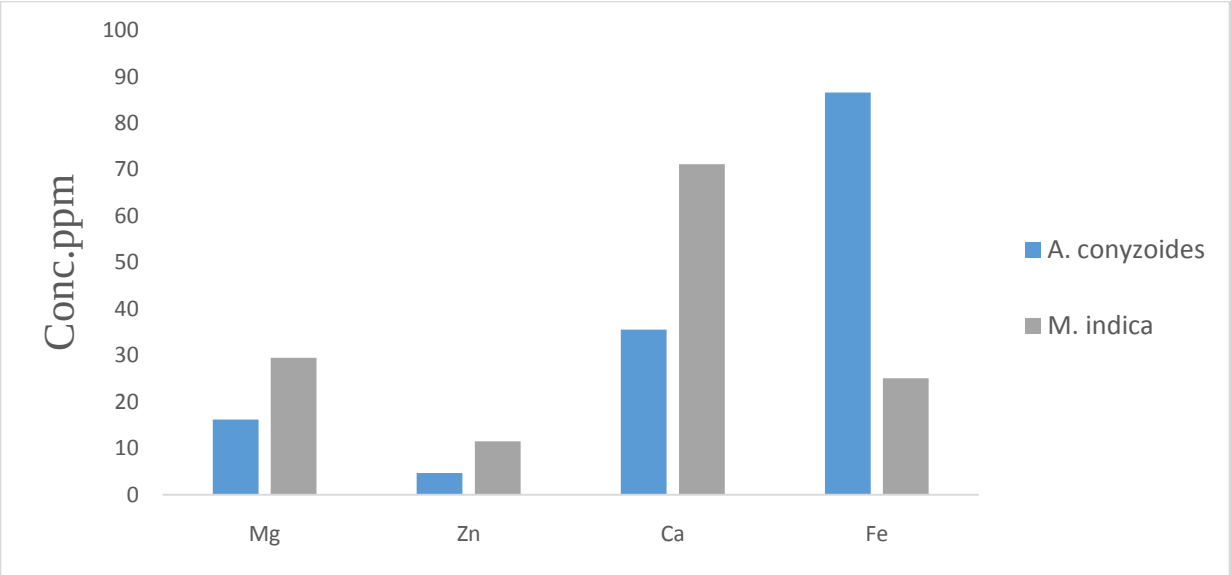


Figure 23:Compositionof elements in most active fractions of *A.conyzoides*and *M. indica* stem bark(mg/kg)



INFORMED CONSENT FORM FOR SICKLE CELL DISEASE PATIENTS ATTENDING AHMADU BELLO UNIVERSITY TEACHING HOSPITAL, ZARIA

Name of principal investigator	Ibrahim, Murja Danja
Name of Organisation	Department of Biochemistry, Ahmadu Bello University, Zaria
Name of proposal	Studies on the*In vitro*Anti-sicklingeffect and Activity Guided Fractionation of *Ageratum conyzoides* and *Mangifera indica*Methanolic Extracts on human sickle cells
Phone	O8065962249

Introduction: I am Murja Ibrahim Danja of Biochemistry Department, Ahmadu Bello University Zaria, doing a research on sickle cell anemia, a disease which is very common in our country. I am going to give you information and invite you to be part of the research. Before you decide, you can talk to any one you feel comfortable with about the research. If there is any thing you do not understand, please feel free to ask questions at any time.

Purpose of research: Sickle cell anemia (SCA) is a fatal disease which is common in Nigeria. Some of the drugs used in managing people with SCA are expensive and unsafe after long period of usage or high dosage. This research aims to find less expensive and safer way of managing the disease.

Type of research intervention: The research would involve the collection of 5ml of blood from the arm. You may feel a slight pain in the process, but it will not cause you any harm. The blood will be collected only once throughout the research period which will last for about six months.

Participant selection: Patients between the ages of 11-44 attending usual clinic follow-up at ABUTH are to participate in this research.

Voluntary participation: Your participation in this study is totally voluntary and you may refuse to participate or you may withdraw from participation at any time without losing the medical care you are entitled to receive.

