

***IN VITRO* ANTISICKLING ACTIVITY OF CRUDE AND PARTIALLY
PURIFIED FRACTIONS OF AQUEOUS AND METHANOLIC EXTRACTS OF
LEAVES, SEEDS AND STEM OF *TELFERIA OCCIDENTALIS* (FLUTED
PUMPKIN)**

By

SAMUEL ATABO

DEPARTMENT OF BIOCHEMISTRY

FACULTY OF SCIENCE

**AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA.**

MAY, 2013

IN VITRO ANTISICKLING ACTIVITY OF CRUDE AND PARTIALLY PURIFIED
FRACTIONS OF AQUEOUS AND METHANOLIC EXTRACTS OF LEAVES, SEEDS
AND STEM OF *TELFERIA OCCIDENTALIS* (FLUTED PUMPKIN)

By

Samuel ATABO, B.Sc. BIOCHEMISTRY (Bayero University, Kano) 2008
MSc/Sci/09513/09-10

A THESIS SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES,
AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD
OF
MASTER DEGREE IN BIOCHEMISTRY.

DEPARTMENT OF BIOCHEMISTRY,
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

MAY, 2013

DECLARATION

I declare that the work in this thesis entitled “*In vitro* Antisickling Activity of Crude and Partially Purified Fractions of Aqueous and Methanolic Extracts of Leaves, Stem and Seeds of *Telferia Occidentalis* (Fluted Pumpkin)” has been carried out by me in the Department of Biochemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any institution.

ATABO, SAMUEL

Name of Student

Signature

May, 2013

Date

CERTIFICATION

This Thesis titled “*In vitro* Antisickling Activity of Crude and Partially Purified Fractions of Aqueous and Methanolic Extracts of Leaves, Stem and Seeds of *Telferia occidentalis* (Fluted Pumpkin)” meets the regulations governing the award of the degree of Master in Science of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Chairman, Supervisory Committee
Dr. I.A.Umar

Signature

(Date)

Member, Supervisory Committee
Dr. B.D. James

Signature

(Date)

Member, Supervisory Committee
Prof. A. Mamman

Signature

(Date)

Head of Department
Dr. H.M Inuwa

Signature

(Date)

Dean, School of Postgraduate Studies
Prof. A. A. Joshua

Signature

(Date)

DEDICATION

This research work is dedicated to God Almighty and to my beloved parents and siblings.

ACKNOWLEDGEMENT

All thanks to God Almighty for His invaluable help in the course of this research.

My utmost gratitude goes to my Supervisors Dr. I.A. Umar, Dr. B.D. James and Prof. A. Mamman for guiding me through the thick and thin of this research, despite their very tight schedules. They have always insisted on the best, no matter how tedious and uncomfortable it may seem. I will specially want to acknowledge the way Dr. I.A. Umar, will give me the drive and courage to do things I never thought I could, and the least I can do is to say “May God Reward You”.

I sincerely thank the Head, Biochemistry Department, Dr. H.M. Inuwa and the academic staff, especially Prof. S.E. Atawodi, Prof. D.A. Ameh, Dr. Ibrahim Sani, Prof. H.C. Nzelibe and Dr. A.S. Balarabe for their invaluable contributions in my study.

My immeasurable gratitude goes to my Parents, Mr. and Mrs. Patrick Yahaya Atabo; Siblings, Ali, Friday, Janet, Sunday, Maria, Dinah, Martha and Rita – Atabo for their prayers and support in all forms.

My deep sense of gratitude goes to Mr. and Mrs. Matthew Abu, Mr. and Mrs. Adams Ekele, Mr. and Mrs. Nache and all the families that aided my stay in Zaria.

I humbly express my appreciation to Ihuoma Onyeyirichi, for her wonderful support all through the research. Special thanks to my colleagues especially Okpe Oche, Abdulmalik Salman, Ali Sani Suleiman, Ogechi Nkeonye, Mayowa Adeniyi, Kingsley Okoyomo, Amaya Habila., Victoria Ayuba, Nweke Ogechi, Karima Mohammed., Maimuna Zubairu, Fatima Hassan, Monday Ejeh, Stella Njoku, Emeka Dim, and others.

In all sincerity, I thank you all.

ABSTRACT

This study was carried out to investigate the antisickling activity, membrane stabilizing potential and the effect of extracts on $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio in sickled haemoglobin (Hb S). Partial purification, quantitative phytochemical and mineral element analysis of plant extracts were also carried out. The antisickling activity of both aqueous and methanolic extracts of leaf, seeds and stem of *Telferia occidentalis* at 10.0, 1.0 and 0.1 mg/ml, revealed that the aqueous leaf extracts (10 mg/ml) exhibited highest antisickling activity (64.03%) which was significantly higher ($p < 0.05$) from that of the stem (47.30%) and seed (37.50%). Thin layer chromatography (TLC) of partially purified fractions showed single and double spots of different RF values. They were then pulled together in that respect. Partially purified fractions obtained, recorded improved antisickling effect, with Fraction 3 (a combination of the 31st and 32nd eluents from column chromatography) showing the maximum antisickling activity of 74%. Para-hydroxybenzoic acid and normal saline were used as positive and negative controls respectively. In the membrane stabilizing assay, the methanolic and aqueous stem extracts showed the highest effect of 71.85% and 61.29% respectively. The methanolic leaves extract demonstrated the highest potential decrease in the methemoglobin (mHb) concentration (4.88%) compared with the control that revealed (13.04%). The results of the phytochemicals (quantitative) in plant parts revealed the presence of saponins, alkaloids, flavonoids, cyanogenic glycosides and total phenolics in both extracts of various plant parts. The quantification of mineral composition of plant parts revealed the presence of sodium, potassium, zinc, calcium, magnesium and iron, with a predominant quantity of K, Mg and Fe shown across all extracts. The results provide scientific evidence for the use of *Telferia occidentalis* in the management of sickled cell disease (SCD) and its possible use in drug development.

TABLE OF CONTENTS

Cover Page.....	<i>i</i>
Fly Leaf.....	<i>ii</i>
Title Page.....	<i>iii</i>
Declaration.....	<i>iv</i>
Certification.....	<i>v</i>
Dedication.....	<i>vi</i>
Acknowledgement.....	<i>vii</i>
Abstract.....	<i>viii</i>
Table of Contents.....	<i>ix</i>
List of Tables.....	<i>xiii</i>
List of Figures.....	<i>xiv</i>
List of Plates.....	<i>xv</i>
List of Abbreviations.....	<i>xvi</i>
List of Appendices.....	<i>xviii</i>

CHAPTER ONE

1.0 Introduction.....	1
1.1 Statement of Research Problem.....	5
1.2 Justification.....	5
1.3 Aim and Objectives.....	6
1.3.1 Aim.....	6
1.3.2 Specific Objective.....	7

CHAPTER TWO

2.0 Literature Review.....	8
----------------------------	---

2.1	Occurrence and Distribution of <i>Telferia occidentalis</i>	8
2.1.1	Nutritional value of <i>Telferia occidentalis</i>	8
2.2	History of Sickle Cell Disease.....	11
2.2.1	Prevalence of Sickle Cell Anaemia.....	11
2.3	Red Blood Cells.....	12
2.3.1	Hemoglobin Structure.....	13
2.3.2	Classification of Sickle Cell Disease.....	14
2.4	Pathophysiology of Sickle Cell Disease.....	16
2.4.1	Hemolysis.....	16
2.4.2	Vaso-occlusion.....	17
2.4.3	Polymerization of Sickled Erythrocytes.....	20
2.5	Erythropoiesis.....	25
2.5.1	Effect of Sickle Cell Disease on Infection.....	27
2.5.2	Effect of Infection on Sickle Cell Disease.....	29
2.6	Malaria in Sickle Cell Disease.....	30
2.7	Oxidative Stress in Sickle Cell Disease.....	31
2.7.1	Sources of Reactive Oxygen Species in SCD.....	32
2.7.2	Consequences of oxidative stress in SCD.....	34
2.8	Bone Problem in SCD.....	36
2.8.1	Causes of Acute Chest Syndrome.....	38
2.9	Role of Nutrition in Sickle Cell Disease.....	41
2.9.1	Macronutrient Deficiencies.....	41
2.9.2	Micronutrient Deficiencies.....	42

CHAPTER THREE

3.0	Materials and Methods.....	46
3.1	Materials.....	46
3.1.1	Equipment, Chemical and Reagent.....	46
3.1.2	Collection and Identification of Plant Materials.....	47
3.2	Methodology.....	47
3.2.1	Preparation and Extraction of Plant Material.....	47
3.2.2	Sample Size.....	49
3.2.3	Blood Collection and Preparation	49
3.2.4	Bioassay of Plant Extracts for Antisickling Activity	49
3.2.5	Assay of Membrane Stabilizing Activity.....	51
3.2.6	Determination of the Effect of Extract on Fe^{2+}/Fe^{3+} Ratio in Sickle Cell Blood....	52
3.2.7	Phytochemical Test.....	53
3.2.8	Mineral Analysis.....	57
3.2.9	Thin Layer Chromatography (TLC)	57
3.2.10	Partial Purification of Crude Extract (Column Chromatography).....	58
3.3	Statistical Analysis.....	59

CHAPTER FOUR

4.0	Results.....	60
4.1	<i>In vitro</i> Antisickling Effects of Aqueous and Methanolic Extracts	60
4.2	Membrane Stabilizing Effect of Extracts.....	67
4.3	Effect of Extracts on the Fe^{2+}/Fe^{3+} Ratio.....	67
4.4	Phytochemical Constituent of the Plant Extracts.....	74
4.5	Mineral Element in Leaves, Seeds and Stem Extracts.....	74

4.6	Partial Fractionation of Active Principle with Antisickling Activity.....	80
4.6.1	Results of Thin Layer Chromatography (TLC).....	80
4.6.2	Results of Effect of Partially Purified Fractions.....	87
CHAPTER FIVE		
5.0	Discussion.....	92
CHAPTER SIX		
6.0	Summary, Conclusion and Recommendation.....	98
6.1	Summary.....	98
6.2	Conclusion.....	98
6.3	Recommendations.....	99
	References.....	100
	Appendices.....	115
	Informed Consent.....	121
	Ethical Clearance.....	123

LIST OF TABLES

Tables	Page
Table 2.1: Clinical Consequences of Haemolysis and Vaso-occlusion in SCD.....	19
Table 4.1: Peak Antisickling Effect of Crude Aqueous and Methanolic Extracts of <i>Telferia occidentalis</i>	64
Table 4.2: Phytochemical Constituents of <i>T. occidentalis</i>	75
Table 4.3: Quantitative Phytochemical Constituent of Aqueous Extracts of <i>T. occidentalis</i> ...	76
Table 4.4: Quantitative Phytochemical Constituent of Methanolic Extract of <i>T. occidentalis</i> ..	77
Table 4.5: Quantitative Mineral Elements in Aqueous Extract of <i>T. occidentalis</i>	78
Table 4.6: Quantitative Mineral Elements in Methanolic Extract of <i>T. occidentalis</i>	79
Table 4.7: Retention Factor (RF) Values of Various Fractions of Crude Extracts Subjected to Thin Layer Chromatography.....	86
Table 4.8: Peak Antisickling Effect of Partially Purified Fractions of Crude Aqueous Leaves Extracts of <i>T. occidentalis</i>	88

LIST OF FIGURES

Figures	Page
Figure 2.1: Change in erythroid cell type, site of erythropoiesis and globin-chain synthesis during human development	15
Figure 2.2: Pathophysiology of Sickle Cell Disease.....	25
Figure 2.3: The Vicious Cycle of the Acute Chest Syndrome.....	40
Figure 3.1: Experimental Design.....	48
Figure 4.1: Percentage Number of Cells Unsickled with Time by 10mg/ml Extracts of Leaf, Seed and stem.....	61
Figure 4.2: Percentage Number of Cells Unsickled with Time by 1mg/ml extracts of Leaf, Seed and Stem.....	62
Figure 4.3: Percentage Number of Cells Unsickled with Time by 0.1mg/ml extracts of Leaf, Seed and Stem	63
Figure 4.4: Membrane Stabilizing Activity of Aqueous and Methanolic Extracts of Leaves of <i>Telferia occidentalis</i>	68
Figure 4.5: Membrane Stabilizing Activity of Aqueous and Methanolic Extracts of Seeds of <i>Telferia occidentalis</i>	69
Figure 4.6: Membrane Stabilizing Activity of Aqueous and Methanolic Extracts of Stem of <i>Telferia occidentalis</i>	70
Figure 4.7: Percentage Methemoglobin in Blood in the Presence of Leaves Extract of <i>Telferia occidentalis</i>	71
Figure 4.8: Percentage Methemoglobin in Blood in the Presence of Seed Extract of <i>Telferia occidentalis</i>	72
Figure 4.9: Percentage Methemoglobin in Blood in the Presence of Stem Extract of <i>Telferia occidentalis</i>	73

Figure 4.10: Antisickling Activity of Partially Purified Leaves Extract (10 mg)	89
Figure 4.11: Antisickling Activity of Partially Purified Leaves Extract (1 mg)	90
Figure 4.12: Antisickling Activity of Partially Purified Leaves Extract (0.1 mg)	91

LIST OF PLATES

Plates	Page
Plate 2.1: <i>Telferia occidentalis</i> Leaves, Seeds and Pod.....	10
Plate 2.2: Hand-foot Syndrome in Patient Aged 14 Months with Homozygous Sickle Cell Disease.....	37
Plate 4.1: Partially Lysed RBC's by Effect of 10mg/ml (30 min) Methanolic Seed Extract...	65
Plate 4.2: Completely Lysed RBC's by Effect of 10mg/ml Methanolic Seed Extract.....	66
Plate 4.3: TLC of Fractions 1 – 3 (Flow Through)	82
Plate 4.4: TLC of Fractions 4 – 20.....	83
Plate 4.5: TLC of Fractions 21 – 37.....	84
Plate 4.6: TLC of Fractions 28 – 55.....	85

LIST OF ABBREVIATION

Abbreviation	Translation
SCD	Sickle Cell Disease
HbSS	Sickle Cell Haemoglobin
Hb S	Sickled Haemoglobin
Hb F	Fetal Haemoglobin
HbAA	Normal haemoglobin
ISC	Irreversible Sickled Cells
RBC	Red Blood Cell
TLC	Thin layer Chromatography
DPG	Diphosphoglycerate
e.g.	For example
i.e.	That is
KCl	Potassium chloride
NIPRD	National Institute for Pharmaceutical Research and Development
Ca	Calcium

K	Potassium
Na	Sodium
Zn	Zinc
Mg	Magnesium
Fe	Iron
$[Ca^{2+}]_i$	Intracellular calcium
ROS	Reactive oxygen species
GSH	Reduced glutathione
XO	Xanthine oxidase
NOS	Nitric oxide synthase
COX	Cyclo-oxygenase
SOD	Superoxide dismutase
ETC	Electron Transport Chain
HUVEC	Human umbilical vein endothelial cells

LIST OF APPENDICES

Appendix	Page
Sickle Cell Morphology after Treatment.....	113
Plate I: Sodium Metabisulphite Induced Sickling.....	113
Plate II: PABA-treated Sickled Cells.....	114
Plate III: Aqueous Leaves Extract-treated Sickled Cells.....	115
Plate IV: Methanolic Leaves Extract-treated Sickled Cells.....	116
Plate V: Normal Saline Treated Sickled Cells (Negative Control)	117
Plate VI: Purified Fractions-treated Sickled Cells.....	118

CHAPTER ONE

INTRODUCTION

Plants provide an alternative source for new drugs search. There is a rich abundance of plants reputed in traditional medicine to possess protective and therapeutic properties. It is likely that plants will continue to be a valuable source of new compounds which may, after possible chemical manipulation, provide new and improved drugs (Shah *et al.*, 2006).

The World Health Organisation (2008) defines traditional medicine as the health practices, approach, knowledge and belief, incorporating plant, animal and mineral-based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination, to treat, diagnose or prevent illness or maintain well being. It further describes medicinal plant as any plant which one or more of its organ contains substances that can be used for the synthesis of useful drugs. This definition distinguishes those plants whose therapeutic properties and constituents have been established scientifically and plants that are regarded as medicinal but which have not yet been subjected to thorough investigation (WHO, 2008). Furthermore, WHO (2001) defines medicinal plants as herbal preparations produced by subjecting plant materials to extraction, fractionation, purification, concentration or other physical or biological processes which may be produced for immediate consumption or as a basis for herbal products.

Medicinal plants contain biologically active chemical substances such as saponins, tannins, essential oils, flavonoids, alkaloids and other chemical compounds (Harborne, 1973; Sofowora, 1996) which are presumably capable of ameliorating or curing disease conditions. These complex chemical substances of different compositions are found as

secondary plant metabolites in one or more of these plants. Tyler (1999) has reported that plants also contain other compounds that moderate the effects of the active ingredients.

Reports of biological significance of leaves, stem and seeds of *Telfairia occidentalis* is no longer new. It has been linked to free radical scavenging, boosting of blood levels, anti-plasmodial, antimicrobial and aphrodisiac properties (Tindall, 1968; Fasuyi, 2006; Toyin *et al.*, 2008; Oyewole and Abalaka, 2012). However, substantial scientific evidence on its antisickling activity is subtle, despite its recommendation for consumption (traditionally) by individuals suffering from SCD in south-eastern Nigeria. Series of plants established to have antisickling properties are found to owe this effect to bioactive components such as zinc, iron, amino acids and phytochemicals (Prasad, 2002; Nwaoguikpe and Ejele, 2010). Investigation in to bioactive components of the leaves, stem and seeds of *Telferia occidentalis* could therefore offer a clue on its propensity of being an antisickling agent.

Sickle-cell anaemia is caused by a point mutation in the β -globin chain of haemoglobin, causing the hydrophilic amino acid - glutamic acid to be replaced with the hydrophobic amino acid - valine at the sixth position. The β -globin gene is found on the short arm of chromosome 11 (Driscoll, 2007).

The association of two wild-type α -globin subunits with two mutant β -globin subunits forms haemoglobin S (HbS). Under low-oxygen conditions, the absence of a polar amino acid at position six of the β -globin chain promotes the non-covalent polymerisation (aggregation) of haemoglobin, which distorts red blood cells into a sickle shape and decreases their elasticity.

The loss of red blood cell elasticity is central to the pathophysiology of sickle-cell disease. Normal red blood cells are quite elastic, which allows the cells to deform to pass through

capillaries. In sickle-cell disease, low-oxygen tension promotes red blood cell sickling, and repeated episodes of sickling damage the cell membrane and decrease the cell's elasticity. These cells fail to return to normal shape when normal oxygen tension is restored (ISC's). As a result, the rigid RBC's are unable to deform as they pass through narrow capillaries, leading to vessel occlusion and ischaemia (Driscoll, 2007).

The actual anaemia of the illness is caused by haemolysis, the destruction of the red cells inside the spleen, because of their mis-shape. Although the bone marrow attempts to compensate by creating new red cells, it does not match the rate of destruction. Healthy red blood cells typically live 90–120 days, but sickle cells only survive 10–12 days (Serjeant, 2001).

Normally, humans have Haemoglobin A, which consists of two alpha and two beta chains ($\alpha_2\beta_2$), Haemoglobin A₂ ($\alpha_2\delta_2$), which consists of two alpha and two delta chains and Haemoglobin F ($\alpha_2\gamma_2$), consisting of two alpha and two gamma chains in their bodies. Of these, Haemoglobin A makes up around 96-97% of the normal haemoglobin in humans. Various approaches have been adapted in an effort to find agents that inhibit the polymerization of haemoglobin and hence prevent or reduce the occurrence of crises in sickle cell disease (SCD) (Iyamu *et al.*, 2002). In this regard, oxygen, carbon monoxide and sodium nitrite were used to reduce the amount of deoxy-haemoglobin. Iyamu *et al.*, (2002) however, reported that these approaches did not give the much needed beneficial effects. This has therefore led to the use of bone marrow transplantation and drugs such as clotrimazole, hydroxyurea and erythropoietin. Cost, availability and accessibility remains a limitation to these remedies, especially in developing countries.

The use of phytomaterials such as *Piper guinensis*, *Pterocarpa osun*, *Eugenia caryophylla* and *Sorghum bicolor* extracts for the treatment of SCD has been reported by Wambebe *et al.*, (2001). The extracts of *Pterocarpus santolinoides* and *Aloe vera* were reported to increase the gelling time of sickle cell blood and inhibit sickling *in vitro* (Ugbor, 2006). Sofowora and Isaac- Sodeye (1971) reported the reversal of sickling by root extracts of *Fagara zanthoxylloides*. *Terminalia catappa* could be effective antisickling agents that inhibit osmotically-induced haemolysis of human erythrocytes (Mgbemene and Ohiri, 1999).

Research on phytomedicine for the treatment of SCD has led to the development of Niprisan (a herbal based drug) which has been patented by the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria and produced to meet increasing global demand by sufferers of SCD. This development indicates that more of such herbal based drugs could be consequent upon scientific investigations on plants that are used in folklore medicine.

This work is therefore intended to ascertain, compare and examine the antisickling effects of the leaves, seeds and stem of *Telfairia occidentalis*.

1.1 STATEMENT OF RESEARCH PROBLEM

Sickle cell disease (SCD) is a serious global problem, management or treatment of which still poses difficult challenges to medical practitioners worldwide. It is a devastating genetic disorder affecting 2.3% of the world population, mainly in Africa (WHO, 2011),

and responsible for about 2% death in under 5 children in sub-sahara Africa (WHO, 2011). In Nigeria, prevalence of SCA is about 20 – 30 per 1000 births (WHO, 2011), and in June 19, 2012, the World Health Organization (WHO) ranks Nigeria as the country with the highest number of Sickle Cell anaemia sufferers in the world (WHO, 2012).

Various approaches have been adapted in an effort to find agents that prevent or reduce crises in SCD. In this regard sodium nitrite was used, but did not give much needed beneficial effects. Further techniques, which directly affects the Haemoglobin molecule such as Bone marrow Transplantation, blood transfusion and even the use of chemical drugs such as clotrimazole, hydroxyurea, senicapoc and valproate have been advocated but are very expensive, inaccessible, mutagenic and may expose patients to iron overload, even though medical practitioners have argued that the complications in SCD far outweighs the effect of the drugs. This has stimulated the current efforts directed at searching for more effective remedies for the disease from the plant kingdom.

1.2 JUSTIFICATION

While orthodox medicine has achieved some level of success in SCD, cure for patients is yet to be found. In the recent years, bone marrow transplantation has been found to be an efficient way of treating SCD. However, the cost implications, unavailability of necessary expertise and the problems of finding suitable donors constituted a major setback to this approach in developing countries (Mpiana *et al.*, 2007). In clinical practice, clotrimazole, hydroxyurea and erythropoietin are used in SCD management, but the side effects of these drugs limit their clinical use (Mehanna, 2001; Eliot *et al.*, 2006).

On the understanding that herbal remedies and medicinal plants products from indigenous flora have long been used in folk medicine in the management of SCD, it appears that proper and intense scientific investigation on such medicinal plants could be of tremendous help in developing efficacious and safer drugs for SCD treatment. Research on phytomedicine has led to the development of Niprisan (a herbal based drug used in the treatment of SCD). Other indigenous plants have gained scientific backing as antisickling agent (*cajanus cajan*, *carica papaya*, *Fagara zanthoxylloides*).

Telferia occidentalis which has been linked to free radical scavenging, boosting of blood levels, anti-plasmodial and antimicrobial effects (Fasuyi, 2006; Toyin *et al.*, 2008; Oyewole and Abalaka, 2012) is now being speculated in this study as a possible therapeutic agent for SCD management, owing to its endorsement for consumption by SCD patients in south-eastern Nigeria..

.

1.3 AIM AND OBJECTIVES OF THE STUDY

1.3.1 Aim

The aim of this work is to investigate and compare the antisickling potentials of the leaves, seeds and stem of *Telfairia occidentalis*.

1.3.2 Specific Objectives

The specific objectives of this study include:

1. To determine the *in vitro* antisickling effect of aqueous and methanolic extracts of leaves, stem and seeds of *Telfairia occidentalis*.
2. To evaluate the membrane stabilizing effect of extracts of the plant part extracts.

3. To assess the effect of extracts on the $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio in sickle cell blood.
4. To identify and quantify phytochemical constituents present in the plant extracts
5. To quantify the mineral element content of extracts of the plant parts
6. To partially purify the most active crude extract and test the fractions for antisickling activity.

CHAPTER TWO

LITERATURE REVIEW

2.1 Occurrence and Distribution of *Telfairia occidentalis*

Telfairia occidentalis (Hook f.) commonly called Fluted pumpkin occurs in the forest zone of West and Central Africa, most frequently in Republic of Benin, Nigeria and Cameroon.

It is a popular vegetable all over Nigeria. It has different traditional names; among the Igbos, it is known as “Ugu, Iroko” or “Aporoko” in Yoruba, “Ubong” in Efik, “Umee” in Urhobo and “Umeke” in Edo (Akoroda, 1990a; Badifu and Ogunsina, 1991).

Telfairia is classified in the tribe *Joliffieae* of the subfamily *Cucurbitaceae*. It comprises 3 species, of which *Telfairia pedata* (Sm. ex Sims) Hook. (Oyster nut) is much cultivated for its seed oil in East Africa. The names *Telfairia pedata* and oyster nut are often erroneously used for *Telfairia occidentalis*. Cultivars of *Telfairia occidentalis* are distinguished by seed colour, thickness of vine, size of leaf, growing vigour, days to flowering and succulence. In Nigeria the two main cultivars are *ugu-ala*, characterized by succulent, broad leaves, small black seeds, thick stem and slow growth and *ugu-elu* which has a high growth rate, large brownish seeds with high viability and thin stem with small leaves. The seed is often polyembryonic, which is useful for multiplication and in breeding (Akoroda, 1990b).

2.1.1 Nutritional value of *Telfairia occidentalis*

Telfairia occidentalis is an important staple vegetable grown in Nigeria. Recent studies have shown that *Telfairia occidentalis* leaf is rich in mineral elements (such as iron, potassium, sodium, phosphorus, calcium and magnesium), antioxidants, vitamins (such as thiamine, riboflavin, ascorbate), nicotinamide and phytochemicals Longe *et. al.*, (1983).

The amino acid profile of *T. occidentalis* seeds had also been shown to be very rich and include alanine, aspartate, glycine, glutamine, histidine, lysine, methionine, tryptophan, cystine, leucine, arginine, serine, threonine, phenylalanine, valine, tyrosine and isoleucine (Tindall, 1968; Fasuyi, 2007).

Following a line of investigation, facts have been established on the biological significance of *Telfairia Occidentalis* seeds. The seeds are highly medicinal as well as nutritious and are eaten, either roasted or boiled. They are also sometimes used as soup thickeners (Okoli and Mgbeog, 1983). The seed is very rich in oil, especially unsaturated fatty acids which form 61% of the oil (Odoemena and Onyeneke, 1998; Okoli and Nyanayo, 1988). Akubue *et al.*, (1980) and Taylor *et al.*, (1983) have documented that Fluted pumpkin seeds are a good source of four mineral elements (Ca, K, Na, Zn) required in human nutrition. The report showed that the seed contained 29% oil and 30% protein. Asiegbu (1987) reported Fluted pumpkin seed contain 47% oil and 31% protein. The protein was said to be markedly deficient in the sulphur-containing amino acid.

Longe *et al.*, (1983) reported that Fluted pumpkin seeds had 53% fat, 22% protein, 3% fibre, 15% carbohydrates and 2% ash. Oyolu (1980) observed that vegetables will continue to remain the primary source of proteins, minerals and vitamins in African countries, he noted that leaves and edible shoots of Fluted pumpkin together contain 85% moisture, while the dry portion of what is usually consumed contains 11% crude protein, 25% carbohydrate, 3% oil, 11% ash and as much as 700 ppm iron.



Telfairia occidentalis
(Leaves)



Telfairia occidentalis(Seeds)



Telfairia occidentalis
(Pods)

Plate 2.1: *Telferia occidentalis* Leaves, Seeds and Pod

2.2 History of Sickle Cell Disease

Sickle-cell disease is a multisystem disease, associated with episodes of acute illness and progressive organ damage. It is one of the most common severe monogenic disorders worldwide (Weatherall, 2005). Herrick (1910) first described the characteristic sickle-shaped erythrocytes. The understanding of the disease has gradually increased since then. Pauling *et.al*, (1949) identified electrophoretic abnormalities in sickle haemoglobin (HbS) and coined the term “molecular disease” in 1949. The haemoglobin biophysics and genetics underlying the disease have been extensively studied and have helped the understanding of other molecular diseases. However, clinical management of sickle-cell disease is still basic and, although some evidence lends support to the use of blood transfusion, and hydroxycarbamide in some circumstances, it is not without serious level limitations.

In sickle cell disease, two abnormal alleomorphic haemoglobin genes are inherited, of which at least one must be the sickle haemoglobin. In the homozygous sickle cell disease (HbSS), both abnormal haemoglobins are HbS. A normal adult haemoglobin is made from a combination of two β -globin protein chains with two α -globin chains and heme. The β_1 -globin gene is located on the short arm of chromosome 11. Approximately 150 diseases have been linked to this same chromosome 11 (Geoffrey *et al.*, 2009).

2.2.1 Prevalence of Sickle Cell Anaemia

Sickle-cell anaemia is particularly common among people whose ancestors come from sub-Saharan Africa, India, Saudi Arabia and Mediterranean countries. Migration raised the frequency of the gene in the American continent. In some areas of sub-Saharan Africa, up to 2% of all children are born with the condition. In broad terms, the prevalence of the sickle-cell trait (healthy carriers who have inherited the mutant gene from only one parent)

ranges between 10% and 40% across equatorial Africa and decreases to between 1% and 2% on the North African coast and <1% in South Africa. This distribution reflects the fact that sickle-cell trait confers a survival advantage against malaria and that selection pressure due to malaria has resulted in high frequencies of the mutant gene especially in areas of high malarial transmission. In West African countries such as Ghana and Nigeria, the frequency of the trait is 15% to 30% (WHO, 2006), whereas in Uganda it shows marked tribal variations, reaching 45% among the Baamba tribe in the west of the country. Frequencies of the carrier state determine the prevalence of sickle-cell anaemia at birth. For example, in Nigeria, by far the most populous country in the sub-region, 24% of the population are carriers of the mutant gene and the prevalence of sickle-cell anaemia is about 20 per 1000 births. This means that in Nigeria alone, about 150, 000 children are born annually with sickle-cell anaemia (WHO, 2006).

2.3 Red Blood Cells (Erythrocytes)

The major function of red blood cells, also known as *erythrocytes*, is to transport *haemoglobin*, which in turn carries oxygen from the lungs to the tissues. The red blood cells have other functions besides transport of haemoglobin. For instance, they contain a large quantity of *carbonic anhydrase*, an enzyme that catalyzes the reversible reaction between carbon dioxide (CO_2) and water to form carbonic acid (H_2CO_3), increasing the rate of this reaction several thousand fold. The rapidity of this reaction makes it possible for the water of the blood to transport enormous quantities of CO_2 in the form of bicarbonate ion (HCO_3^-) from the tissues to the lungs, where it is reconverted to CO_2 and expelled into the atmosphere as a body waste product. The haemoglobin in the cells is an excellent *acid-base*

buffer, so that the red blood cells are responsible for most of the acid-base buffering power of whole blood (Guyton and Hall, 2006).

2.3.1 Haemoglobin Structure

Haemoglobin is a globular protein molecule composed of two polypeptide chains, each folded around a heme molecule. The types of polypeptide chains vary with different stages of intra-uterine development and are designated by Greek letters alpha (α), beta (β), gamma (γ), delta (δ), epsilon (ϵ) and zeta (ζ). The epsilon (ϵ) and zeta (ζ) and some alpha (α) chains are synthesized in early embryonic life, alpha (α) and gamma (γ) chains in fetal life, and alpha (α), beta (β), delta (δ) chains predominate in postnatal life. The polypeptide chains available determines the type of haemoglobin molecule produced. In earliest embryonic life, zeta (ζ) and epsilon (ϵ) combines to form Hb Gower I ($\zeta_2\epsilon_2$), α and ϵ chains form Gower II ($\alpha_2\epsilon_2$), and ζ and γ chains form Hb Portland ($\zeta_2\gamma_2$). In fetal life, the predominant molecule is HbF ($\alpha_2\gamma_2$) whereas in postnatal life, fetal haemoglobin is replaced by HbA ($\alpha_2\beta_2$) and HbA₂ ($\alpha_2\delta_2$). During development, there is a coordinated switching of haemoglobin synthesis, affecting both the sites of erythropoiesis as well as the types of polypeptide chains and hence haemoglobin synthesized.

The different polypeptide chains manifest many similarities in their molecular structure, and on comparison of amino acid composition are clearly divided into two groups and α and ζ chains and the β , γ , δ , and ϵ chains. The different globin chains have probably arisen by successive gene duplications from an ancestral α -like globin structure, and the close similarity between the structures of the β and the δ chains suggests that they have diverged recently in evolutionary terms. The α and ζ chains contain 141 amino acids and the β , γ , δ , and ϵ chains, 146 amino acids.

The constraints and dimensions of peptide bonds cause polypeptide chains to coil in to globular subunits in which helical sections are separated by less well-organised segments of polypeptide chain. The subunits are then paired to form dimmers which associate to form the tetramer haemoglobin. The molecules of HbA dissociates easily in to $\alpha\beta$ dimmers at an interface called $\alpha_1\beta_1$ and under extreme conditions in to the constituent monomers (individual chains) at a second interface called $\alpha_1\beta_2$.

2.3.2 Classification of Sickle Cell Disease

The term sickle-cell disease is used to refer to all the different genotypes that cause the characteristic clinical syndrome, whereas sickle-cell anaemia, the most common form of sickle-cell disease, refers specifically to homozygosity for the β^S allele. Only a little evidence for the management of other types of sickle-cell disease has been reported. In populations of African ethnic origin, sickle cell anaemia typically accounts for 70% of cases of sickle cell disease, with most of the remainder having haemoglobin SC disease (HbSC disease) owing to the coinheritance of the β^S and β^C alleles (Nagel *et al.*, 2003) The third major type of sickle-cell disease occurs when β^S is inherited with a β -thalassaemia allele, causing HbS/ β -thalassaemia; this is a variable disorder dependent on the type of the β -thalassaemia mutation (Serjeant,2001). Apart from the many different types of HbS/ β -thalassaemia, ten further genotypes that cause sickle-cell disease have been described, although most are rare (David *et al.*, 2010).

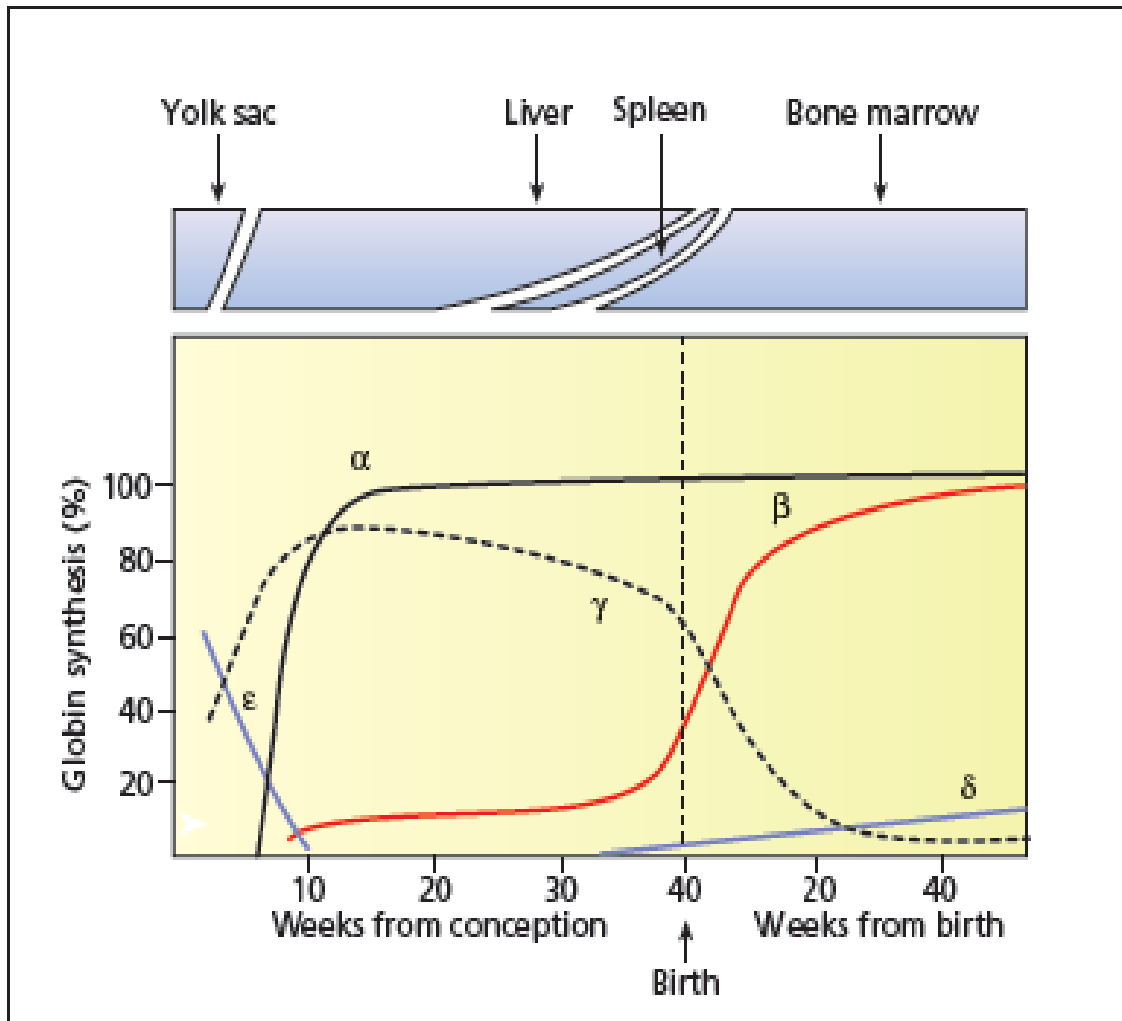


Figure 2.1: Change in erythroid cell type, site of erythropoiesis and globin-chain synthesis during human development (Culled from Wood W.G., 1976).

2.4 PATHOPHYSIOLOGY OF SICKLE CELL ANAEMIA

The anaemia from which sickle cell anaemia is explained as an uncompensated hemolytic anaemia, in which a markedly shortened overall red cell (RBC) survival (increased rate of RBC destruction) is insufficiently balanced by the increase in production (erythropoiesis) to maintain normal levels of total RBCs and hemoglobin concentrations. Although reduced RBC survival is usually considered primary in SS, the central features of HbSS and its secondary effects result in a sub-maximal (below normal) erythropoietic response, thereby contributing to the anaemia. The major dynamics in pathophysiology of SCA include:

- i. Haemolysis
- ii. Vaso-occlusion
- iii. Polymerisation

2.4.1 Hemolysis

The lifespan of a red blood cell is decreased from a normal 120 days to 10–12 days in most patients with SCD. A new haematological equilibrium is formed with haemoglobin levels of 6–9 g/dL and reticulocyte counts of 5–15% (Serjeant, 1997).

According to Robert (1996), the shortened lifespan results from two apparently independent properties of HbSS: (1) the propensity of the concentrated haemoglobin to polymerize, resulting in morphologic sickling, RBC dehydration, and a marked decrease in RBC deformability could lead to ‘despication’ (breaking-off) of sickled RBC’s hence haemolysis; and (2) the instability of the sickled haemoglobin due to its ability to undergo auto-oxidation results in haemolysis. Although these two properties contribute in different ways to the cells’ early demise, resulting in both intravascular and extravascular hemolysis, (Harris, 1956) they may act in concert.

2.4.2 Vaso-occlusion

Impaired perfusion of blood vessels by stiff sickled red cells may lead to ischemia or necrosis of the tissue supplied, the symptoms being determined by the vessels affected.

Basic Pathophysiology of Vaso-occlusion

1. Prolongation of the erythrocyte microvascular transit time caused by:

Red cell adhesion to vascular endothelium as well as hetero-cellular aggregate formation clogs the blood vessels. Additional abnormal cation homeostasis and red cell dehydration promotes the formation of dense cells and irreversible sickled cells, which also obstructs the microvasculature. Also, abnormal vasomotor tone via nitric oxide, endothelin-1 and eicosanoid dysregulation does favour vasoconstriction. These factors promote vaso-occlusion by prolongation of transit time for erythrocytes across the microvascular system.

2. Reduction in delay time to HbS polymer formation caused by:

Under hypoxic condition, red cells are deoxygenated. The deoxygenation increases intracellular sickled haemoglobin (HbS) concentration resulting in the formation of sickle-haemoglobin-polymers (i.e. the cells polymerize). The rapidity of polymerization is seen when the protective haemoglobin (HbF, HbA₂) concentration as well as PH is low. This reduces the delay time for sickle-haemoglobin-polymer formation, thus increasing the obstruction of the vessels (vaso-occlusion) by already formed HbS polymers.

3. Miscellaneous potential modulators

Release of free radicals in addition to sudden restoration of blood flow (reperfusion) may cause injuries to associated tissues, by constriction of blood vessels.

Coagulation activation with the formation of pro-adhesive thrombin also crowds the microvasculature and result in vaso-occlusion.

Central to much of the early pathology of sickle-cell disease is damage to the splenic vasculature by these poorly compliant cells. The spleen acts like a filter, removing damaged red cells and bacteria from the bloodstream. In sickle-cell disease the damaged red cells obstruct this filtration system, rendering children prone to overwhelming infections, especially pneumococcal septicaemia, acute enlargement (acute splenic sequestration), chronic enlargement (hypersplenism), and eventually a progressive splenic fibrosis and atrophy. The risks of pneumococcal septicaemia may be reduced by prophylactic penicillin (Gaston, *et al.*, 1986)

Table 2.1 Clinical Consequences of Haemolysis and Vaso-occlusion in Sickle Cell

Disease

Haemolysis	Vaso-occlusion	Bone pain crises	Others
<ul style="list-style-type: none"> • Megaloblastic erythropoiesis • Aplastic crisis • Clinical jaundice and gallstones 	<ul style="list-style-type: none"> • Splenic manifestations (pneumococcal septicaemia, splenic sequestration, hypersplenism) • Stroke • Sickle retinopathy • Impaired growth • Complications in pregnancy 	<ul style="list-style-type: none"> • Painful crisis • Dactylitis 	<ul style="list-style-type: none"> • Acute chest syndrome • Genitourinary problems • Leg ulcers

Culled from Sickle-cell disease seminar by Graham (1997)

2.4.3 Polymerization of Sickled erythrocytes

Polymerization is the aggregation of sickled erythrocytes resulting from deoxygenation.

This feature of deoxy-HbS has direct deleterious effect on the cells' survival. Its major effects include;

1. A dramatic increase in cell viscosity, which makes the cells much more rigid and therefore much less deformable, and consequently at great risk of being trapped in the microcirculatory beds;
2. Various degrees of morphologic "sickling", that is, formation of pointed projections of polymer bundles that protrude through the membrane's cytoskeleton along with an envelope of the "intrinsic membrane" (Liu *et al.*, 1991) and tend to break off ("despiculation"), resulting in cell fragmentation and mechanical fragility;
3. Membrane permeabilization to cations, which triggers the process of RBC dehydration.

Dehydration of the sickled RBCs, which increases haemoglobin concentration and density (often with the persistent morphologic deformations of ISCs) seem to play an important role in both the shortened RBC survival (Bertles and Milner, 1968) and the anaemia (Serjeant and Serjeant, 1969). There has recently been considerable effort directed toward understanding the mechanisms underlying SS cell dehydration, with the hope of developing therapies aimed at preventing or reversing the dehydration. Therefore, this process is considered in further detail.

Polymerization of Hb S upon deoxygenation causes a nonselective increase in membrane cation permeability to Na^+ , K^+ , Mg^{2+} , and Ca^{2+} (Bookchin, 1987), resulting in net movements of these ions across the cell membrane along their normal gradients. The increase in permeability is relatively small, sustained during the period of sickling and is

fully reversed on re-oxygenation. When sickling is reduced or prevented, as in cells with a high content of Hb F, there is a corresponding change in permeability and dehydration (Bookchin *et al.*, 1991). From this and other evidence, it is generally believed that polymer-induced permeabilisation is the prime trigger of cell dehydration. The nature of the permeability pathway generated by the polymer as well as the degree to which the extent of polymerization correlates with the extent of permeabilisation is still unknown. But mechanisms by which the original ion-gradient dissipation may cause cell shrinkage have been well characterized.

Three main transport pathways have been identified and are designated here according to the main transporters involved:

- i. Ca^{2+} -activated K^+ efflux (Gardos) channel
- ii. The K:C1 co-transport pathway, and
- iii. The Na^+/K^+ pump.

The Ca^{2+} -dependent process is complex and involves the participation of at least six membrane transporters: The polymer-induced permeability allows extracellular Ca^{2+} to leak into the cells down its huge inward gradient, thereby increasing the cytoplasmic ionized calcium concentration ($[\text{Ca}^{2+}]_i$). The red cell cytoplasm has a very low buffering capacity for Ca^{2+} , leading to a small net influx which can produce a significant elevation in ionized calcium concentration. But the powerful ATP-fueled Ca^{2+} -extrusion pump of the cell membrane opposes Ca^{2+} entry. However, the little increase in ionized calcium concentration is just enough to cause a detectable activation of Ca^{2+} -sensitive K^+ channels, as measured by K^+ fluxes or more indirect means (Etzion *et. al*, 1993).

When this K^+ channels are maximally activated *in vitro* by increasing ionized calcium concentration ($[Ca^{2+}]_i$) to saturating levels using ionophores, red cells can dehydrate to the levels of the densest HbSS cells in 15 to 20 minutes. Thus, small but repeated channel activation in the circulation should result in dehydration of HbSS cells to high densities over periods of a few days (Robert *et al.*, 1996).

2.4.3.1 Ca^{2+} -dependent K^+ -channel activation cause cell dehydration

Red cells have very high Cl^- and water permeability, and their distributions across the cell membranes rapidly re-adjust when their electrochemical and osmotic equilibria are perturbed. When the K^+ permeability is increased, the membrane potential becomes more negative, driving Cl^- from the cells, together with K^+ , down their gradients. The net loss of KCl is accompanied by water to preserve water (and osmotic) equilibrium.

Sickled cells were shown to have highly increased calcium levels, (Bookchin *et al.*, 1980) whose extent correlated with cell density (over 200 micromoles per liter cells in the densest SS cells), so they must have been gaining calcium in the circulation. This suggested that Ca^{2+} -mediated dehydration could be important, but the original observations created more questions than answers. The main puzzles concerned findings of cell calcium accumulation in the face of normal-functioning SS Ca^{2+} pumps, and no detectable Ca^{2+} -sensitive K^+ channel activity despite normal function of that channel. Among the possibilities considered were that the high Ca^{2+} might be contained at extremely high levels only in a few dying cells, or that it could be trapped in cytoplasmic vesicles, inaccessible to pumps and channels. Red cells were assumed to be devoid of organelles or structured intracellular compartments, but this proved to be wrong both for normal and SS cells. The increased calcium of sickle cells is reported to be at high concentrations (largely in reversible

precipitates with phosphate) within intracellular endocytic (inside-out) vesicles, whose Ca^{2+} pumps were thus directed inward, making them capable of ATP-dependent Ca^{2+} accumulation (Lew *et al.* 1985). Furthermore, all of the excess SS cell calcium could be removed from the cells when they were permeabilized with calcium ionophores in the presence of extracellular Ca^{2+} chelators. Together, this proved that the high calcium contained in SS cells was compartmentalized, and it explained why the pumps and channels did not "see" it.