Confidentiality: We would not share the identity of those participating in the research. However, the data collected from this research may be published in order to allow other people to benefit from it.

SECTION B: Certificate of Consent

I hereby consent to the collection of the blood sample of myself/my child or ward, and have the understanding that this sample is being collected solely for the purpose of research and no harm shall come to me (my child/ward) by providing the samples. The results of the study may or may not be of immediate benefit to the patient.

The above statement has been read out or explained to me, and having understood the same, I put my signature or thumb impression

Print Name of Participant_____

Signature of Participant _____

Date _____

Day/month/year

If illiterate

I have witnessed the accurate reading of the consent form to the participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness_____ or Thumb print of participant

Signature of witness _____

Date _____

Day/month/year

Statement by the researcher

1. 5ml of blood will be collected from the participant.
2. I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked were answered to the best of my ability.
3. I confirm that the individual is not forced into giving consent, and the consent was given freely and voluntarily.
4. A copy of this informed consent form was provided to the participant.

Name of researcher ----------

Signature ----------

Date ----------

Ahmadu Bello University Teaching Hospital

P.M.B. 06, Shika - Zaria, Kaduna State, Nigeria. 069-876305

website: www.abuth.org abuthshika@yahoo.com Abuthshika@gmail.com

Chairman of Board:

Chief Medical Director: **DR. LAWAL KHALID,** *MBBS, FMCS, FWACS, FRCS(ED) mni*

Chairman, Medical Advisory Committee: **DR. ABDULLAHI MOHAMMED,** *MBBS, FWACP FICS*

Director of Administration: **BARR. ISHAK BELLO,** *LL.B, BL, LL.M, PGDM, AHAN, FCAI*

Our Ref: ABUTH/HREC/TRG /36

3th October, 2013

ABUTH HREC FULL ETHICAL CLEARANCE CERTIFICATE

Re: *"ANTI SUCKLING EFFECT AND ACTIVITY GUIDED FRACTIONATION OF AGERATUM COYZOIDS METHANOLIC EXTRACT ON HUMAN SICKLED RED BLOOD CELL IN VITRO"*

ABUTH Ethics Committee assigned number:	-	ABUTH/HREC/G07/2013
Name of the principal Investigator:	-	Dr Ibrahim Murja Danja
Address of the Principal Investigator:	-	Dept. of Biochemistry ABU
Date of receipt of valid application:	-	3rd July, 2013
Date of meeting when final determination on ethical approval was made:	-	3rd October, 2013 to 3rd October, 2014

This is to inform you that the research described in the submitted protocol, the consent forms, and other participant information materials have been reviewed and ***given full approval by the ABUTH Ethics Committee.***

Please note: this approval dates from *4th October, 2013* to *4th October, 2014*.

No participant recruitment into this research may be conducted outside these dates.

Ahmadu Bello University Teaching Hospital

P.M.B. 06, Shika - Zaria, Kaduna State, Nigeria. 069-876305
website: www.abuth.org abuthshika@yahoo.com Abuthshika@gmail.com

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Our Ref: ABUTH/HREC/TRG /36 3th October, 2013

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Please note: this approval dates from ***4th October, 2013*** to ***4th October, 2014***.

No participant recruitment into this research may be conducted outside these dates.

All informed consent forms in this study must carry the ABUTH HREC number assigned to this research and the duration of ABUTH HREC approval of the study.

This HREC expects that you submit your application as well as an annual report for ethical clearance renewal 3 months prior to expiration of study dates. This is to enable you obtain renewal of your approval and avoid interruption of your research.

If there is delay in starting the research, please inform the ABUTH HREC so that starting dates can be adjusted accordingly.

No changes are permitted in the research without prior approval by ABUTH HREC, except in circumstances outlined in national code for Health Research Ethics: http://www.nhrec.net.

ABUTH HREC reserves the right to conduct compliance assessment visits to your research site without prior notification.

Prof. A. I. Mamman
Chairman, ABUTH HREC