Normal red cells have also been shown to contain some endocytic vesicles, but they do not accumulate excess calcium. Therefore, the elevated calcium of the SS cells must serve as a cumulative record of Ca^{2+} -permeabilization episodes in the circulation. In each sickling episode, $[\text{Ca}^{2+}]_i$ is transiently increased, and a "tug of war" occurs between cell membrane pumps extruding Ca^{2+} from the cells and vesicle pumps adding to the vesicular Ca^{2+} . It is clear that dehydration mediated directly by the K^+ -channel occurs only during deoxygenation, while the Ca^{2+} permeability is increased and $[\text{Ca}^{2+}]_i$ remains elevated. In vitro, single sickling pulses with reticulocyte-rich light SS density fractions, and recurrent sickling pulses in discocyte-rich density fractions, generated some dehydrating cells only in the presence of Ca^{2+} (Bookchin *et al.*, 1991). Preliminary results of clinical trials with a K^+ -channel blocker, clotrimazole, indicate reduced shrinkage of sickle cells, a direct demonstration of K^+ -channel involvement in vivo (Brugnara *et al.*, 1996). The extent of in vivo dehydration by this mechanism could not be accurately estimated, but an important side effect of dehydration by KCl loss is cell acidification. This is relevant as it describes the link between Ca^{2+} -driven dehydration with dehydration mediated by K:Cl co-transport.

2.4.3.2 K:Cl co-transport pathway

The KCl co-transporter is active mainly in reticulocytes and young red cells from a variety of mammalian species (Lauf, 1992). In human red cells, it inactivates during cell maturation (Canessa *et al.*, 1997). Expression of this transporter in SS reticulocytes and young cells is very variable (Brugnara *et al.*, 1986). Cells with high K:Cl transporter expression, if maximally stimulated, can dehydrate substantially within 1 or 2 hours. Cell acidification is a powerful stimulus to KCl-mediated transport; cell swelling also stimulates, and cell shrinkage is inhibitory to, this co-transport. In at least some cells, the stimulatory effect of acidification appears to exceed the inhibitory effect of shrinkage, because the cells can reach high density.

Another important regulator of K:Cl activity is the concentration of intracellular Mg^{2+} ($[Mg^{2+}]_i$); elevated $[Mg^{2+}]_i$ is inhibitory (Canessa *et al.*, 1987). $[Mg^{2+}]_i$ oscillates in red cells as they are oxygenated and deoxygenated, because ATP and 2,3-DPG, the main cytoplasmic Mg^{2+} buffers, are bound much more by deoxy-Hb than by oxy-Hb (Flatman, 1980). $[Mg^{2+}]_i$ is therefore increased during deoxygenation, and K:Cl-mediated transport is inhibited, compared with the oxygenated state. Thus, unlike Ca^{2+} -driven dehydration, K:Cl-mediated dehydration should operate preferentially in the oxygenated state, although in both mechanisms dehydration involves a net loss of K:Cl and water with associated cell acidification. K:Cl-driven dehydration requires a stimulated activity of this transporter for the normal pump-leak balance of the cells to be perturbed persistently in favor of net K:Cl transporter leak.

2.4.3.3 The Na⁺/K⁺ pump in Sickle cell anaemia

Sickling-induced permeabilization of Na⁺ and K⁺ produces balanced gradient-dissipation which alone would cause no volume change of the cells; however, the increased intracellular Na⁺/K⁺ concentration ratio stimulates the Na⁺-pump. The pump tends to restore the normal Na⁺/K⁺ gradients, but because it extrudes 3Na⁺ out for each 2K⁺ in, the result is a net loss of NaCl over KCl gain (in red cells, the charge-balancing anion is Cl⁻ owing to its high permeability). The initially balanced dissipation of Na⁺ and K⁺ gradients can therefore produce delayed dehydration (Joiner *et al.*, 1986). Estimates of the contribution of this mechanism to overall SS cell dehydration suggest that it is minor, and also that the cation composition of the dense cells cannot be explained by Na⁺-pump-driven dehydration (Bookchin *et al.*, 1989).

2.5 ERYTHROPOIESIS

Sickle cell anaemia is an "uncompensated" hemolytic anaemia; the extent of increased red cell production is insufficient to balance its increased rate of destruction. Compared with similar levels of anaemia in otherwise normal persons, the erythropoietic response appears to be sub-maximal in SS patients. At least part of this is due to secondary effects of Hb S in the red cells.

The normal response to anaemia by erythroid progenitor cells depends on an increased level of erythropoietin. This in turn depends on tissue sensors of the hypoxia resulting from the anaemia, that is, decreased oxygen delivery to the tissues due to a reduced quantity of circulating Hb. Some of these mechanisms are clearly offset in SS cells, which, for reasons outlined later, have a lower oxygen affinity than that found with comparable levels of anaemia from most other causes.

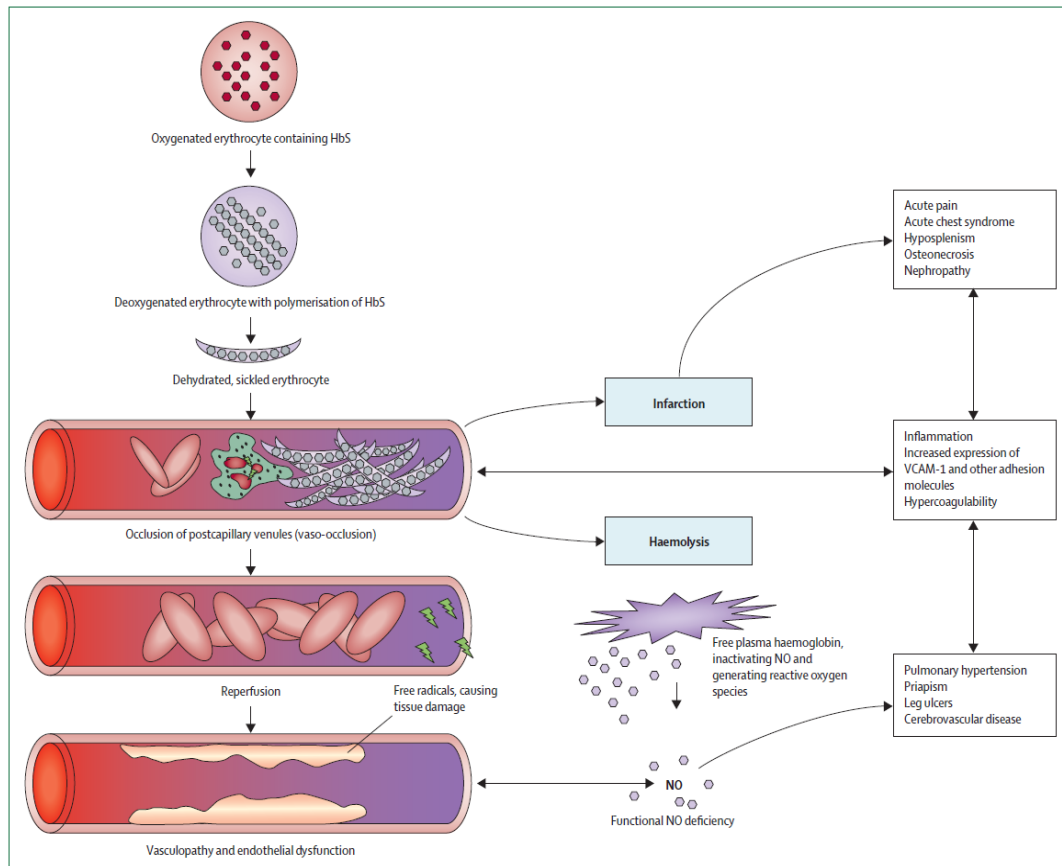


Figure 2.2: Pathophysiology of sickle-cell disease: *Culled from sickle-cell disease seminar by David et al., 2010*

The roles of HbS polymerization, hyperviscosity, vaso-occlusion, haemolysis, and endothelial dysfunction are shown. Deoxygenation causes HbS to polymerise, leading to sickled erythrocytes. Vaso-occlusion results from the interaction of sickled erythrocytes with leucocytes and the vascular endothelium. Vaso-occlusion then leads to infarction, haemolysis, and inflammation; inflammation enhances the expression of adhesion molecules, further increasing the tendency of sickled erythrocytes to adhere to the vascular endothelium and to worsen vaso-occlusion. Reperfusion of the ischaemic tissue generates free radicals and oxidative damage. The damaged erythrocytes release free haemoglobin in to the plasma, which strongly bind to nitric oxide, causing functional nitric oxide deficiency and contributing to the development of vasculopathy. HbS=sickle haemoglobin. NO=nitric oxide. VCAM=vascular cell-adhesion molecule.

2.5.1 Effect of Sickle Cell Disease on Infection

Sickle cell disease has been shown to influence some disease conditions, which further compromises the fragile health condition of sickle cell disease patients by promoting susceptibility to a range of attack by bacteria and other micro organisms, thereby resulting in infections.

a. Impaired splenic function as an effect of sickle cell disease in infections

The spleen has a key role in the increased susceptibility to certain bacterial infections seen in SCD. It functions as a phagocytic filter, removing old and damaged cells and blood borne microorganisms, and also produces antibodies. Some bacteria can be recognized directly by macrophages, but many first require opsonization - coating of the microbial surface by complement components (especially C3b) or other molecules, which in turn interact with receptors on phagocytes (Janeway and Travers, 1997).

The spleen is the site of synthesis of *tuftsin*, an immune-stimulatory peptide, and *properdin*, which participates in complement activation (William and Corazza, 2007). Opsonized bacteria are removed efficiently by macrophages in the spleen or liver, but poorly opsonized bacteria are only cleared effectively by the spleen. Such pathogens include encapsulated bacteria, in particular *Streptococcus pneumoniae* (pneumococcus) and *Haemophilus influenzae*.

Repeated episodes of sickling and ischemic damage with progressive sclerosis of arterioles lead to multiple infarcts of spleen tissue. Unable to regenerate, the spleen becomes scarred and atrophied, culminating in 'autosplenectomy', where the organ shrinks to a small remnant and the individual is rendered *effectively asplenic* (Lucas, 2004). In HbSS this sequence develops from the age of 6 months to 3 years (William and Corazza, 2007).

b. Defects in complement activation as an effect of sickle cell disease in infections

The complement system involves a large number of plasma proteins that are cleaved sequentially by protease enzymes to generate active fragments. These function as opsonins or chemo-attractants, and the terminal components can kill some pathogens directly by creating pores in their membranes. The cascade can be activated either via the classical pathway, following binding of IgM or IgG to surface antigens, or the alternative pathway, in which C3b interacts directly with the pathogen cell surface, then recruiting further downstream components (Janeway and Travers, 1997). Studies have not consistently demonstrated any deficiencies in the amount of complement components, but early work did suggest a reduced functional activity of the alternative pathway, with lower levels of the active form of factor B (the first protein recruited by C3b) and impaired opsonization of yeast *in vitro* (Larcher and Wyke, 1982). In some studies, reduced leukocyte function in SCD, particularly neutrophil killing ability, has been shown to correlate with clinical severity of the disease. However, this has not been a consistent finding.

c. Deficiencies in micronutrients as an effect of sickle cell disease in infections

Zinc is known to be important for immune function, so low levels in SCD have been suggested as a contributory factor in susceptibility to infection. Zinc deficiency is associated with lymphopenia, possibly due to activation of the hypo-thalamo-pituitary-adrenocortical axis, causing chronic glucocorticoid production, which stimulates apoptosis of B and T cells in bone marrow and the thymus (Fraker *et al.*, 2000). It has also been linked with reduced production of interleukin (IL)-2 (a cytokine needed for expansion and maintenance of thymocytes and peripheral T cells), reduced natural killer (NK) cell lytic activity, low thymulin activity, reduced CD4:CD8 ratio, and impaired Th1 cell function

(Prasad *et al.*, 1999). Zinc deficiency may affect 60 - 70% of SCD patients. High protein turnover increases requirements, while hemolysis releases zinc, which is lost via the kidneys as renal tubular damage impairs reabsorption. At the same time poor diet and inadequate intestinal absorption could reduce intake. A study in 21 zinc deficient children suggested that giving supplements increased IL-2 levels, reduced the incidence of bacterial infections and cut hospital admissions ((Prasad *et al.*, 1999). Therefore, if such a simple measure as a mineral supplement could improve quality of life, the issue may warrant further exploration.

2.5.2 The Effect of Infection on Sickle Cell Disease

Vaso-occlusion, initially assumed to be due to passive mechanical blockage by sickled RBCs, is in fact a complex, dynamic process involving active interaction between adhesion molecules on the vascular endothelium (e.g., intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (V-CAM1), and $\alpha_5\beta_3$ integrin) and both RBCs and leukocytes. Red blood cells attach via surface ligands including $\alpha_4\beta_1$ integrin, basal cell adhesion molecule (BCAM), phosphatidylserine, and sulfated glycans (Okpala, 2004). This could contribute directly to occlusion, but also acts via slowing transit of RBCs through the microvasculature.

During infection with any pathogen, changes occur at a cellular level, which predispose to crises. Levels of circulating leukocytes and inflammatory cytokines increase, with elevated expression of adhesion molecules on both the vascular endothelium and leukocytes themselves. This occurs locally in infected tissues and systemically. Neutrophils, basophils, and monocytes attracted to sites of inflammation produce cytotoxic proteins such as proteases, collagenase, and elastase and generate reactive O₂ radicals, which cause

oxidative damage. This promotes further endothelial activation and cell adhesion (Frenette and Atweh, 2007). Adrenaline produced in times of stress can increase the adhesion of *laminin* to RBC BCAM (Stuart and Nigel, 2004).

2.6 Malaria in Sickle cell disease

The sickle-cell gene has become common in Africa because the sickle-cell trait confers some resistance to falciparum malaria during a critical period of early childhood, favouring survival of the host and subsequent transmission of the abnormal haemoglobin gene.

Although a single abnormal gene may protect against malaria, inheritance of two abnormal genes leads to sickle-cell anaemia and confers no such protection, rather, malaria is a major cause of ill-health and death in children with sickle-cell anaemia (Catherine *et al.*, 2010).

The presence of HbS is associated with reduced parasitic invasion of erythrocytes, impaired multiplication, and accelerated clearance of parasites by the spleen, as RBC infection produces intracellular hypoxia, provoking sickling and hence splenic filtration of parasitized cells (Makani *et. al*, 2007). It might be assumed that homozygous SCD would confer greater resistance to malaria, however co-existence of the two is associated with increased mortality and morbidity, and malaria is the most common precipitating cause of crisis in endemic countries (Oniyangi and Omari, 2006). This mainly reflects the general effects of systemic infection, including massive release of inflammatory cytokines. The metabolic activity of parasites within RBCs causes hypoxia, acidosis, and hence sickling (Onwubalili, 1983). Red cells containing schizonts adhere to the capillary endothelium even in normal individuals, causing obstruction. In SCD patients, the deleterious effects of this are magnified. The spleen plays an important role in the control of malaria, removing damaged and parasitized RBCs from the circulation, 'pitting' infected cells (removing

parasites and returning the cells to the circulation intact), and generating specific B and T cell responses. Splenectomized individuals with *P. falciparum* have reduced clearance of parasitized RBCs, but it is unclear whether they suffer more severe malarial symptoms (Engwerda *et al.*, 2005).

2.7 OXIDATIVE STRESS IN SICKLE CELL DISEASE

Under normal physiological conditions, anti-oxidant enzymes and oxygen radical scavengers ensure that basal fluxes of reactive oxygen species (ROS) do not injure the host organism. Major ROS defence mechanisms include enzymatic (superoxide dismutase (SOD), catalase, glutathione peroxidase) and non-enzymatic systems (reduced glutathione (GSH), ubiquinols, uric acid, vitamins C and E, lipoic acid, selenium, riboflavin, zinc, carotenoids), as well as metal-binding proteins (Chan *et al.*, 1999). An oxidatively stressed environment results when the production of ROS overwhelms these endogenous anti-oxidant defence mechanisms (Halliwell and Whiteman, 2004). Oxidative stress can damage specific molecular targets (lipids, proteins, carbohydrates), resulting in cell dysfunction and/or death. Enzymes that participate in ROS production include xanthine oxidase (XO), NADPH oxidase, nitric oxide synthase (NOS), cytochrome P450, cyclo-oxygenase (COX) and lipoxygenase.

2.7.1 Sources of Reactive Oxygen Species (ROS) in Sickle Cell Disease

i. Sickle haemoglobin auto-oxidation

Hebbel *et al.*, (1988) first demonstrated that HbS exhibits an enhanced rate of auto-oxidation compared with HbA (normal Hb) in the presence of oxygen. Hence, HbS has been implicated in the increased levels of superoxide and hydroxyl radical detected in the RBC of SCD patients.

ii. Xanthine oxidase

Several lines of evidence support a role for xanthine oxidase (XO) in SCD, including increased XO activity in aortic endothelium (Aslan *et al.*, 2001) and attenuation of SCD-associated blood cell–endothelial cell adhesion in the venules of allopurinol-treated SCD mice (Kaul *et al.*, 2004). Although vascular endothelial cells can produce XO and its precursor xanthine dehydrogenase (XD) and hypoxia/re-oxygenation is an established stimulus for XD to XO conversion (Parks, *et al.*, 1988), it has been suggested that the increased endothelial cell XO activity that accompanies SCD may also reflect the binding of liver-derived XO (Aslan *et al.*, 2001). The relative importance of soluble circulating XO and endothelial-associated XO to ROS formation in the microvasculature during SCD remains unclear. Because endothelial cells in some tissues (e.g. brain) exhibit little or no XO activity, whereas other tissues (e.g. intestine) exhibit high endothelial XO activity, the importance of this enzyme as a source of ROS in SCD is likely tissue specific (Katherine and Granger, 2007).

iii. NADPH oxidase

Leucocytes, which exist in the blood in higher numbers and produce twice the fluxes of superoxide in SCD, represent potentially important sources of ROS in this disease.

The contributions of leucocyte-derived ROS to the haemolysis associated with infections or Vaso-occlusive crisis and the apparent anti-oxidant effects of hydroxyurea therapy in SCD

patients suggests that, like sickle RBC, leucocytes may serve both as targets of (L-selectin activation) and sources of (lipid peroxidation, haemoglobin oxidation) the oxidative environment in SCD (Jison *et al.*, 2004). NADPH oxidase, the major superoxide-producing enzyme in leucocytes, is a potentially noteworthy source of ROS in SCD. Although phagocytic NADPH oxidase has been eliminated as a contributor to the adhesion of sickled RBC in microvessels (Haynes and Obiako, 2002). Recent evidence implicates endothelial cell-associated NADPH oxidase-derived superoxide in the adhesion of leucocytes and platelets in cerebral venules of SCD mice (Wood *et al.*, 2005).

iv. Other sources of ROS

Homocysteine, mitochondria and other sources may also contribute to enhanced ROS production in SCD. Hyperhomocysteinaemia, which occurs in SCD, appears to enhance the production of ROS by a mechanism that promotes the formation of asymmetric dimethylarginine (ADMA), which competes with L-arginine as the substrate for nitric oxide synthase (NOS). In the absence of L-arginine, NOS becomes 'uncoupled' and preferentially produces superoxide rather than nitric oxide (Forstermann and Munzel, 2006; Dawson *et al.*, 1996). The role of mitochondria in SCD associated oxidative stress has gone unexplored. A large fraction of circulating blood cells in SCD patients is composed of reticulocytes, which possess mitochondria and exhibit evidence of lipid peroxidation. Mitochondrial membrane leakiness, dispersion of the proton motive force and electron transport chain (ETC) uncoupling could underlie a contribution of mitochondria to enhanced production of superoxide in SCD (Katherine and Granger, 2007).

2.7.2 Consequences of Oxidative Stress in SCD

2.7.2.1 Hemolysis

The enhanced oxidative stress experienced by sickle RBC has both autocrine and paracrine effects that begin with lipid peroxidation-mediated senescence and end in lysis of sickle RBC. Sickled RBC shows increased susceptibility to membrane rigidity and mechanical instability as a consequence of ROS generation (Kuypers *et al.*, 1990). Attempts at reproducing the sickle RBC phenotype with exogenously supplied superoxide (phenazine methosulphate) revealed that ROS dose-dependently induce membrane rigidity, reduce RBC elasticity and oxidize membrane-associated heme and thiols (Hebbel *et al.*, 1990).

2.7.2.2 Red blood cell adhesion

Extracellularly generated superoxide has been shown to promote RBC adhesion and a corresponding auto-oxidation of normal haemoglobin, suggesting that the pro-adhesive phenotype of sickle RBC may be related to oxidative stress. An *in vitro* study noted that adhesion of sickle RBC to cultured human umbilical vein endothelial cells (HUVEC) is linked to increased thiobarbituric acid-reactive substances formation, NF- κ B activation & elevated vascular cell adhesion molecule expression in endothelial cells, which were attenuated by treatment with SOD, catalase or the anti-oxidant probucol (Sultana *et al.*, 1998).

2.7.2.3 Leucocyte adhesion

Several groups have reported corresponding increases in superoxide production and numbers of adherent leucocytes in venules of the cremaster and brain of SCD mice. Kaul *et al.*, (2004) noted enhanced leucocyte rolling and adherence in cremasteric venules of unstimulated and hypoxia/re-oxygenation-stimulated SCD mice, which were normalized by treatment with sulfasalazine, an NF- κ B inhibitor with anti-oxidant properties. A recent study of leucocyte adhesion in the cerebral venules of a chimeric SCD mouse, under similar conditions of hypoxia/re-oxygenation stimulation, demonstrated elevated leucocyte

adhesion to venular endothelium that was normalized by genetic deficiency of gp91phox (NADPH oxidase) or over-expression of cytosolic SOD (SOD1) in vascular endothelium (Wood *et al.*, 2005), indicating an important role for NADPH oxidase-derived superoxide and its products in SCD-associated cerebral vasculopathy.

2.7.2.4 Platelet adhesion

The recent observation that SCD is associated with adhesion of platelets in the microcirculation is unsurprising given previous reports describing an activated coagulation pathway (Berney *et al.*, 1992), increased P-selectin and glycoprotein IIb/IIIa expression on circulating platelets (Tomer *et al.*, 2001_a), the presence of platelet/RBC aggregates (Wun *et al.*, 1997) and microthrombi in SCD patients (Martin *et al.*, 1996). We recently reported that platelet adhesion in the cerebral microvasculature of SCD is increased four- to fivefold compared with wild-type counterparts and that this exaggerated platelet adhesion in SCD is independent of the platelet phenotype (wild type or sickle), which implicates another cell type in the genesis of the procoagulant phenotype (Wood *et al.*, 2004). Furthermore, the adhesion of platelets is normalized in chimeric SCD mice with either vascular NADPH oxidase deficiency or genetic over-expression of vascular SOD1 (Wood *et al.*, 2005). Combined with an earlier observation that vascular P-selectin mediates platelet adhesion in SCD mice (Wood *et al.*, 2004), the findings are consistent with a mechanism wherein oxidative stress signals increased expression of P-selectin on endothelial cells, which, in turn, mediates recruitment of both platelets and leucocytes (Katherine and Granger, 2007).

2.8 BONE PROBLEM IN SCD

Bone involvement is the commonest clinical manifestation of sickle cell disease both in the acute setting such as painful vaso-occlusive crises, and as a source of chronic, progressive disability such as avascular necrosis.

Acute bone problems in sickle cell disease: The most frequent complications requiring hospital admissions for patients with sickle cell disease are painful vaso-occlusive crises and osteomyelitis (Neonato *et al.*, 2000). Other acute bony problems that have been described in sickle cell disease are stress fractures (Bahebeck *et al.*, 2002), orbital compression syndrome because of orbital bone infarction (Naran and Fontana, 2001), dental problems (Demirbas *et al.*, 2004), vertebral collapse (Emodi and Okoye, 2001) and bone marrow necrosis (Ataga and Orringer, 2000).

- *Dactylitis:* In children under the age of 7 years, particularly those aged 1–2 years, vaso-occlusive crises frequently occur in the small bone of the hands and feet, still containing haemopoietic bone marrow, at this age in children with SCD (Kim and Miller, 2002). Clinically, Dactylitis presents with acute, painful swelling of one or more of the digits.
- *Osteomyelitis:* The increased susceptibility of sickle cell disease patients to infections including osteomyelitis, has long been recognized with several mechanisms postulated. These mechanisms include hyposplenism, impaired complement activity and the presence of infarcted or necrotic bone. The most common cause of osteomyelitis in sickle cell disease is *Salmonella* (non-typical serotypes - *typhimurium*, *enteritidis*, and *paratyphi B*), followed by *Staphylococcus aureus* and gram-negative enteric bacilli (Atkins *et al.*, 1997; Burnett *et al.*, 1998), perhaps because intravascular sickling of the bowel leads to patchy ischaemic infarction.



Plate 2.2: Hand-foot Syndrome in Patient Aged 14 Months with Homozygous SCD

Culled from clinical review "Management of patients with sickle cell disease" by Sally and Oni, (1997)

- *Acute chest syndrome:* Acute chest syndrome is the second most common cause of hospital admission in patients with sickle-cell disease. It is a form of acute lung injury and is defined as the development of a new alveolar pulmonary infiltrate involving at least one lung segment (Gladwin and Vichinsky, 2008). This syndrome is caused by a combination of infection, fat embolism, and vaso-occlusion of the pulmonary vasculature. Severity varies, but 13% of patients require mechanical ventilation and 3% die (Vichinsky *et al.*, 2000). Treatment involves broad-spectrum antibiotics, bronchodilators, and oxygen. If haemoglobin concentrations decrease substantially or the patient's clinical condition deteriorates, blood transfusion is commonly given. Transfusion corrects anaemia, decreases the percentage of HbS, suppresses HbS synthesis and reduces haemolysis, all of which are of potential benefit.

2.8.1 Causes of the Acute Chest Syndrome

Three major causes of the acute chest syndrome have been proposed: pulmonary infection, embolization of bone marrow fat and intravascular pulmonary sequestration of sickled erythrocytes, resulting in lung injury and infarction.

a. Pulmonary Infection

The most common cause of the acute chest syndrome in children and adults is pulmonary infection by a community-acquired pathogen, which incites an excessive inflammatory response to what should have been a mild upper respiratory infection.

b. Fat Emboli

The second major cause of the acute chest syndrome is the fat emboli syndrome. It is associated with a severe vaso-occlusive pain crisis involving multiple bones, especially the pelvis and femur, which results in infarction and edema of the bone marrow. The bone

marrow undergoes necrosis, and its contents, including fat, cells, and even bony spicules, are released into the bloodstream and travel to the lung, where they cause acute pulmonary hypertension, severe lung inflammation, and hypoxemia(Case Records of the Massachusetts General Hospital, “Case 34-1997” and “Case 52-1983”; Gladwin and Rodgers, 2000)

c. Pulmonary Infarction

Pulmonary infarction or vaso-occlusion, may also contribute to the development of the acute chest syndrome. In a small number of patients, wedgeshaped lung infarction, sometimes followed by central cavitation, develops (Vichinsky *et al.*, 2000; Bellet *et al.*, 1995).

Chronic bone problems in sickle cell disease

Chronic skeletal problems are common in sickle cell disease. Many patients suffer from chronic pain because of avascular necrosis (AVN), vertebral collapse and/or chronic arthritis. In addition, hyperplasia of the bone marrow may cause osteopenia and growth disturbance (Claster and Vichinsky, 2003).

- *Osteonecrosis and Chronic arthritis*: Osteonecrosis or AVN occurs when vaso-occlusion results in the infarction of the articular surfaces and heads of the long bones. A recent cohort study over four decades in 284 patients with sickle cell disease found that osteonecrosis was present in 15% of the cohort (Powars *et al.*, 2002). Symptomatic patients complain of painful, limited motion of the affected joint, occasionally with pain at rest.

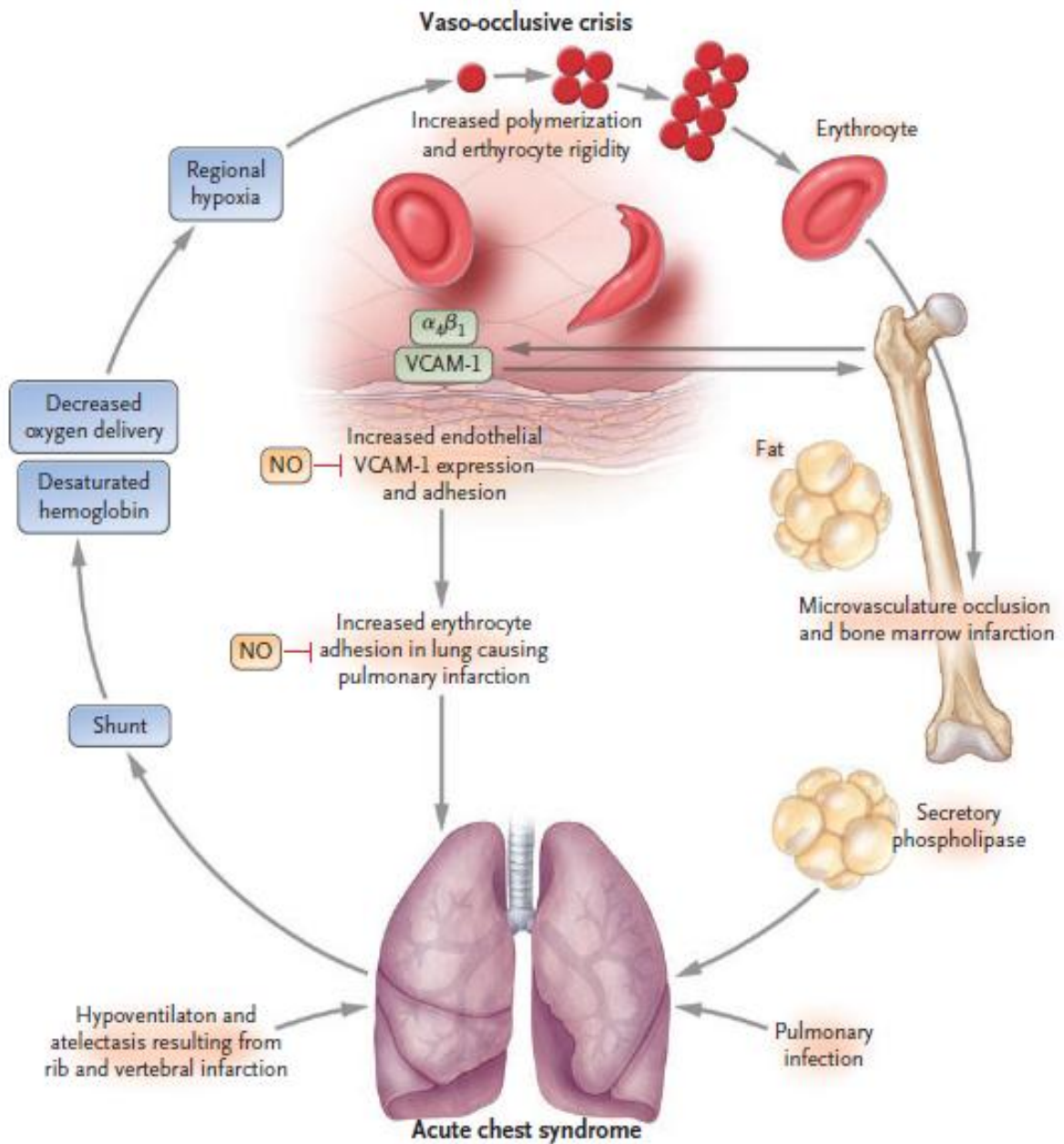


Figure 2.3: The Vicious Cycle of the Acute Chest Syndrome.

The acute chest syndrome is a lung injury syndrome initiated by three major triggers, all related to vaso-occlusion by sickle cells: infection, embolization of bone marrow fat, and intravascular sequestration of red cells, all of which cause lung injury and infarction. Lung injury results in ventilation–perfusion mismatch and hypoxemia, which leads to increased deoxygenation of haemoglobin S, followed by haemoglobin polymerization and erythrocyte vaso-occlusion, which in turn promote bone marrow infarction and pulmonary vaso-occlusion. NO denotes nitric oxide, and VCAM-1 vascular-cell adhesion molecule. Culled from Vichinsky et al., 2000.

- *Osteoporosis and Osteopenia:* Several studies have shown an overall reduction in bone mineral density, attributed to marrow hyperplasia, in patients with sickle cell disease (Brinker *et al.*, 1998 and Nelson *et al.*, 2003). Compared with normal subjects from the general population, Brinker *et al.*, (1998) found that the patients with sickle cell disease had lower bone mineral density values in all scan regions (6–21% lower than expected). Patients may go on to develop vertebral collapse either from the osteoporosis or as a result of vertebral infarction. Vertebral collapse is often asymptomatic but may cause acute and/or long-term pain requiring analgesia and mechanical support such as a brace.

- *Impaired growth:* This is a well recognized complication of sickle cell disease in children (Barden *et al.*, 2002); at least some of this impairment in growth seems to be because of marrow hyperplasia (Claster and Vichinsky, 2003). Marrow hyperplasia can cause ischaemia of the central portion of the vertebral growth plate, leading to disturbance of vertebral growth and resulting in the characteristic ‘H’ shaped vertebrae because of squared-off depression of the vertebral end plates (Williams *et al.*, 2004).

2.9 ROLE OF NUTRITION IN SICKLE CELL DISEASE

Since HbSS was first described in 1910, effort has been made to develop clinical care to lessen the severe clinical problems, mainly frequent hospital admissions for recurrent painful episodes. However, only since the late 1980s has nutrition been identified as a critical feature of sickle cell disease (Al-Saqladi *et al.*, 2008).

2.9.1 Macronutrient Deficiencies in Sickle cell disease

The first and most direct evidence of insufficient macronutrient intake, demonstrated by clinical improvement following dietary intervention, was reported by Heyman *et al.*,

(1985), via a small diet supplementation trial, in which the researchers studied 5 growth-retarded children with HbSS, each below the fifth percentile for both weight and height. Two of the growth retarded children showed clinical improvement and accelerated growth after naso- gastric supplements of protein and calories, in addition to their regular diets. The results showed that protein and energy supplements could improve clinical status and growth in HbSS children, whereas vitamin and mineral supplements alone did not change clinical status or growth. Though Interpretation of these data was limited because only 5 patients (3 to 16 years) were studied, still, these results demonstrated a role for malnutrition as one of the complications in SCD and a possible benefit of routine food supplements.

More recent reports of feeding high protein and L-arginine supplements to sickle mice (Archer *et al.*, 2008; Dasgupta *et al.*, 2006; and Kaul *et al.*, 2002) and n-3 fatty acids to HbSS men (Tomer *et al.*, 2001_b) have shown significant reductions in inflammation, oxidative stress, red cell density and pain episodes, and improved microvascular function. In particular, the feeding of a high protein diet to weanling Berkeley transgenic sickle mice, improved rate of weight gain and reduced circulating levels of inflammatory proteins, C-reactive protein and interleukin-6 (IL-6), compared with sickle mice fed normal dietary protein, thus improving clinical outcome in this sickle mouse model.

2.9.2 Micronutrient deficiencies in Sickle cell disease

Sickle cell disease has been associated with micronutrient deficiencies, including iron, zinc, copper, folic acid, pyridoxine and vitamin E (Reed *et al.*, 1987). The role of these deficiencies has long been extensively studied, including their involvement in immunity (Prasad *et al.*, 1988; Prasad, 1998) and growth (Zemel, 2002).

Several studies have investigated the role of these micromolecules in the pathogenesis of sickle cell disease, which makes it pertinent to discuss.

Iron: Fairly recent reports from India (Mohanty *et al.*, 2008) and Nigeria (Okeahialam and Obi, 1982), describe low iron stores in the bone marrow of 36%–67% of the patients they studied. In contrast, Vinchinsky *et al.*, (1981) reported that only 16% of their non-transfused patients in the United States showed evidence of iron deficiency. This difference could be attributed to the difference in environment, that is (i.e.) developing versus developed country and one could deduce that lower socioeconomic status in developing countries may be associated with a lower dietary iron intake. Although it is an important component of red cells, excess iron (commonly due to chronic blood transfusion) has been shown to contribute to the generation of free radicals, which lead to lipid peroxidation, severe membrane damage and worsening of hemolysis in HbSS patients (King, *et al.*, 2008; McCullough and Wally, 2007).

Zinc: Many health consequences of zinc deficiency have been reported, including immune dysfunction, abnormal or slowed sexual maturation, abnormal growth pattern, poor wound healing and decreased level and activity of zinc metallo-proteins (Prasad, 2008). Interestingly, virtually all of these complications have been associated with the HbS pathophysiology, (Barden, *et al.*, 2002; Hibbert *et al.*, 2005) and Prasad *et al.*, (1975), first reported that HbSS patients had decreased zinc levels in plasma, erythrocytes and hair associated with increased urinary excretion, compared with controls (Prasad *et al.*, 1975). Findings have point to a combination of hyperzincuria, high protein turnover (due to increased hemolysis) and inadequate dietary intake as contributing to the significantly increased zinc requirement demonstrated in the HbSS patients (Prasad, 2002). There was

also a reported increase in serum testosterone (Prasad *et al.*, 1981) and reduced infections and hospital admissions (Prasad, *et al.*, 1999), following zinc supplementation in adult HbSS patients. Zinc remains a vital mineral in sickle cell disease as it has been reported to decrease oxidative stress and inflammatory cytokines and increase anti-inflammatory proteins concomitantly (Bao, *et al.*, 2008).

Magnesium: Some studies have measured normal circulating levels of magnesium (Akenami, *et al.*, 1999; Oladipo, *et al.*, 2005) while others are reported to be low (Zehtabchi, *et al.*, 2004). Low levels of total Mg in sickle cell erythrocytes have been associated with increased sickling due to propensity for red cell dehydration and hence, increased HbS polymerization (De Franceschi, *et al.*, 2000). It has been demonstrated that the dehydration is due to abnormally high red cell permeability and loss of potassium (K^+) via at least three loosely connected pathways, in which the relative contribution of each is not yet known. One of these pathways, the K-Cl co-transport, is abnormally activated by low cell Mg^{2+} (De Franceschi, *et al.*, 2000). This causes rapid irreversible loss of K^+ and Cl^- ions, with water following osmotically. These inferences were derived from studies in which oral Magnesium supplements were observed to improve several hematological indices in adult HbSS patients, including significant improvement of red cell hydration indicated by reduction in number of dense sickle erythrocytes, absolute reticulocyte count and immature reticulocytes while erythrocyte Mg and K content were significantly increased (De Franceschi, *et al.*, 2000). Zehtabchi *et al.*, (2004) subsequently confirmed these postulates when he reported hypomagnesemia and elevated Ca^{2+}/Mg^{2+} ratios, which might benefit from magnesium supplementation.

Copper: There are reports of increased plasma copper levels in individuals with HbSS (Pellegrini *et al.*, 1995; Akenami, *et al.*, 1999). Prasad *et al.*,(1978) observed decreased plasma copper levels in a patient who was receiving zinc as an anti-sickling agent, albeit with some hematologic consequences (microcytosis, and relative neutropenia) which were easily corrected with copper supplementation. It has been suggested that overdose of metals such as zinc might inhibit copper absorption, due to similar valence and competition for the same binding sites, and possibly accounting for the observed inverse relationship (Underwood, 1977). More recently, the interactive roles of copper and zinc have been found to be based mainly on zinc level (King *et al.*, 2006). Hence, a high zinc intake sustained over weeks is reported to induce intestinal synthesis of metallothionein, a copper-binding protein that traps copper within intestinal cells, blocking its absorption.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipment, Chemicals and Reagents

Equipment

Equipment (and models of equipment) used in this research work includes Incubator (GRANT JB SERIES), Light microscope (OLYMPUS CX 21), Atomic Absorption Spectrophotometer (VARIAN AA240FS), Oven (GALLENKAMP), Spectrophotometer (Jenway 6400), Centrifuge (Labofuge 300).

Chemicals

Sodium metabisulphite and Para-hydroxybenzoic acid used were purchased from Sigma chemical Company, Paderborn - Germany. Other chemicals used were of analytical grade.

Reagents

Hexane, Ethyl acetate, ethanol, acetone, and Methanol (Sigma Aldrich, Germany).

3.1.2 Collection and Identification of Plant Material

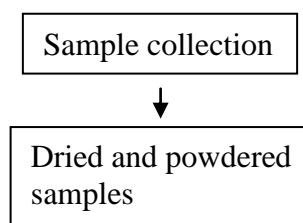
Samples of leaves, seeds and stem of *Telferia occidentalis* were purchased from a Fluted Pumpkin plantation in Kagarko L.G.A of Kaduna state in May, 2011. Plant parts were then taken to the Herbarium unit, Biological Science Department, Ahmadu Bello University Zaria, Nigeria, where the plant was authenticated and identified. Voucher specimen number (3252) was deposited.

3.2 METHODOLOGY

3.2.1 Preparation and Extraction of Plant Material

Leaves, stem and seeds of the plant were collected, shade-dried and powdered using mortar and pestle. The powdered samples were stored in airtight containers and properly labeled. Each of the dried powdered material (500 g) was extracted with 2 L of methanol by cold maceration for 7days in large amber bottles with intermittent shaking. At the end of the extraction, the crude methanolic extract was filtered using Whatman filter paper No 42. The filtrate was concentrated by evaporation (using water bath). In another setup, dried powdered materials were soaked in distilled water for three days using the same proportion used for methanol extraction. It was overlaid with chloroform to prevent fermentation. This was followed by evaporation to dryness to obtain aqueous extract. Filtrates obtained in both cases were reconstituted in normal saline and its antisickling property evaluated.

Further fractionation of each extract in column chromatography using silica gel as adsorbent was carried out. Fractions obtained were used to assay for antisickling potency independently.



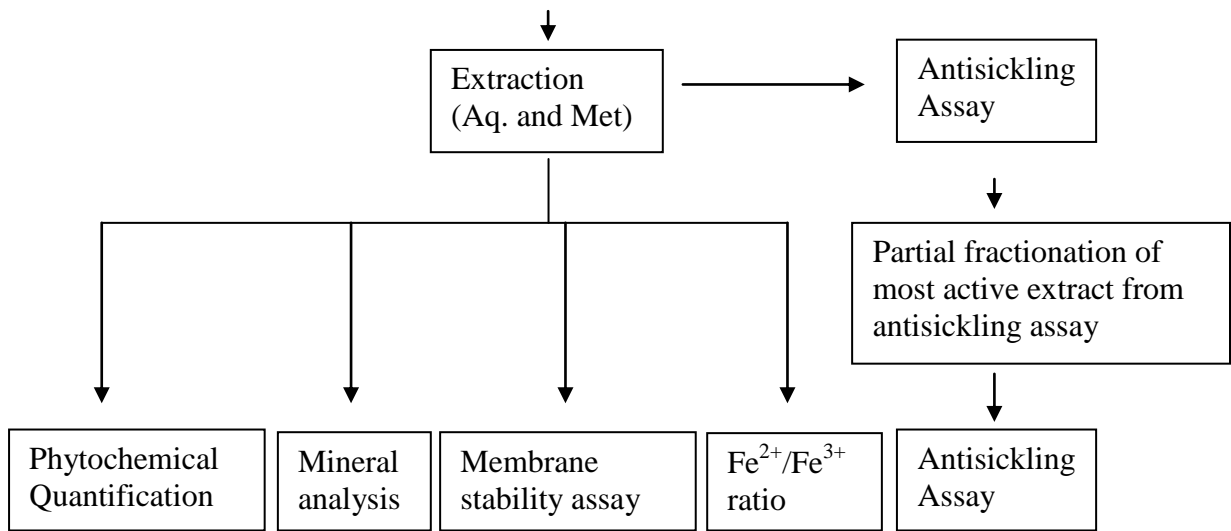


Figure 3.1: Experimental Design Showing Different Stages of the Research

3.2.2 Sample size

The minimum sample size in this study is determined by the formula of James *et al.*, (2001)

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

where n = minimum sample size

z = is the value of the normal curve corresponding to 95% confidence interval = 1.96

p = prevalence of sickle cell disease = 2.3% and q = 1- p i.e. 1 - 0.023 = 0.977

d = level of significance or error margin = 5%

$$n = 1.96^2 \times 0.023 \times 0.977 / 0.05^2 = 0.088 / 0.0025 = 35.2$$

Therefore the minimum sample size was approximately 35.

3.2.3 Blood Collection and Preparation

Five milliliters of blood was obtained by venipuncture from a sickle cell disease patient (HbSS), not in crisis. The blood was placed in sodium EDTA bottles and thoroughly mixed by gently rolling the bottle. The blood sample was then centrifuged to remove plasma and the packed erythrocytes obtained. The resulting packed erythrocytes were washed 3 times with same normal saline quantity of decanted plasma. The samples were then centrifuged each time for 5 min at a speed of 626 (xg) to remove the supernatant (Egunyomi *et al.*, 2009). The washed packed cells were used for the antisickling assay.

3.2.4 Bioassay of Plant Extracts for Antisickling Activity

The bioassay of both methanolic and aqueous extracts of the plant materials for antisickling activity were carried out by measure of level of reversal of sickled RBC's.

3.2.4.1 Evaluation of antisickling activities (Reversal of Sickling)

Evaluation of different plant extracts for sickling reversal activity was carried out according to the procedure of Oduola *et al.*, (2006).

Principle: Washed erythrocytes were mixed with 2% sodium metabisulphite. Sodium metabisulphite reduces oxygen tension thereby inducing the typical sickle-shape of red blood cells. This then followed by introduction of test extract in the test tube. The degree of reversal of sickling were measured by counting the number of cells unsickled under the light microscope.

Procedure: Half a milliliter of the washed erythrocytes were mixed with 0.5 ml of freshly prepared 2% sodium metabisulphite in a clean test tube and incubated in water-bath at 37°C for 30 minutes. A drop of the mixture was then viewed under the microscope. Equal volumes each of normal saline and the extracts were added to the blood- metabisulphite mixture in different test tube and incubated at 37°C for another 30 minutes. Aliquots were taken at 30 minutes interval for up to 2 hours.

The procedure described by Egunyomi *et al.*, (2009) was used for smear preparation and counting of sickled and unsickled cells. In this, each sample was smeared on microscope slide, fixed with 95% methanol, dried and stained with giemsa stain. It was then examined under an oil immersion microscope and counting red blood cells was carried out by viewing from different fields (4 Fields) across the slide. The number of both sickled and unsickled red blood cells were counted and the percentage of cells unsickled determined.

$$\text{Percentage of Cells Unsickled} = \frac{\text{Number of cells unsickled}}{\text{Total number of sickled cells}} \times 100$$

Antisickling Experimental Control

Two types of controls were employed in this bioassay. A positive control using p-hydroxybenzoic acid at 5 mg/ml and normal saline as negative control.

3.2.5 Assay of Membrane Stabilizing Activity

The membrane stabilizing assay method was based on the procedure described by Falade *et al.*, (2005).

The assay mixture consisted of 2ml of 0.25% (w/v) sodium chloride, 1.0 ml of 0.15M sodium phosphate buffer (pH 7.4), 0.75 ml of various concentrations (0.5, 1.0, 1.5, 2.0, 2.5 mg/ml) of plant extracts and 0.5 ml of (2% v/v) erythrocyte suspension. The control was prepared as above, but without drug or extract (blood control), while the drug control was without extract. The Standard drugs used were Indomethacin and Ibuprofen. The reaction mixtures were incubated at 56⁰C for 30 minutes, cooled under running water and then centrifuged at 3913 x g.

The principle behind this assay is the spectrophotometric measurement of the amount of haemoglobin released (read at 560nm) by sickled erythrocytes, which is dependent on the extent of stabilization of sickled red blood cells' membrane exerted by the test extract.

The percentage membrane stability was estimated thus;

$$\% \text{ Membrane stability} = \frac{100 - (\text{Absorbance drug}_{\text{test}} - \text{Absorbance drug}_{\text{control}}) \times 100}{\text{Absorbance Blood}_{\text{control}}}$$

3.2.6 Determination of the effect of extract on the Fe²⁺/Fe³⁺ ratio in sickle cell blood

The analysis of Fe²⁺/Fe³⁺ (Iron II/Iron III) concentration in sickle cell whole blood (HbSS) was carried out as described by Tietz (1976). The analysis was done within 48 hours of collecting the blood samples. The principle of this determination is based on the fact that haemoglobin and methaemoglobin absorb light at different wavelengths (540nm and 630nm) as their respective peak absorbance.

Control

In a test tube containing 5.0 ml of distilled water, 0.02ml of whole blood was added. Normal saline (0.02ml) was added to the mixture to serve as negative control, while vitamin C (0.2-0.8% w/v) was the positive control. The mixture was allowed to stand for 60 minutes at room temperature, and the absorbance was read at two different wavelength maximum (λ_{\max}), 540 nm and 630nm, using a spectrophotometer.

Test

The effect of each extract on haemoglobin and methaemoglobin concentration was carried out by introducing 0.02ml of the specified concentrations (0.2-0.8% w/v) of each extract into separate test tubes. This was followed by the addition of 5ml of distilled water and 0.02ml of whole blood sample. The mixture was allowed to stand for 60 minutes at room temperature, after which, the absorbance was read at 540 nm and 630nm using a spectrophotometer. The percentage haemoglobin and methaemoglobin was obtained with the formula:

$$\text{Percentage haemoglobin (Fe}^{2+}\text{)} = \frac{(A_{540})^2}{(A_{540})^2 + (A_{630})^2} \times 100$$

where A_{540} and A_{630} are absorbance at λ_{\max} of 540 nm and 630 nm, respectively.

3.2.7 Phytochemical Tests

3.2.7.1 Qualitative Phytochemical Constituents Determination

3.2.7.1.1 Test for Alkaloids

- A) Mayer's Test; 1 ml of the extract was treated with few drops of Mayer's reagent giving rise to a cream or pale yellow precipitate which indicate presence of alkaloids.
- B) Dragendoff's Test; Another 1 ml of extract solution was treated with a few drops of Dragendoff's reagent giving rise to an orange precipitate indicating the presence of Alkaloids (Jaliwala *et al.*, 2011).

3.2.7.1.2 Test for Saponins

Froth test; The extract was dissolved in 3ml of ethanol and mixed with 10ml of distilled water in a test tube. The tube was shaken vigorously and allowed to stand for 30 min. Honey comb froth was observed (Akindakun, 2005).

3.2.7.1.3 Test for glycosides

Glycosides: The extracts were hydrolyzed with HCl and neutralized with NaOH solution. A few drops of Fehling solution A and B were added. Red precipitate indicates the presence of glycosides, (Mohammad *et. al.*, 2011)

Cyanogenic glycoside: Extract (5 g) was mixed with 20 mL of water and heated in water bath with sodium picrate paper. A change from yellow to orange is positive for cyanogenic glycoside (Emeka, 2009).

3.2.7.1.4 Test for Flavonoids

- A) Sodium hydroxide test; To a 2ml of the extract, 5 ml of 10% sodium hydroxide was added, yellow colouration indicates presence of flavonoids.

B) Schinodo's Test; Small amount of magnesium chips were added to the 2ml of the extract solution followed by a few drops of concentrated hydrochloric acid. Appearance of an orange, pink or red colour indicate the presence of flavonoids (Maras, 2011).

3.2.7.1.5 Test for phenols

Ferric Chloride Test: Extracts (2g) were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

3.2.7.1.6 Test for Triterpenes

Lieberman- Burcherd's Test; 2ml of the extract was mixed with 1ml of acetic anhydride followed by addition of 1ml concentrated sulphuric acid down the side of the test tube. A green layer indicate the presence of terpenes, (Akindakun, 2005).

3.2.7.1.7 Test for phlobatannins

Extract (0.5g) was boiled with 1% aqueous hydrochloric acid. Deposition of a red precipitate is an evidence for the presence of phlobatinins (Mohammad, 2011)

3.2.7.2 Quantitative Phytochemical Constituents Determination

3.2.7.2.1 Alkaloid Determination

The alkaloid content of the plant sample was determined according to the method of Harborne, (1998).

Procedure: Five (5) g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 4 hours. This was filtered and the extract concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was then added drop-wise to the extract until precipitation was complete. The whole solution was allowed to settle and the precipitate collected and washed with dilute ammonium hydroxide and then filtered. The residue was the alkaloid, which was allowed to dry and then weighed.

3.2.7.2.2 Tannin Determination

The tannin concentration was estimated according to the method described by Van-Burden and Robinson (1981).

Procedure: Exactly 500 mg of the sample was weighed into a 50 ml plastic bottle. About 50 ml of distilled water was added and shaken for 1 hour in a mechanical shaker. This was then filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtrate was pipetted out into a test tube and mixed with 2 ml of 0.1M FeCl₃ in 0.1N HCl and 0.008M potassium ferrocyanide. The absorbance was then measured at 120nm within 10 minutes.

3.2.7.2.3 Saponin Determination

Saponin concentration was determined using the method of Obadoni and Ochuko (2001)

Procedure: Exactly 20 g of the ground plant sample was transferred into a conical flask and 100 ml of 20% aqueous ethanol added. This was heated over a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60 ml of n-butanol added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples was dried in the oven to a constant weight; the saponin content was calculated as percentage.

3.2.7.2.4 Flavonoids Determination

Flavonoid concentration was determined according to the method of Boham and Kocipai (1974).

Procedure: Exactly 10 g of the plant material was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No 42 (125 mm). The filtrate was later transferred into a crucible, evaporated to dryness over a water bath and weighed to a constant weight.

3.2.7.2.5 Determination of Total Phenolic Contents

The total polyphenol content of the plant extracts was estimated using Folin-Ciocalteu Phenol Reagent according to the method described by Lakshman, (2012).

Procedure: An aliquot of 0.1 ml of 0.5 mg of extract dissolved in 1 ml methanol was transferred into test-tubes and the volumes made up to 0.5 ml with distilled water. Then, 0.25 ml of Folin-Ciocalteu phenol reagent (10% v/v in distilled water) and 1.25 ml of 20% aqueous Sodium Carbonate solution were added, the tubes were vortexed and allowed to stand for 40 minutes before taking absorbance at 725nm against a blank solution containing 0.1 ml of the solvent instead of the extract. The determination was done in triplicate. The standard curve was prepared using 0.1-0.5 mg/ml solutions of Gallic acid in methanol and the total phenolics content was expressed as Gallic Acid Equivalent (GAE) in milligrams per gram of dry matter of sample.

3.2.8 Mineral Element Analysis

Atomic Absorption Spectroscopy was used to determine the quantity of mineral elements in the extract of the powdered plant parts.

3.2.9 Thin Layer Chromatography (TLC)

Commercially prepared TLC aluminum sheets of 20 x 20cm lined with silica gel were used. The plate was cut to size of 5 x 5cm.

The extract was dissolved in 95% methanol and spotted at the bottom of the TLC plate (about 0.5cm from the base) using a micro haematocrit capillary tube. The plate was placed in a chromatographic tank and eluted with a mixture of different solvents, with hexane/ethylacetate giving the best resolution. Hexane / ethyl acetate at different ratios (100% hexane, 9:1 v/v, 8:2 v/v, 7:3 v/v, 6:4 v/v, 5:5 v/v, 4:6 v/v, 3:7v/v, 2:8 v/v, 1:9 v/v, 100% ethyl acetate) were used. Thereafter the plate was removed, air-dried and developed

by spraying with 10% sulphuric acid in methanol. It was then viewed under UV light. Spots where seen, hence adjudged the solvent system of choice.

3.2.10 Partial Purification of Crude Extract (Column Chromatography)

Partial purification of active crude extracts using a modified method of Usha *et. al* (2011) was employed. In this method, the crude extract of *T. occidentalis* was separated using silica gel (as adsorbent, 70-230 mesh) packed in a chromatographic column. The adsorbent was overlaid with cotton wool before and after the application of plant extract. The cotton wool is to prevent direct contact or mixing of extract with the silica gel, to absorb shock (that may cause crack in the loaded adsorbent) when solvent is being poured in to the chromatographic column and to serve as filter for particles that may lurk within the solvent. A slurry of finely powdered silica gel (in hexane) was packed in glass column (50cm) to a height of about 12cm (and diameter of 3cm) and loaded with 10ml of the extract dissolved in methanol and separated by gradient elution with solvent system of choice (100% hexane 9:1 v/v, 8:2 v/v, 7:3 v/v, 6:4 v/v, 5:5 v/v, 4:6 v/v, 3:7v/v, 2:8 v/v, 1:9 v/v, 100% ethyl acetate) at different proportion and in order of their increasing polarity. Fractions of 10ml aliquots were collected into different beakers. The solvent was allowed to evaporate and the content spotted on TLC chromatographic sheet to view the level of resolution on the basis of spots. Fractions that showed same number of spots having the same colour and same retention factor (RF) values were pulled together and used for anti-sickling assay.

3.3 Statistical analysis

Results were presented as mean \pm standard deviation (SD). Within and between the groups, comparisons were performed by analysis of variance (ANOVA) (using SPSS 17.0 computer software package). Significant differences were compared using Duncan Multiple Range Test (DMRT), and a probability level of less than 5% ($p < 0.05$) was considered significant.

CHAPTER FOUR

RESULTS

4.1 *In vitro* Antisickling Effects of Aqueous and Methanolic Extracts of Leaves, Seeds and Stem of *Telferia occidentalis*

Data on *in vitro* studies of the antisickling activity of *Telferia occidentalis* extract carried out on blood samples collected from non-crisis sickle cell patients showed reversal of sickle cells at different rates and at different extract concentrations. The leaf extracts - aqueous and methanolic, at 10mg/ml exhibited the highest unsickling activity of $64.03\% \pm 1.69$ and $57.79\% \pm 2.61$, respectively (Figure 4.1); compared to the positive control (p-hydroxybenzoic acid) which showed activity of up to $72.25\% \pm 1.85$. Aqueous and methanolic crude extracts of the stem demonstrated a dose-dependent steady increase in reverting sickled cells back to normal, with time. The methanolic extract of seed resulted in partial lysis of erythrocytes (Plate 4.1) at the maximum concentration of 10 mg/ml, after 30 minutes of incubation (Figure 4.1). Complete lysis (Plate 4.2) was seen across 60 minutes to 120 minutes, by the effect of 10 mg/ml of methanolic seed extract (Table 4.1). This may imply that at high concentration, and over a long period, seed extract of *Telferia occidentalis* may have a cytotoxic effect.

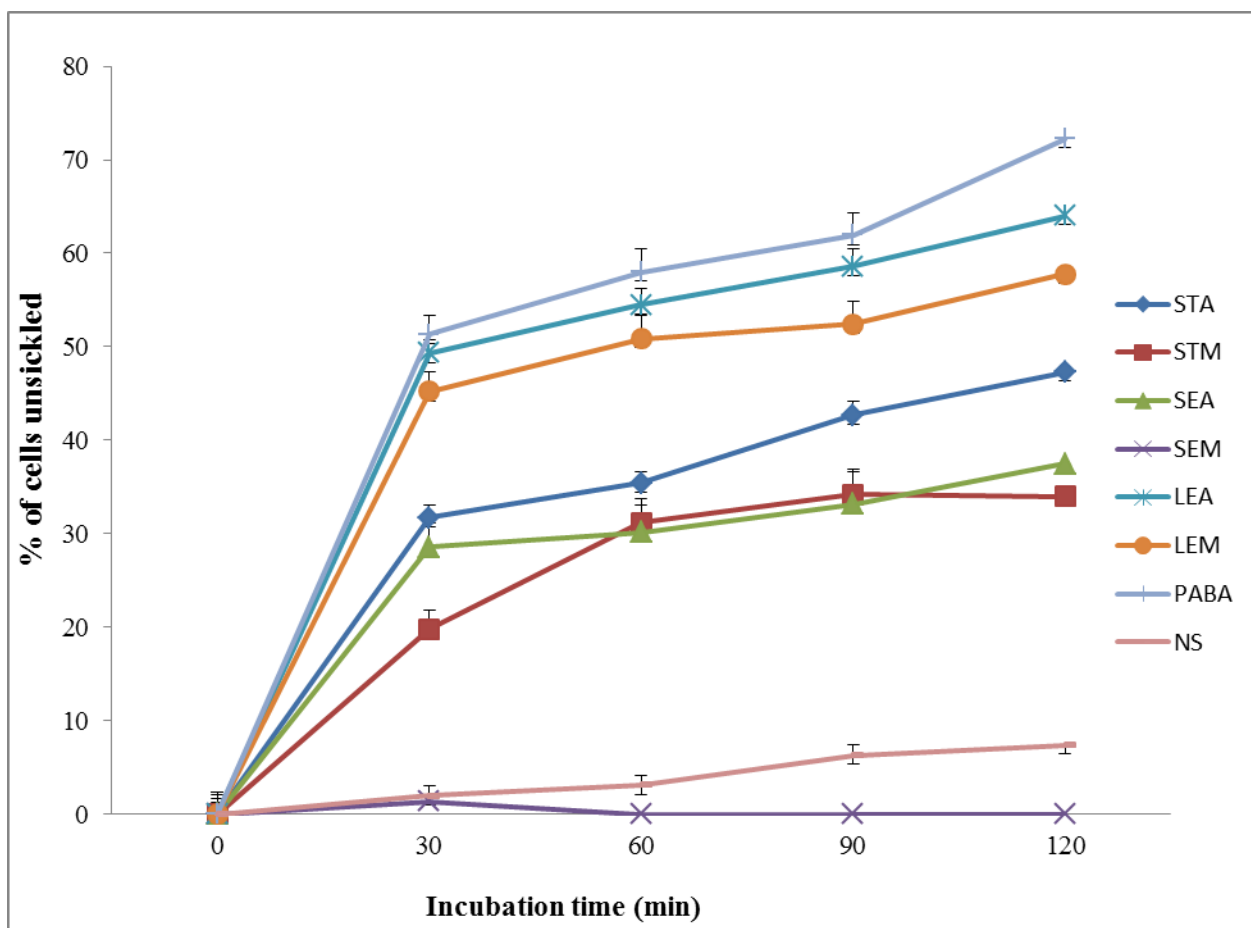


Figure 4.1: Percentage Cells Unsickled with Time by 10mg/ml Extracts of Leaf, Seed and Stem

- LEA = Aqueous Leaves Extract**
- LEM = Methanolic Leaves Extract**
- SEA = Aqueous Seed Extract**
- SEM = Methanolic Seed Extract**
- STA = Aqueous Stem Extract**
- STM = Methanolic Stem Extract**
- NS = Normal Saline**
- PABA = Parahydroxybenzoic acid**

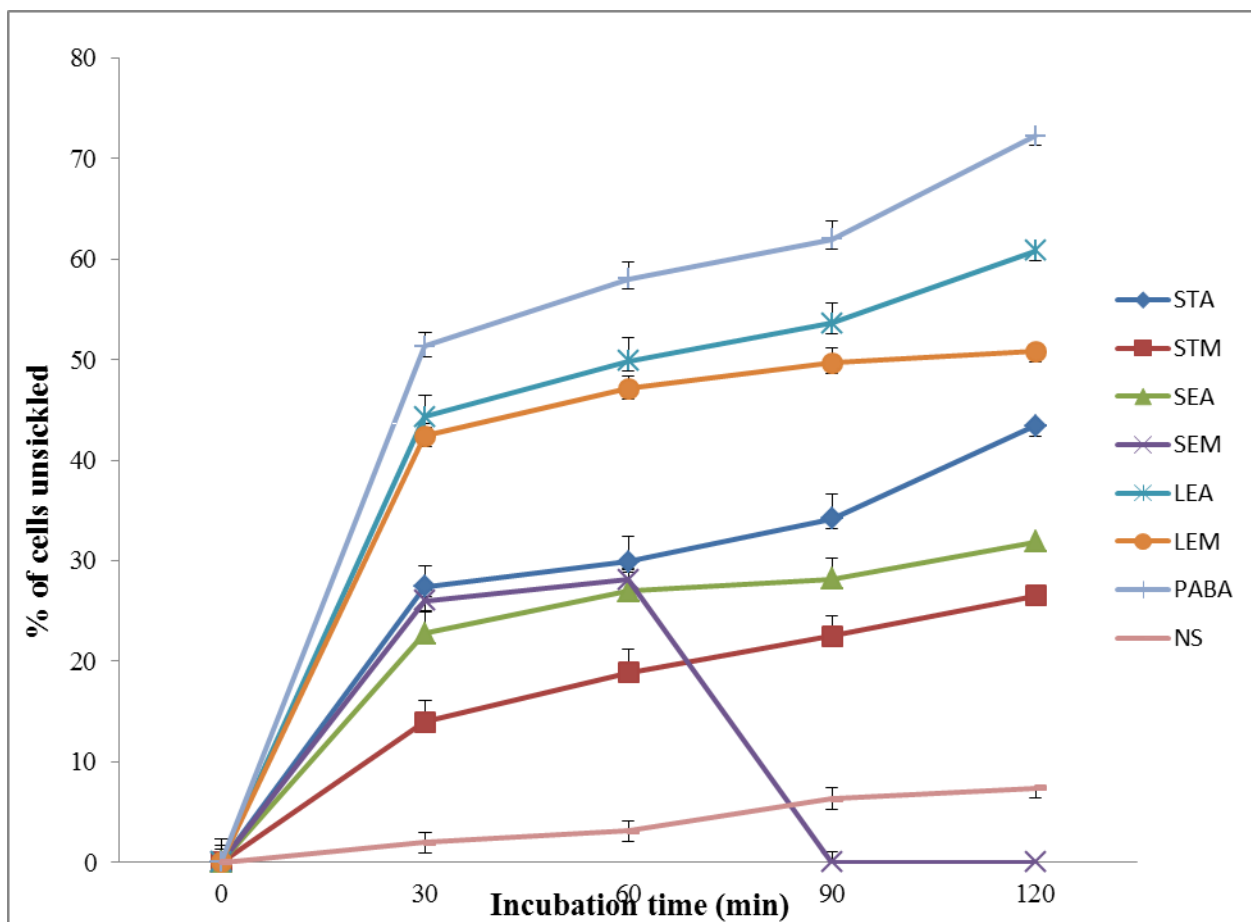


Figure 4.2: Percentage Cells Unsickled with Time by 1mg/ml Extracts of Leaf, Seed and Stem.

- LEA = Aqueous Leaves Extract**
- LEM = Methanolic Leaves Extract**
- SEA = Aqueous Seed Extract**
- SEM = Methanolic Seed Extract**
- STA = Aqueous Stem Extract**
- STM = Methanolic Stem Extract**
- NS = Normal Saline**
- PABA = Parahydroxybenzoic acid**

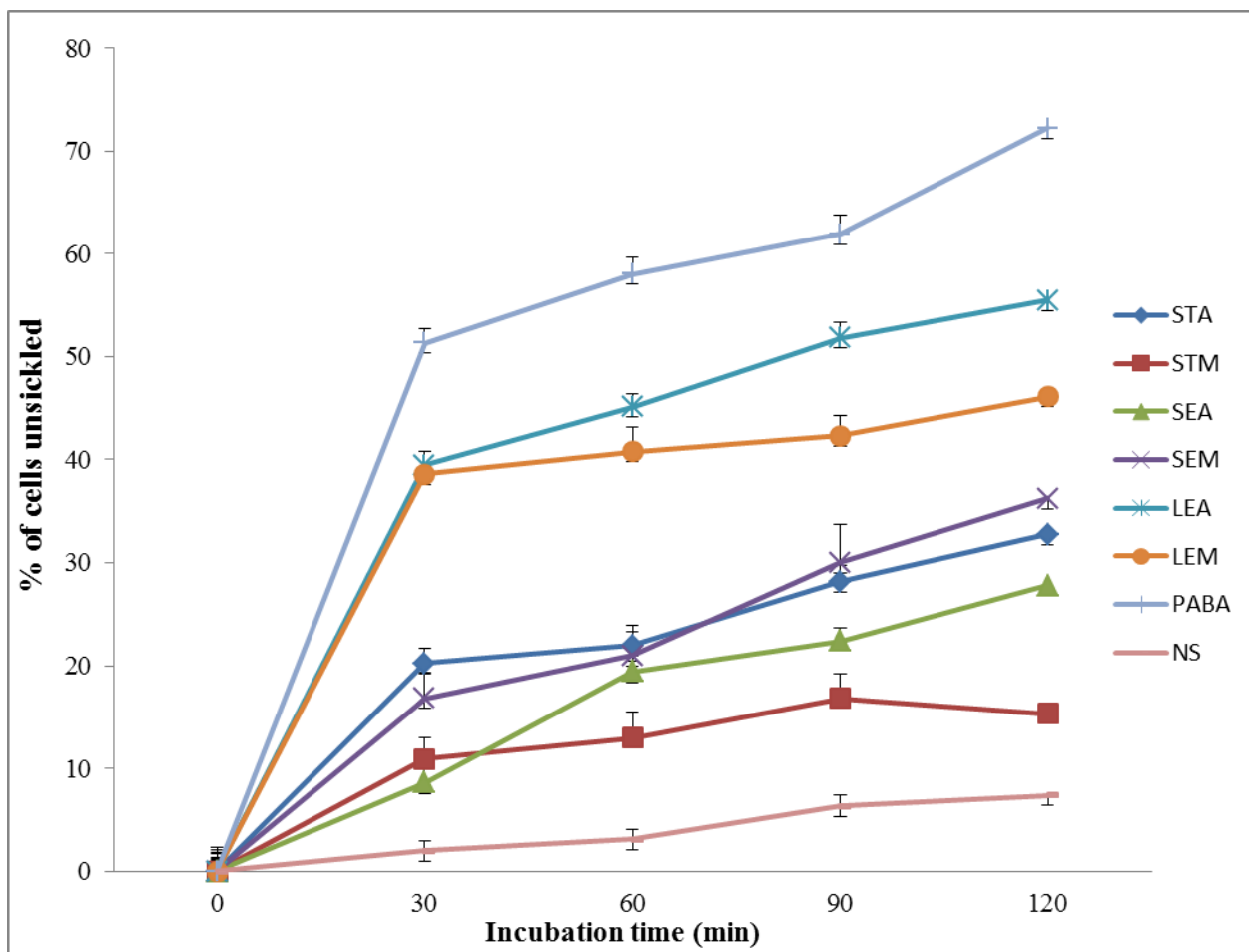


Figure 4.3: Percentage Unsickled with Time by 0.1mg/ml Extracts of Leaf, Seed and Stem.

- LEA = Aqueous Leaves Extract**
- LEM = Methanolic Leaves Extract**
- SEA = Aqueous Seed Extract**
- SEM = Methanolic Seed Extract**
- STA = Aqueous Stem Extract**
- STM = Methanolic Stem Extract**
- NS = Normal Saline**
- PABA = Parahydroxybenzoic acid**

Table 4.1: Peak Antisickling Effect of Crude Aqueous and Methanolic Extracts of *Telferia occidentalis* (Fluted Pumpkin) Leaves, Seeds and Stem.

Crude Extract	% of cells unsickled at 120 min		
	10 mg	1 mg	0.1 mg
LEA	64.03±1.69 ^{c,5}	60.59±1.60 ^{b,5}	55.45±1.46 ^{a,5}
LEM	57.79±2.61 ^{c4}	50.82±1.18 ^{b,4}	46.11±1.72 ^{a,4}
STA	47.33±2.11 ^{b,3}	43.69±3.20 ^{b,3}	32.79±1.80 ^{a,3}
STM	33.98±1.54 ^{c,1}	26.51±1.52 ^{b,1}	15.36±0.41 ^{a,1}
	37.45±1.16 ^{c,2}	31.35±1.20 ^{b,2}	27.48±1.23 ^{a,2}
SEA	*	*	36.23±3.70
SEM			

Values in the same row with different superscripts (a-c) are significantly different at p < 0.05.

Values in the same column with different superscript (1-5) are significantly different at p < 0.05

LEA – Leaves aqueous extract, LEM - Leaves methanolic extract, STA - Stem aqueous extract, STM – Stem methanolic extract, SEA - Seeds aqueous extract, SEM – Seed methanolic extract,.

* - complete lysis of haemoglobin.

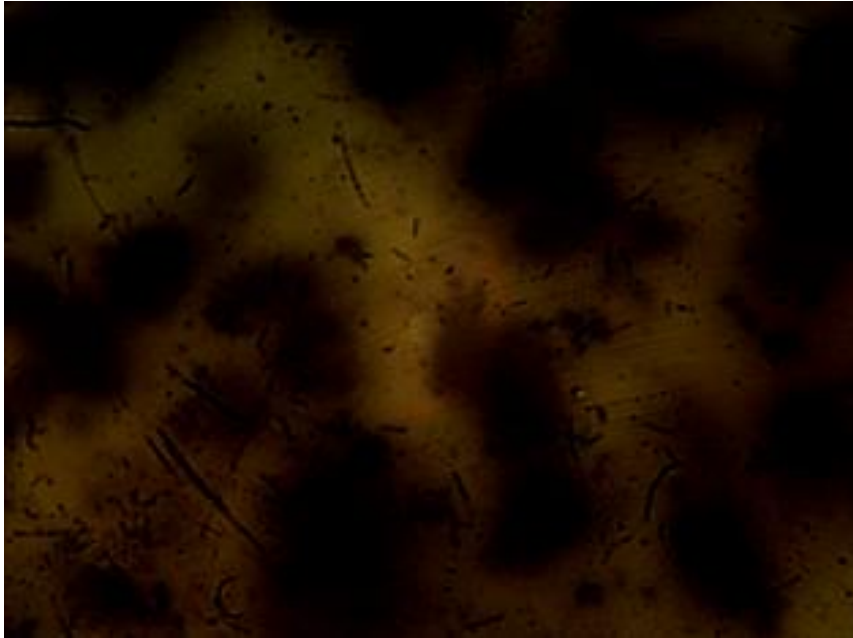


Plate 4.1: Partially Lysed Erythrocytes by the Effect of 10mg/ml (at 30 min)

Methanolic Seed Extract

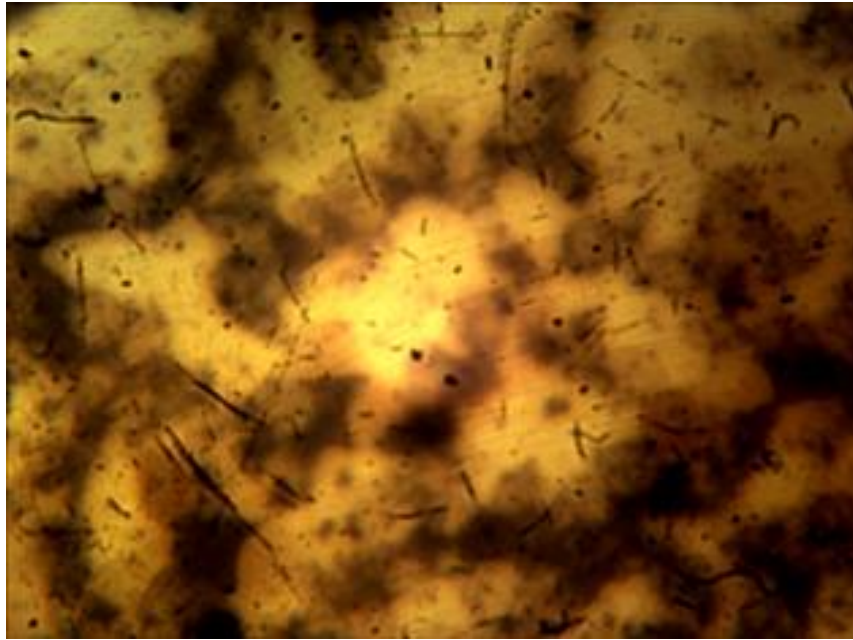


Plate 4.2: Completely Lysed Erythrocytes by Effect of 10mg/ml (> 60 min)

Methanolic Seed Extract

4.2 Membrane Stabilizing Effect of Extracts from the Different Plant Parts

Figures 4.4, 4.5 and 4.6 show the results of membrane stabilizing activity of leaves, seeds and stem extracts of *Telferia occidentalis* as compared with the standard drugs. Both aqueous and methanolic extracts of leaves and seeds of *T. occidentalis* exhibited a maximum inhibition of 27.05% and 32.24% , respectively (Figures 4.4 and 4.5). Figure 4.6 showed that the aqueous and methanolic extracts of the stem exhibited a very high membrane stabilizing activity (61.29% and 71.85%, respectively), which can be compared to the membrane stabilization activity of the standard drugs – Ibuprofen and Indomethacin (62.45% and 81.82%), respectively.

4.3 Effect of extracts on the Fe^{2+}/Fe^{3+} ratio in Sickle Cell Blood

The percentage methaemoglobin concentration of sickled red blood cells in the presence of increasing concentrations (0.2, 0.4, 0.6 and 0.8%) of leaves, seeds and stem extract of *Telferia occidentalis* are presented in figures 4.7, 4.8 and 4.9 respectively. The aqueous seed extract at 0.2% exhibited the lowest capacity to inhibit the build-up of methaemoglobin in sickled erythrocyte, presenting about $11.48 \pm 0.19\%$ methaemoglobin (Figure 4.8). The negative control presented a methaemoglobin concentration of 13.04%. An increase in inhibition of methaemoglobin-generation was generally observed in all plant parts as the concentration of the extract administered was increased. An all high Fe^{2+}/Fe^{3+} ratio (indicating inhibition of methaemoglobin formation and promotion of haemoglobin generation) was demonstrated by methanolic leaves extract (4.88 ± 0.12) at 0.6% (Figure 4.7), as compared to the positive control - Vitamin C (0.2, 0.4, 0.6 and 0.8%) which displayed an Fe^{2+}/Fe^{3+} ratio of 2.64 ± 0.07 .

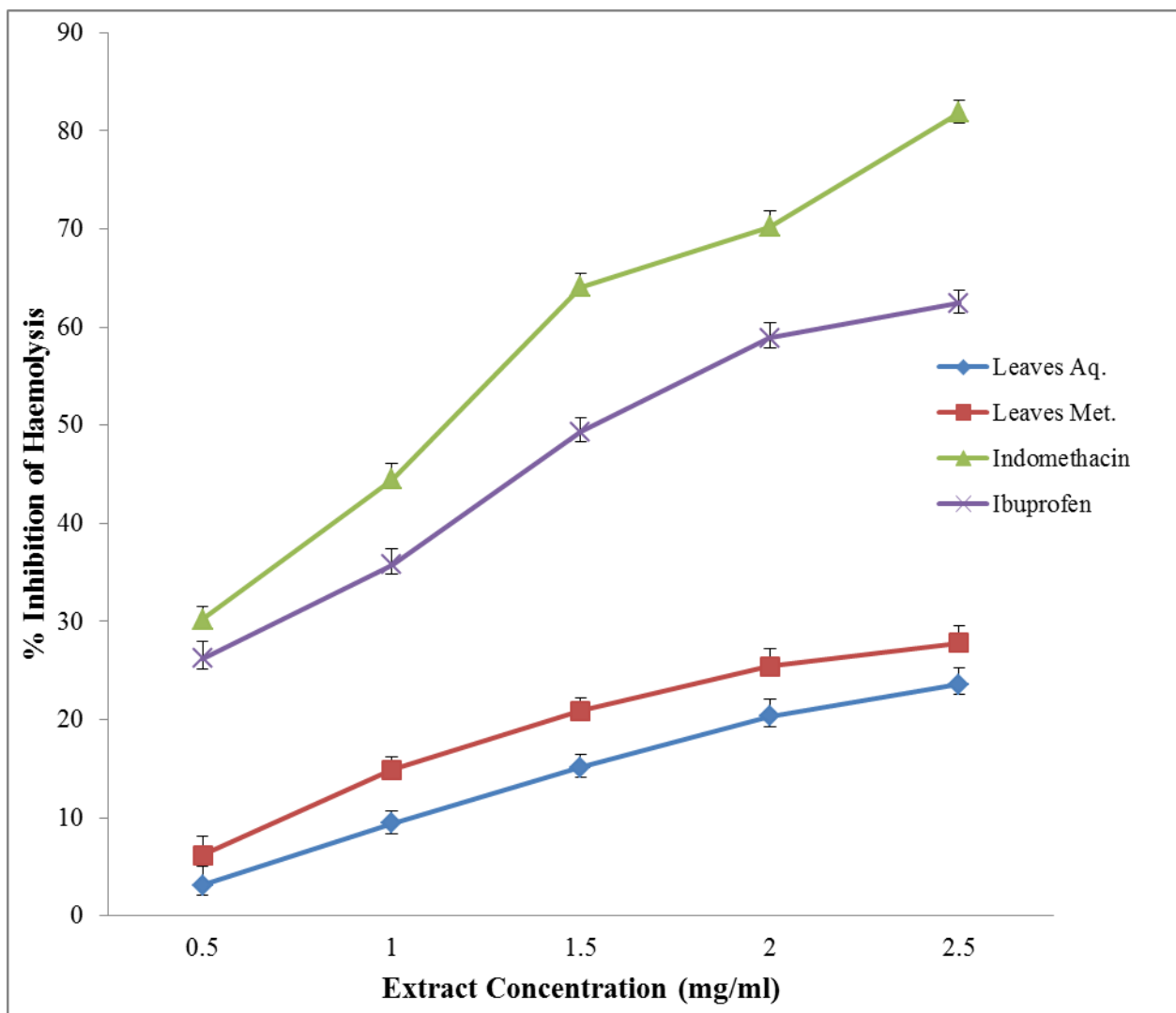


Figure 4.4: Membrane Stabilizing Activity of Aqueous and Methanolic Extracts of Leaves of *Telferia occidentalis*

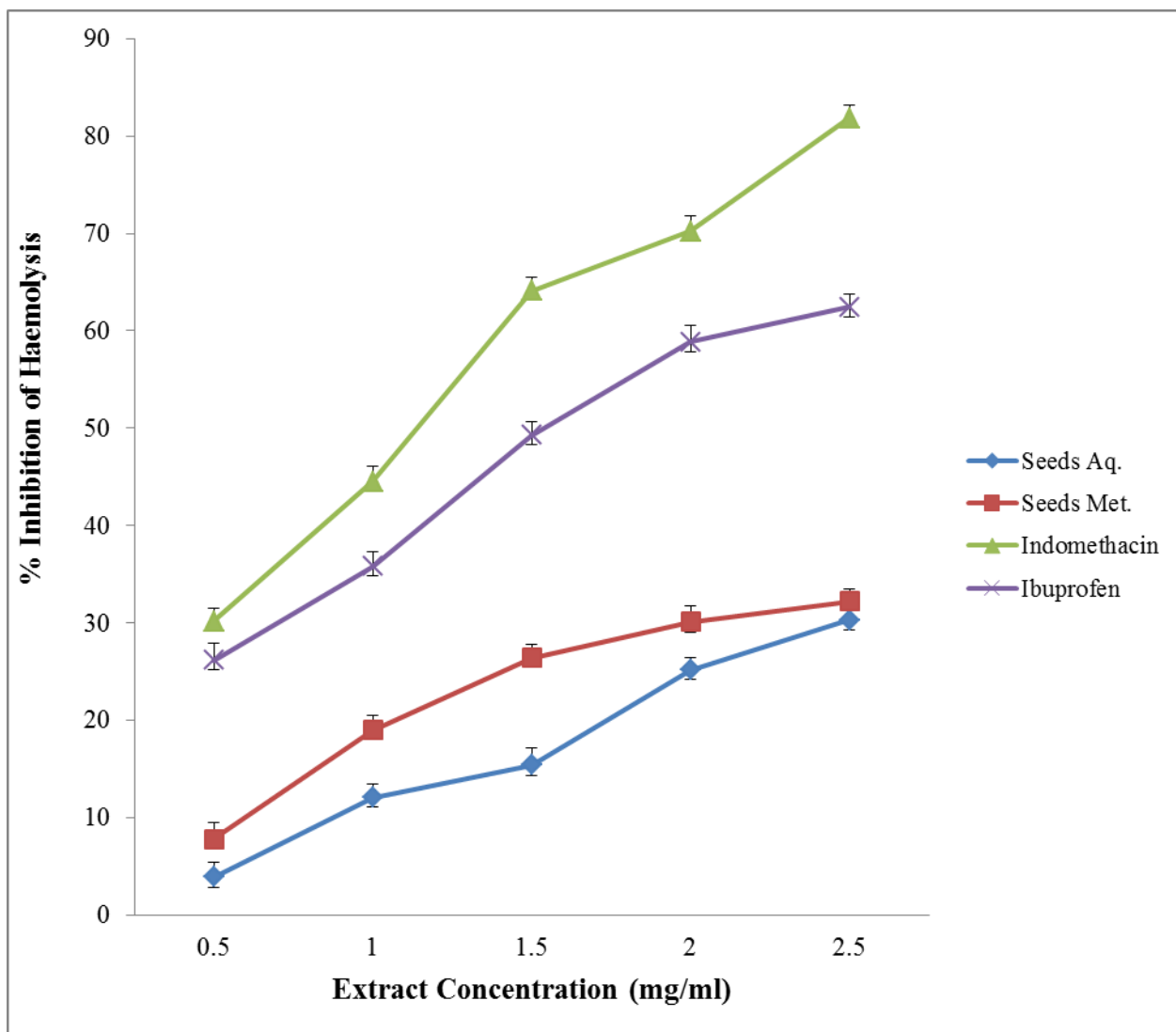


Figure 4.5: Membrane Stabilizing Activity of Aqueous and Methanolic Extracts of Seeds of *Telferia occidentalis*

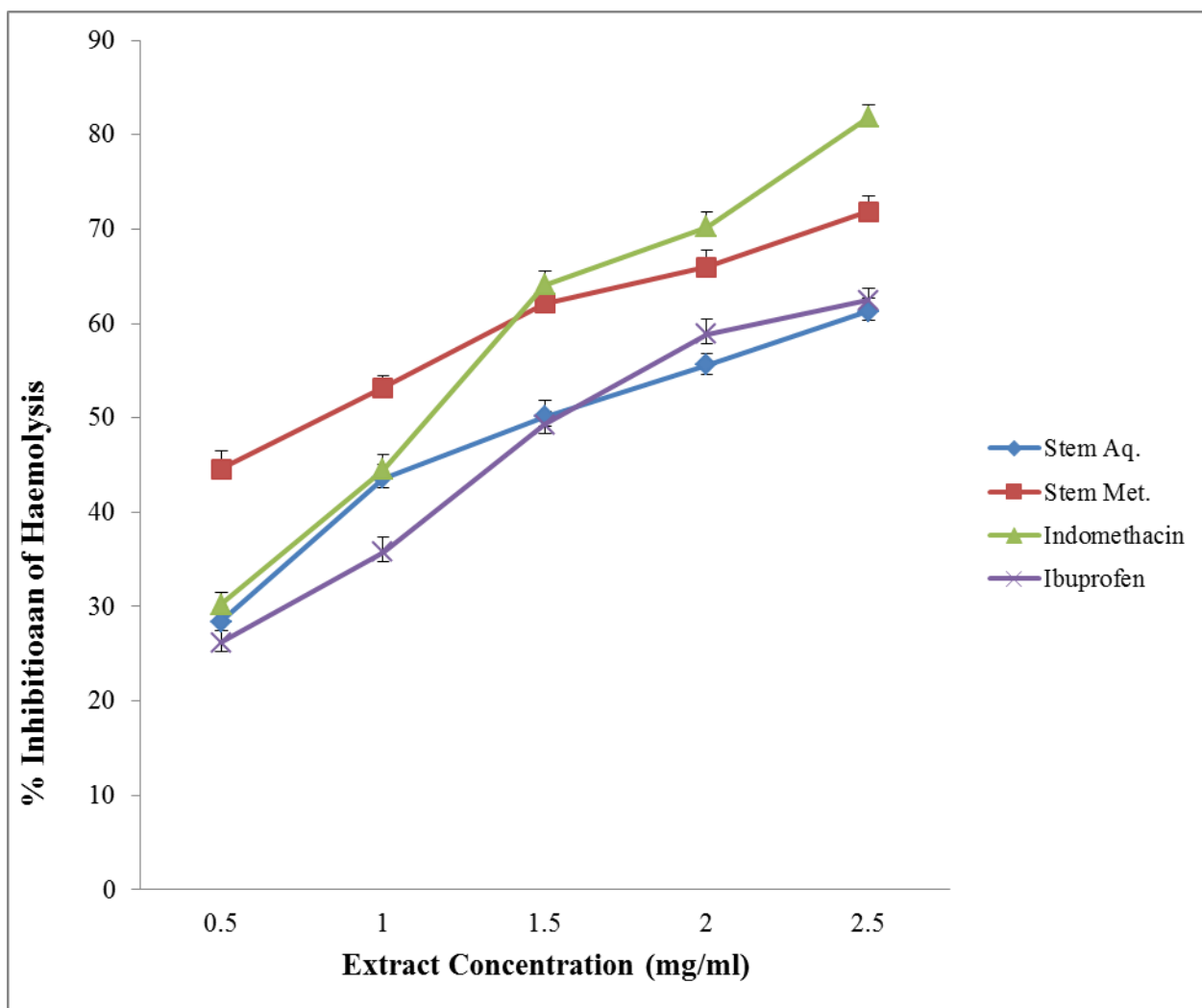


Figure 4.6: Membrane Stabilizing Activity of Aqueous and Methanolic Extracts of Stem of *Telferia occidentalis*

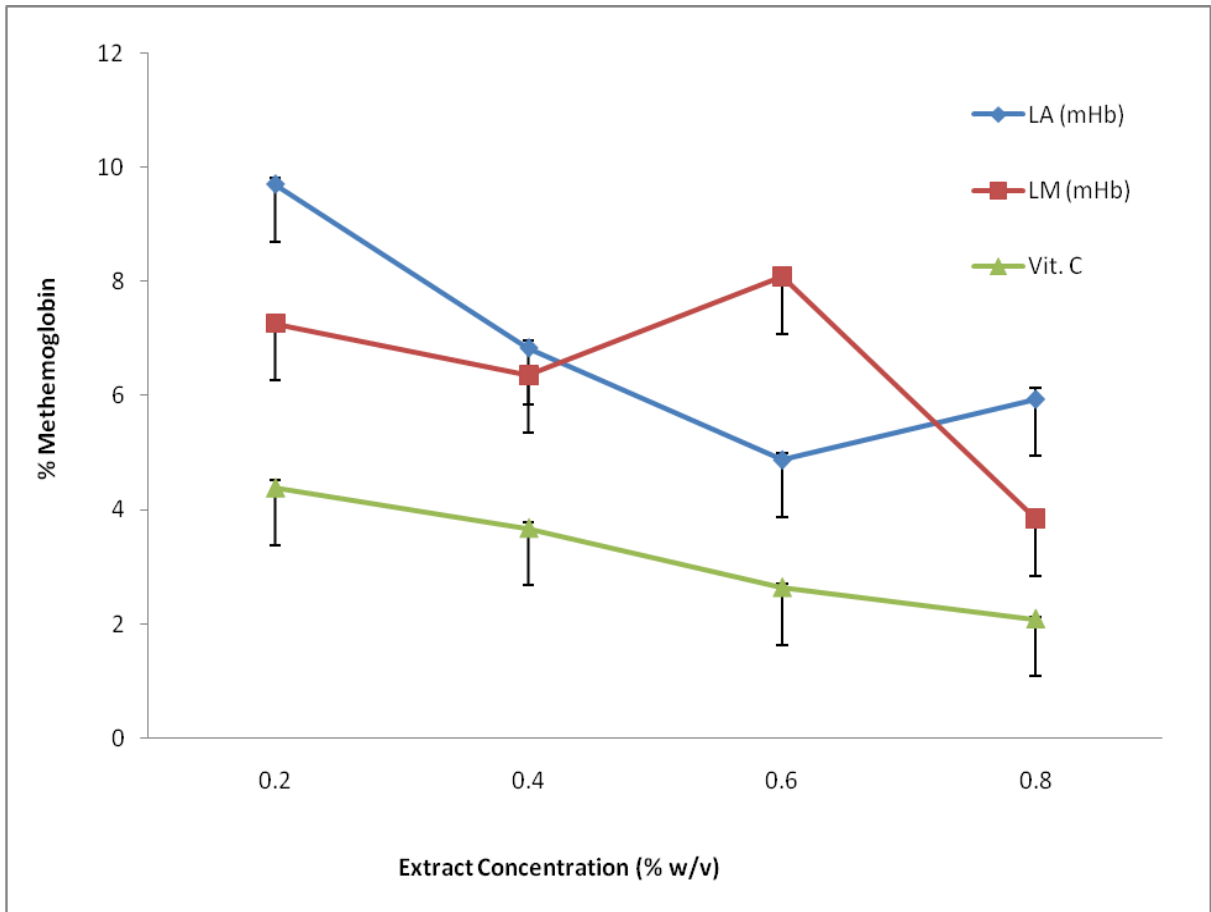


Figure 4.7: Percentage Methaemoglobin in Blood in the Presence of Leaves Extract of *Telferia occidentalis*

LA = Aqueous Leaves Extract

LM = Methanolic Leaves Extract

Vit. C = Vitamin C

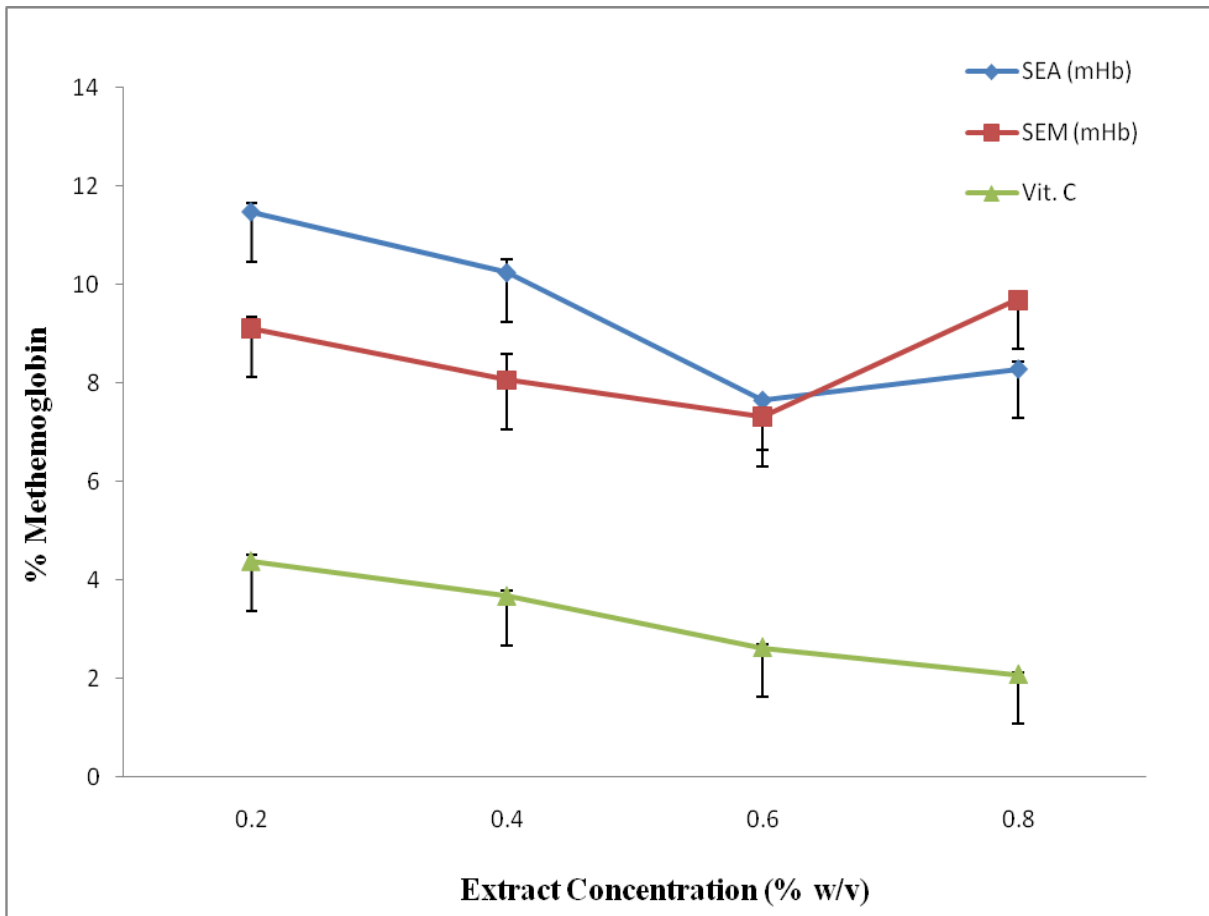


Figure 4.8: Percentage Methaemoglobin in Blood in the Presence of Seed Extract of *Telferia occidentalis*

SEA = Aqueous Seed Extract

SEM = Methanolic Seed Extract

Vit. C = Vitamin C

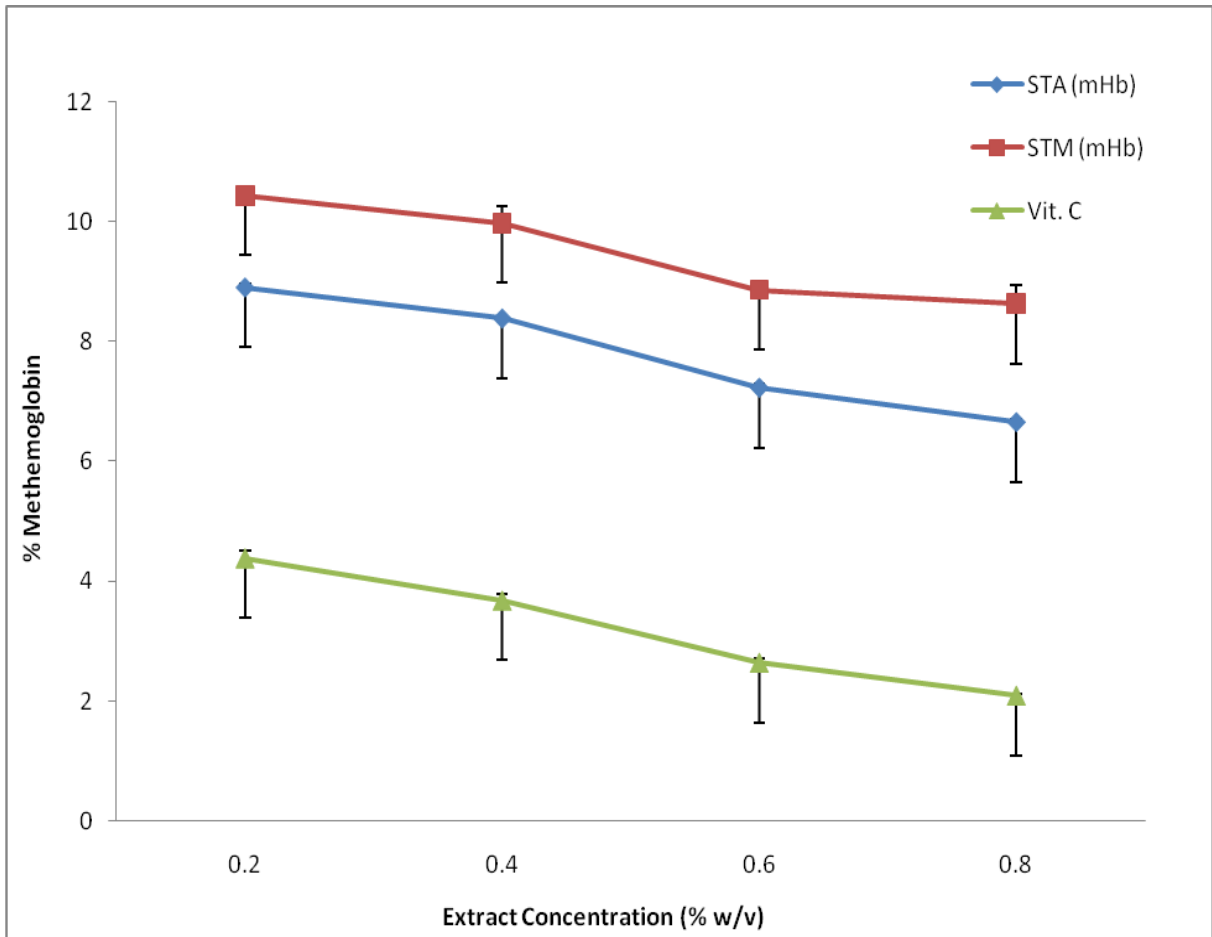


Figure 4.9: Percentage Methaemoglobin in Blood in the Presence of Stem Extract of

Telferia occidentalis

STA = Aqueous Stem Extract

STM = Methanolic Stem Extract

Vit. C = Vitamin C

4.4 Phytochemical Constituent of the Plant Extracts

The Phytochemical screening results of aqueous and Methanolic extracts of *Telferia occidentalis* (Table 4.2) showed that the leaves, stem and seeds contain alkaloids, saponins, cyanogenic glycosides, flavonoids, and total phenolics. The quantitative estimate of the phytochemical constituents as shown in Tables 4.3 and 4.4 indicated that total phenolic content was high in all extracts, followed by the cyanogenic glycosides and saponins.

4.5 Mineral Elements in Leaves, Seeds and Stem Extracts

The quantitative estimates of mineral elements (ppm) in aqueous and methanolic extracts are shown in Tables 4.5 and 4.6. The results of the mineral elements analyses indicated a high amount of Potassium (K) and Magnesium (Mg) in all plant parts. While Iron (Fe), Sodium (Na) and Calcium (Ca) were comparatively low. The zinc content in the leaves was relatively higher, compared to the zinc content in seeds and stem. In all, potassium content was found to be the highest in all extract (Aqueous extract of leaves, stem and seed; 844.81 ± 0.0004 , 507.05 ± 0.0005 , and 426.13 ± 0.0002 ; methanolic extract of leaves, stem and seeds - 555.50 ± 0.0004 , 328.80 ± 0.0001 , 462.56 ± 0.0005 ppm, in that order).

Table 4.2: Phytochemical Constituents of *Telferia occidentalis*

Constituent	Inference
Alkaloids	+
Saponins	+
Cyanogenic glycosides	+
Flavonoids	+
Total phenolics	+
Triterpenes	-
Phlobatannins	-

“+” represents PRESENT, “-” represents ABSENT

Table 4.3: Quantitative Phytochemical Constituents for Aqueous Extracts of *Telfairia occidentalis* (Fluted Pumpkin)

Phytochemical constituents (mg/100g)	Leaves	Stem	Seed
Alkaloids	0.47 ^a ± 0.04	0.40 ^a ± 0.12	0.31 ^a ± 0.07
Saponins	1.73 ^b ± 0.50	0.26 ^a ± 0.03	0.35 ^a ± 0.05
Flavonoids	0.35 ^a ± 0.05	0.59 ^b ± 0.17	0.66 ^b ± 0.14
Cyanogenic glycosides	7.73 ^c ± 0.13	2.39 ^b ± 0.03	1.01 ^a ± 0.01
Total phenolics	69.18 ^c ± 0.38	46.30 ^b ± 0.12	23.09 ^a ± 0.12

Each value represents the mean ± SD from triplicate determinations. Values in the same row with different superscript (a-c) are significantly different at $p < 0.05$

Table 4.4: Quantitative Phytochemical Constituents of Methanolic Extracts of *Telfairia occidentalis* (Fluted Pumpkin).

Phytochemical constituents (mg/100g)	Leaves	Stem	Seed
Alkaloids	0.46 ^a ± 0.14	0.37 ^a ± 0.05	0.41 ^a ± 0.07
Saponins	3.67 ^b ± 0.42	3.37 ^b ± 0.38	0.19 ^a ± 0.06
Flavonoids	0.67 ^a ± 0.21	1.57 ^b ± 0.25	0.52 ^a ± 0.18
Cyanogenic glycosides	2.22 ^c ± 0.61	1.72 ^b ± 0.12	0.72 ^a ± 0.09
Total phenolics	68.67 ^c ± 0.05	46.03 ^b ± 0.48	22.97 ^a ± 0.24

Each value represents the mean ± SD from triplicate determinations. Values in the same row with different superscript (a-c) are significantly different at p < 0.05

Table 4.5: Quantitative Mineral Elements Composition of Aqueous Extract of***Telfairia occidentalis* (Fluted Pumpkin) Leaves, Seeds and Stem.**

Elemental composition (ppm)	Leaves	Stem	Seed
Sodium	17.76 ^a ± 0.0016	25.32 ^b ± 0.0042	13.56 ^a ± 0.0011
Potassium	844.81 ^c ± 0.0004	507.05 ^b ± 0.0005	426.13 ^a ± 0.0002
Zinc	3.32 ^b ± 0.0027	2.88 ^a ± 0.0012	2.69 ^a ± 0.0017
Calcium	12.05 ^b ± 0.0008	27.65 ^c ± 0.0023	3.01 ^a ± 0.0009
Magnesium	138.23 ^a ± 0.0028	138.91 ^a ± 0.0008	212.44 ^b ± 0.0037
Iron	46.58 ^b ± 0.0012	28.37 ^a ± 0.0019	23.97 ^a ± 0.0008

Each value represents the mean ± SD from triplicate determinations. Values in the same row with different superscripts (a-c) are significantly different at $p < 0.05$

Table 4.6: Quantitative Mineral Elements Composition for Methanolic Extract of *Telfairia occidentalis* (Fluted Pumpkin) Leaves, Seeds and Stem.

Elemental composition (ppm)	Leaves	Stem	Seed
Sodium	18.15 ^{ab} ± 0.0073	25.95 ^b ± 0.0017	13.23 ^a ± 0.0024
Potassium	555.50 ^c ± 0.0004	328.80 ^a ± 0.0001	462.56 ^b ± 0.0005
Zinc	2.58 ^b ± 0.0009	2.39 ^b ± 0.0009	0.870 ^a ± 0.0005
Calcium	2.15 ^b ± 0.0002	7.905 ^c ± 0.0002	0.637 ^a ± 0.0003
Magnesium	53.47 ^b ± 0.0005	11.33 ^a ± 0.0002	88.587 ^c ± 0.0032
Iron	34.48 ^c ± 0.0052	22.71 ^a ± 0.0089	26.78 ^b ± 0.0023

Each value represents the mean ± SD from triplicate determinations. Values in the same row with different superscripts (a-c) are significantly different at p < 0.05

4.6 Partial Fractionation of Active Extract with Antisickling Activity

4.6.1 Results of Thin Layer Chromatography (TLC)

Fifty-five (55) fractions were obtained from column chromatography of the most active crude extract (aqueous leaves extract), eluted with different ratios of hexane to ethylacetate. Viewed under the ultra-violet (UV) light showed spots, each of which travelled certain distances (4.08, 4.64, 4.18, 4.20, 4.55 and 3.87). The distance travelled by solvent (solvent front) was also noted (4.90, 5.61, 5.57, 5.51).

Plate 4.3 represents the TLC of the flow through (fractions 1 – 3). Void volume is the early fractions collected from the column (basically the solvent in which the silica gel was suspended), prior to elution of purified extract fractions. TLC of the void volume showed no observable movement of solute (spot) on the chromatographic sheet.

View of fractions 4 – 20 (plate 4.4) showed the presence of single compounds (spots) that travelled the same distance on the chromatographic sheet. Single spots were seen, up to the 30th fraction (plate 4.5). Retention Factor (RF) value was calculated to be 0.83. They all showed light orange colour under UV light. Fractions 31 and 32 (plate 4.5) showed two spots each, with the first spot corresponding with the spots of initial fractions (RF = 0.83, orange colour), while the second spot was light green with RF value of 0.75. Fractions 33 – 40 (plates 4.5 and 4.6) showed single spots with RF values of 0.75 (light yellow colour). Fractions 41 – 55 (plate 4.6) presented double spots of light green and pale yellow with respective RF values of 0.83 and 0.70 (Table 4.7). Fractions with the same RF values, same number of spots and same colour under UV light were pooled together and used to test for antisickling activity.

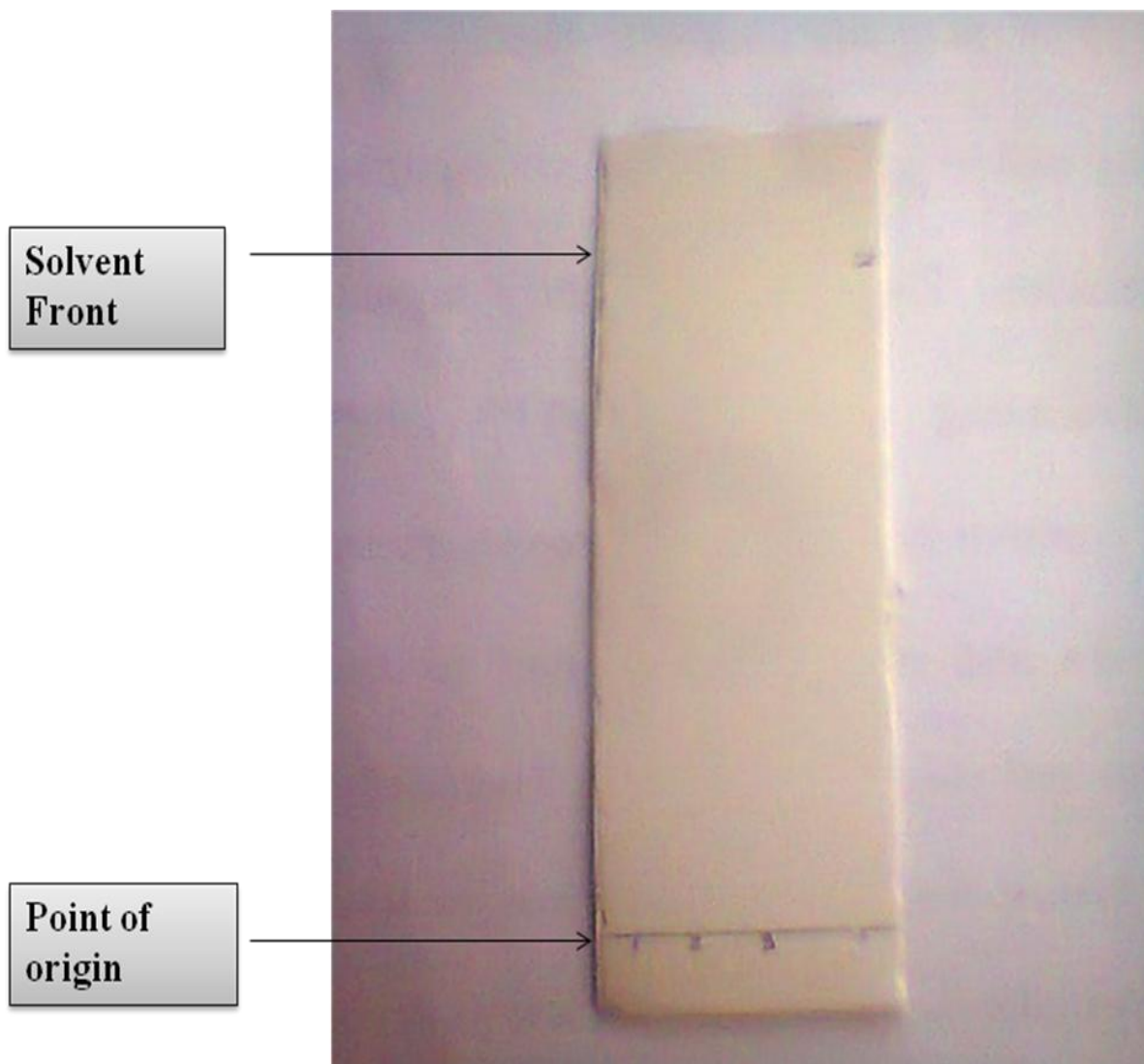


Plate 4.3: Thin layer Chromatogram of Fractions 1, 2 and 3 Obtained From Column Chromatography of Aqueous Crude Leaves Extract (Flow through)

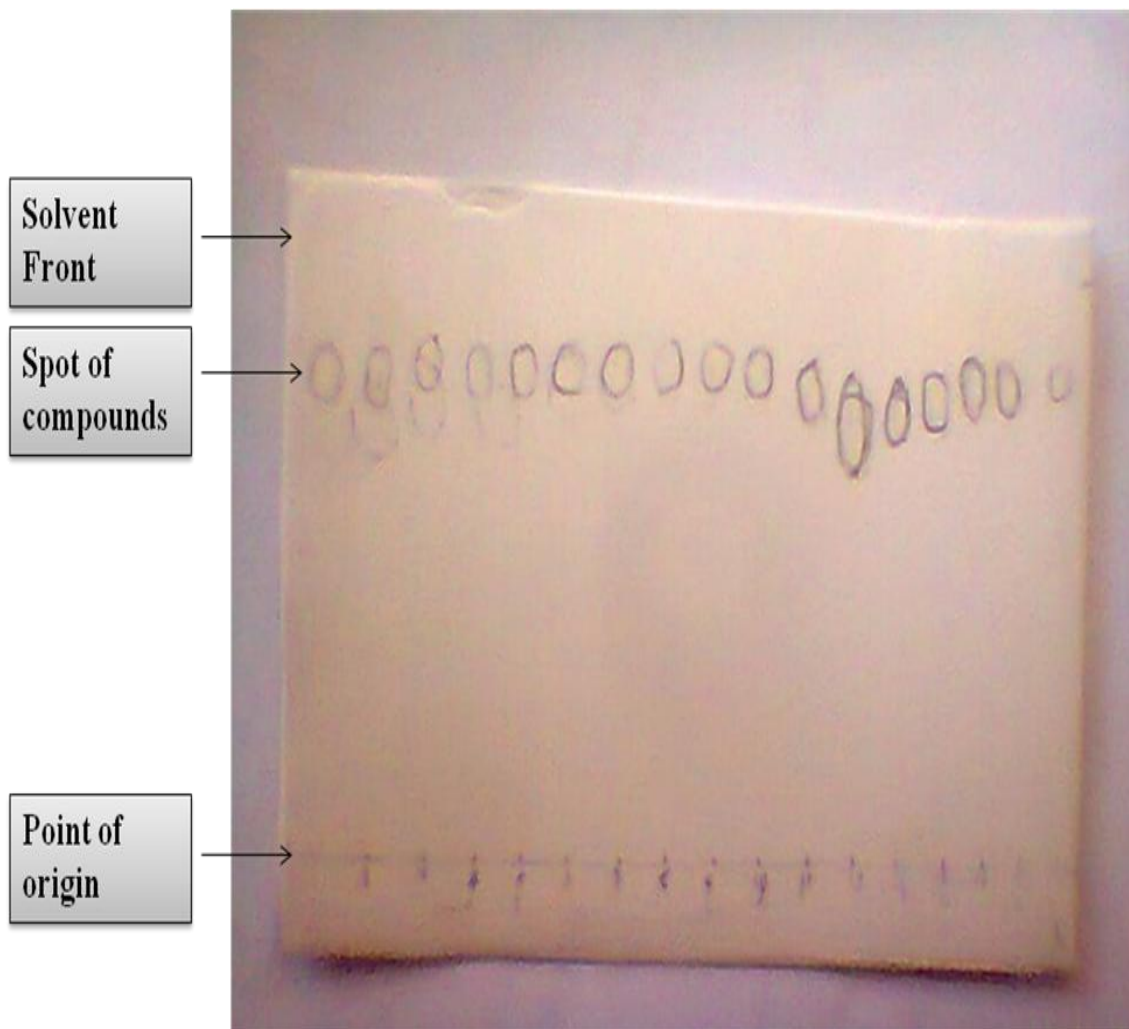


Plate 4.4: Thin layer Chromatogram of Fractions 4 - 20 Obtained from Column Chromatography of Aqueous Crude Leaves Extract

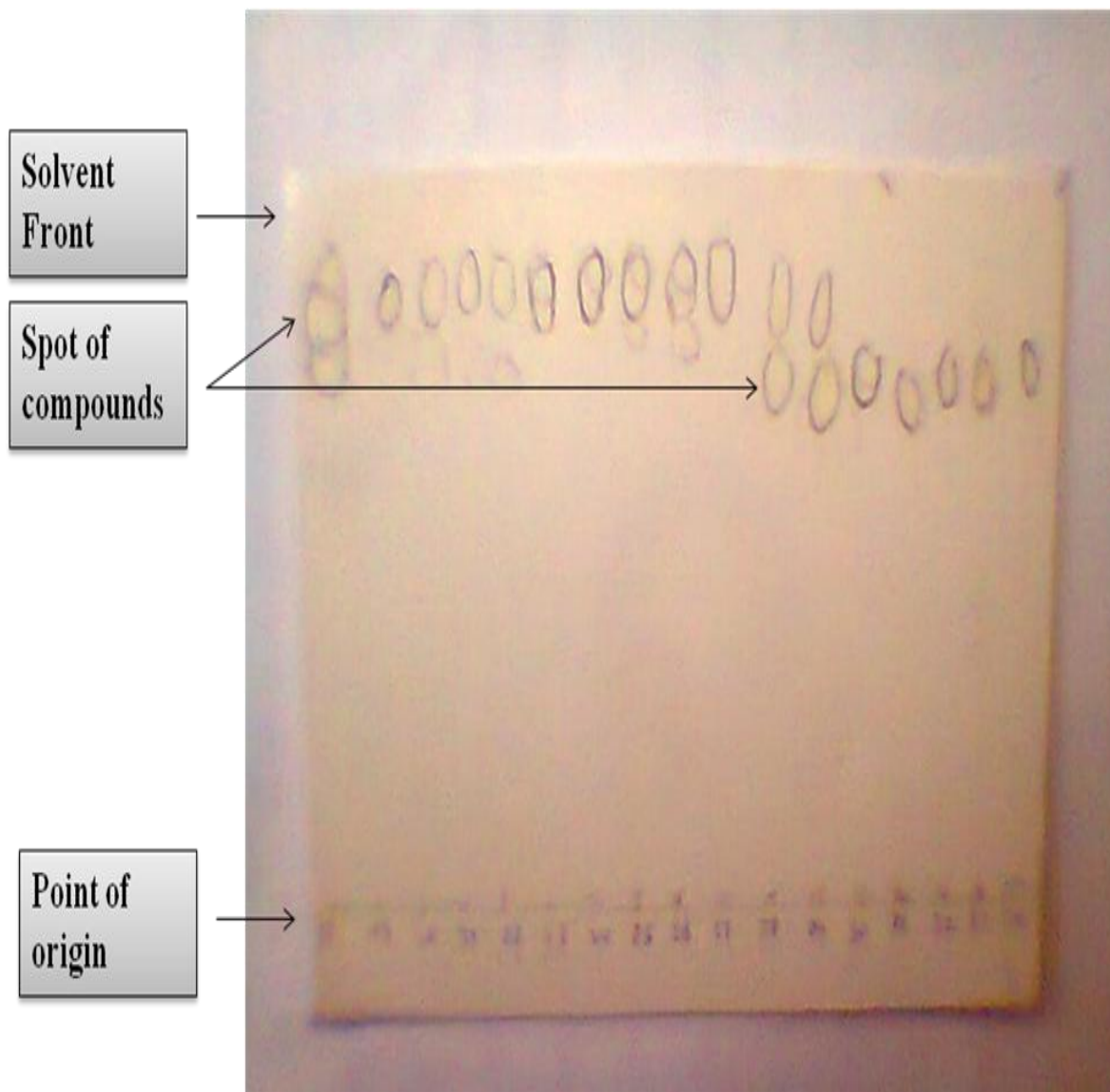


Plate 4.5: Thin layer Chromatogram of Fractions 21 - 37 Obtained from Column Chromatography of Aqueous Crude Leaves Extract

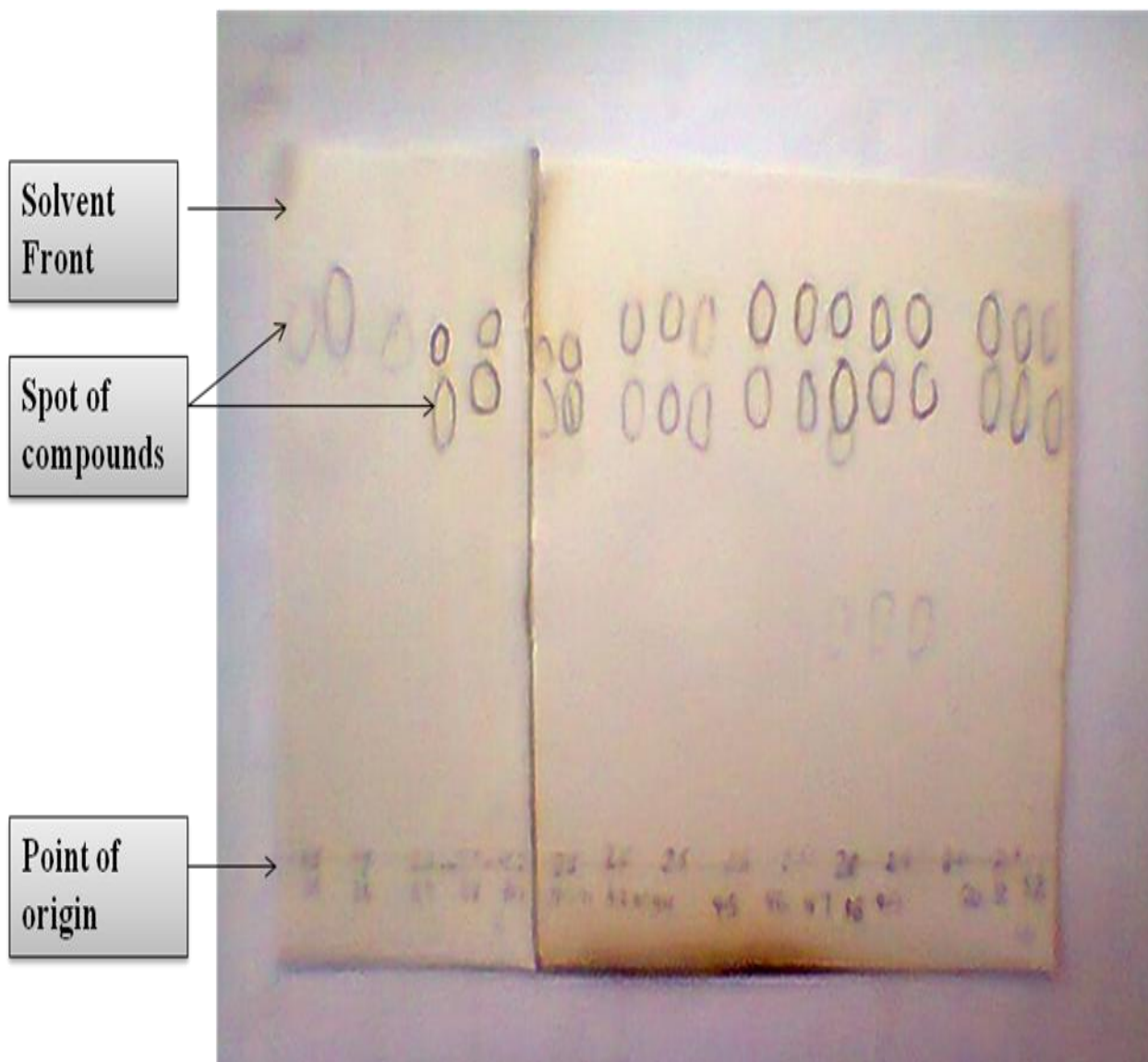


Plate 4.6: Thin layer Chromatogram of Fractions 28 - 55 Obtained from Column Chromatography of Aqueous Crude Leaves Extract

**Table 4.7: Retention Factor (RF) Values of Various Spots of Crude Aqueous Extract
Subjected to Thin Layer Chromatography**

Fraction Number	Fractions Collected	Mean Distance Covered by Solute (cm)	Mean Solvent Front (cm)	RF values
Fraction 1	1 – 3		5.31	
Fraction 2	4 – 20	4.08	4.90	0.83
	21 – 30	4.64	5.61	0.83
Fraction 3	31 – 32	4.64, 4.18	5.61, 5.61	0.83, 0.75
Fraction 4	33 – 37	4.18	5.61	0.75
	38 - 40	4.20	5.57	0.75
Fraction 5	41 - 55	4.55, 3.87	5.51	0.83, 0.70

4.6.2 Results of Antisickling Effect of Partially Purified Fractions

The antisickling effect of partially purified leaves extract of *Telferia occidentalis* showed a dose dependent antisickling activity, with the highest activity shown by the 10mg/ml (Figure 4.10), followed by 1mg/ml (Figure 4.11) and 0.1mg/ml (Figure 4.12).

Fraction 3 (Fractions 31 – 32) showed the highest antisickling activity of 74% compared to fractions 2, 4 and 5 that revealed 61%, 64% and 53% antisickling activity, respectively; at 10mg/ml of purified extract, at 120 minutes (Table 4.8). The flow through (fractions 1 – 3) completely evaporated leaving no visible content in the beaker. Rinsing the beaker with 1 ml normal saline (re-constitution) and testing for antisickling activity showed no observable antisickling effect.

The antisickling effect shown by Para-hydroxybenzoic acid (PABA) – the positive control; at 5mg/ml and at about 120 minutes was 79% while the negative control (normal saline) showed about 7% antisickling activity.

Table 4.8: Peak Antisickling Effect of Partially Purified Fractions of Crude Aqueous Leaves Extracts of *Telfairia occidentalis* (Fluted Pumpkin).

Partially Purified Fractions	% of Cells Unsickled at 120 min		
	10 mg	1 mg	0.1 mg
Fraction 2	61	50	46
Fraction 3	74	67	52
Fraction 4	64	56	48
Fraction 5	53	47	42
PABA (5mg/ml)	79		
Normal saline	7		

Fraction 2 = Fractions 4 - 30
 Fraction 3= Fractions 31 – 32
 Fraction 4= Fractions 33 – 40
 Fraction 5= Fractions 41 – 55

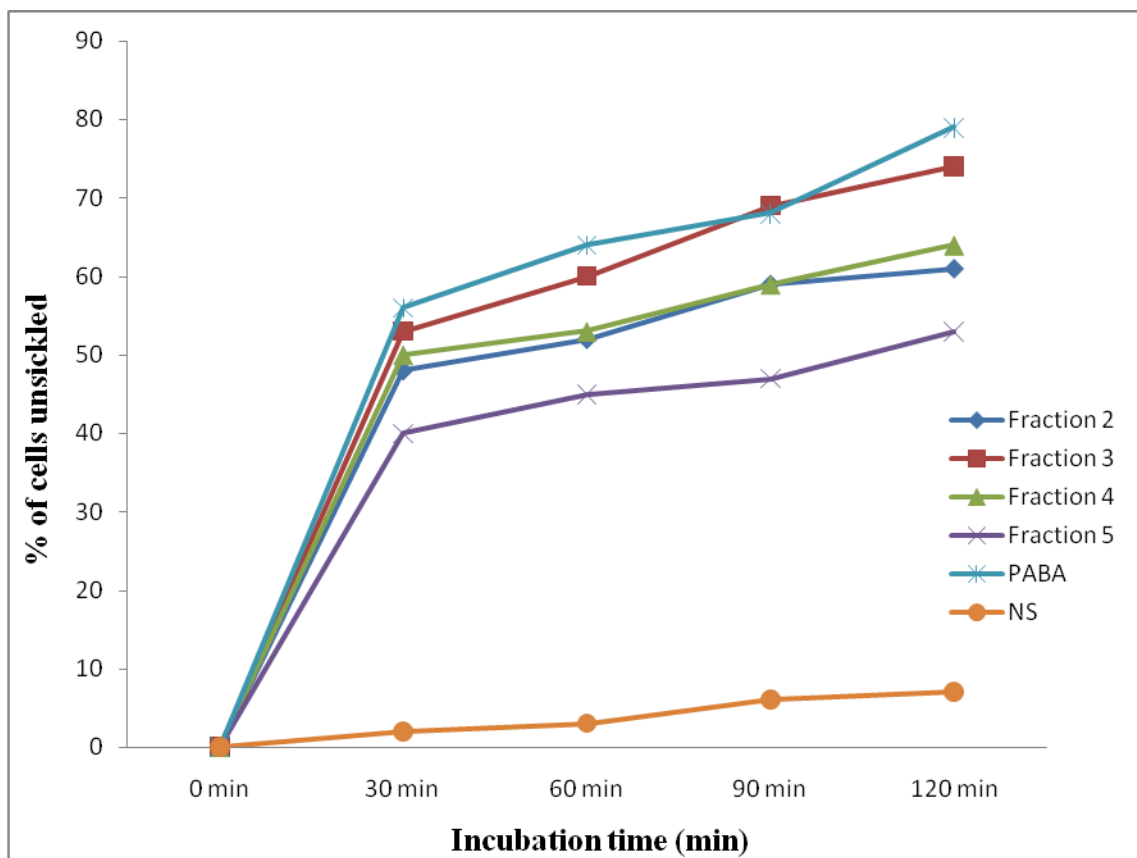


Figure 4.10: Antisickling Activity of Partially Purified Leaves Extract of *T. occidentalis* (10mg)

PABA = Para-hydroxybenzoic acid

NS = Normal Saline

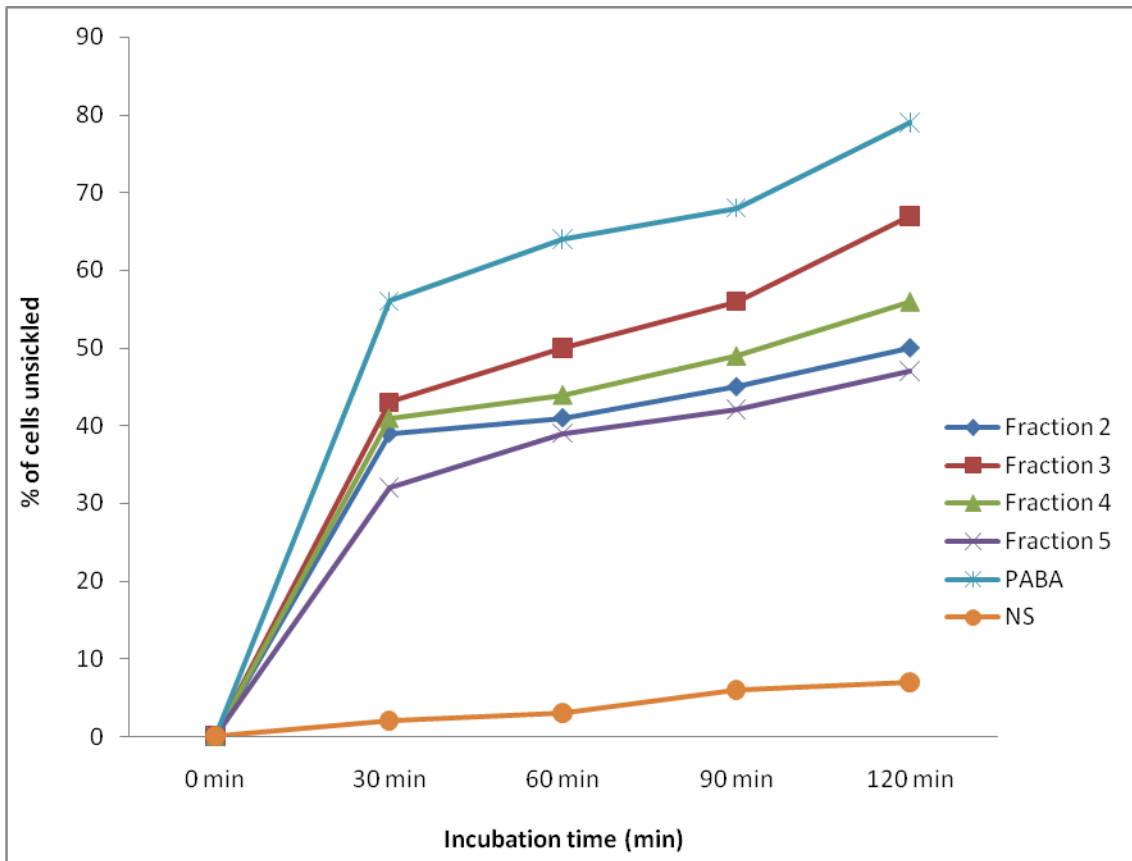


Figure 4.11: Antisickling Activity of Partially Purified Leaves Extract of *T. occidentalis* (1mg)

PABA = Para-hydroxybenzoic acid

NS = Normal Saline

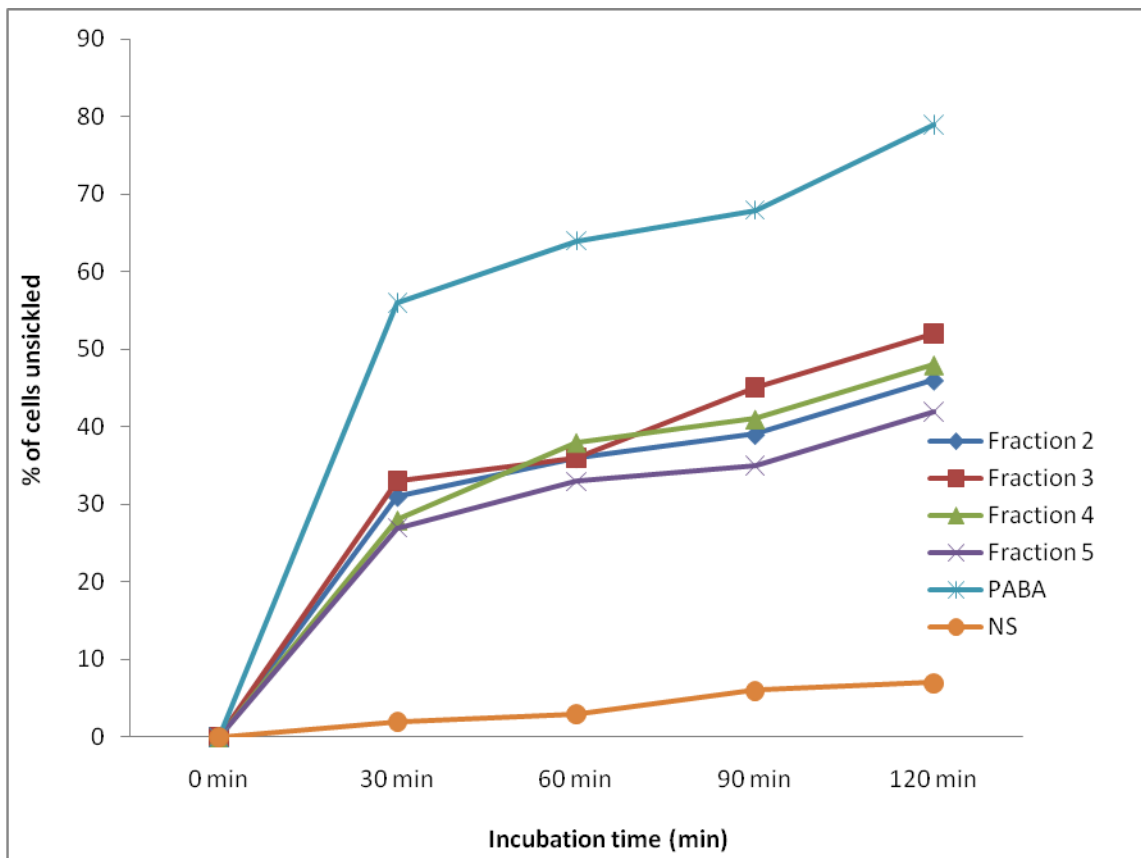


Figure 4.12: Antisickling Activity of Partially Purified Leaves Extract of *T. occidentalis* (0.1 mg)

PABA = Para-hydroxybenzoic acid

NS = Normal Saline

CHAPTER FIVE

DISCUSSION

In the present study, antisickling effect of *Telferia occidentalis* on red blood cells exposed to sodium metabisulphite – promoted sickling may be attributed to synergistic effect of inherent phytochemical constituents and mineral elements. The result of phytochemical screening and quantification revealed the presence of alkaloids, tannins, flavonoids, cyanogenic glycosides and total phenols; while potassium (K^+), sodium (Na^+), magnesium (Mg^{2+}), calcium (Ca^{2+}) and zinc (Zn^{2+}) were found to be present, following mineral element analysis.

Although glycosides and saponins have been documented to significantly increase the proliferation abilities of bone marrow (Li, *et. al.*, 2011; Goa, 1992; Kirby and Bentley, 1991), while alkaloids have been reported to enhance the restoration of hematopoiesis (Boyko and Belskey, 1998) *in vivo*, the mechanism of *in vitro* reversal of sickling by these phytochemicals is still elusive. However, these phytochemical constituents (alkaloids, tannins, flavonoids, phenolics, cyanogenic glycosides) may have *in vivo* therapeutic benefits such as analgesic, anti-plasmodiac, bactericidal, (Stary, 1998; Okwu and Okwu, 2004), antimicrobial (Nwogu *et al.*, 2008), anti-oxidant, (Ihekoronye and Ngoddy, 1985) and anti-cyanotic effects (Okwu and Okwu, 2004), while acting as good sedative (Kenner and Yves, 1996) in sickle cell disease patients.

The mechanism of cell dehydration in sickle cell disease (SCD) is associated with intracellular levels (concentration) of minerals or cation. Dehydration that takes place during sickling and the loss of potassium (K^+) due to the increase in cell membrane

permeability to calcium (Ca^{2+}) activates the Gardos channel and induces diffusion influx of sodium ion (Na^+). The Na^+/K^+ ATPase pump activated by the increase of Na^+ leads to further cell dehydration and potassium efflux (Joiner *et al.*, 1988). Additional K^+ loss occurs as a result of low cellular Mg^{2+} which activates K-Cl co-transport system, leading to further cell dehydration. K^+ , Na^+ , Ca^{2+} , Mg^{2+} and water are the major electrolytes involved in the electrolyte imbalance theory, which by osmolytic and diffusional processes could correct imbalance and reverse physiological processes observed during sickling (Folasade *et al.*, 2006).

All extracts showed high concentration of K^+ and Mg^{2+} , low Ca^{2+} , and average quantity of Na^+ (Tables 4.3 and 4. 4), especially in aqueous leaves extract. High amount of K^+ and Mg^{2+} play an inhibitory role to major mechanism of sickling which include – Calcium activated potassium efflux (Gardos) and K-Cl co – transport channels. An earlier report (De Franceschi, 2000) which revealed that clotrimazole (potent drug used in the treatment of SCD) shown to be a specific inhibitor of Gardos channel, *in vivo* and *in vitro*, increases the red cell K^+ content and reduces dehydration. Dennis and Roberts (1990) proposed a mechanism for antisickling activity of some plant species on sickled erythrocytes. They thought it may be due to inhibition of Ca^{2+} activated K^+ channel. Activation of this channel results in loss of K^+ , accompanied by Cl^- and water from sickled red blood cells (RBC) with subsequent dehydration which brings about increased intracellular concentration of sickled haemoglobin (HbS), leading to polymerization of deoxy HbS with its associated painful episodes. Inhibition of this pathway increases K^+ cell content, especially when there is a concomitant supply of this cation by the plant species. This will result in rehydration of

red blood cells and ensuing increase in haemoglobin levels. This event leads to cell swelling, decreased HbS concentration and decreased sickling.

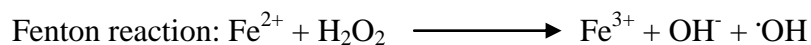
Results obtained from the experiment for sickling reversion indicates that *in vitro* antisickling action of the extracts (leaf, stem and seed), especially the aqueous leaf extract was rapid, and is proposed to likely be due to inhibition of two main erythrocyte sickling pathways; the Ca^{2+} activated K^+ efflux and K-Cl co-transport Channels – principally due to high K^+ and Mg^{2+} content, (which promotes the rehydration of sickle cells) in synergy with protective and curative effects of phytochemicals present (especially the haematopoietic glycosides, saponins and alkaloids) in the plant. A 64.03% reversal of sickled cells was observed in 180 minutes by the effect of the crude leaves extract of *T. occidentalis*. Since the aqueous leaves extract showed the highest antisickling activity compared to other plant parts, it was subjected to partial purification in column chromatography, using silica gel as adsorbent. All the partially purified fractions showed high antisickling activity, with fraction 3 showing the highest level of reversal of sickled erythrocytes (74%), which was also higher than that of the crude extract (64.03%). The higher antisickling activity shown by the partially purified extract may be attributed to the fact that bulky interfering molecules such as impurities, artifacts or contaminants that may be present in the crude extract have been eliminated by the purification process, thereby leaving behind the partially pure bioactive components that are likely responsible for the antisickling effect.

If this action can be reproduced *in vivo*, then the extract may as well hold a lot of promise in the treatment of sickle cell disease. Depending on its half-life, it would be expected that its periodic administration would reduce both frequency and duration of crises.

Pathophysiology of sickle cell disease has been attributed to both sickle haemoglobin and erythrocyte – membrane behavior (Mpiana *et al.*, 2010). Stabilization of erythrocytes membrane exposed to both heat and hypotonic – induced lyses was employed in order to assess the level of protection that can be exhibited by the plant extracts on sickle cell erythrocyte membrane.

Assay of membrane stabilizing effect of various concentrations of aqueous and methanolic (leaf, stem and seed) extracts on sickle red blood cells exposed to both heat and hypotonic induced lyses revealed an overall maximum activity of $71.85 \pm 0.001\%$ in the stem methanolic extract, and minimum activity of $22.60 \pm 0.003\%$ in the aqueous leaf extract. The results showed that both aqueous and methanolic extracts contained principles that protected the erythrocyte membrane effectively. Membrane stabilizing activity was observed to be plant part and concentration dependent, and this may be due to the quantity of inherent flavonoids. Kumar *et al.*, (2008), Okwu, (2007), and Miliauskas *et al.*, (2004), reported that bio-membrane damage can be inhibited by flavonoids, which serve as strong free radical scavengers and singlet oxygen quenchers. This is in conformity with our result where the methanolic extract of the stem (having the peak activity) contains the highest quantity of flavonoids. Also, the aqueous leaf extract showed the least protection on erythrocyte membrane, which may not be unconnected with its elevated levels of tannins, a chaotropic agent that has been reported to compromise erythrocyte membrane integrity (Mota *et al.*, 1985). On the basis of these results, it could be inferred that that the aqueous and methanolic extracts of leaves, stem and seeds of *T. occidentalis* contained principles that were capable of stabilizing sickle erythrocyte membrane against heat and hypotonic-induced lyses.

The extracts, apart from inhibition of polymerization and protection of erythrocyte membrane, improved the $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio, thereby reducing endogenous methaemoglobin concentration. Methaemoglobin is formed when the iron of deoxy-haemoglobin is oxidized from its ferrous (Fe^{2+}) to the ferric state (Fe^{3+}) (Murray *et al.*, 2006). The oxidation of the iron of deoxy-haemoglobin (Fe^{2+}) to iron of methaemoglobin (Fe^{3+}) can arise from auto-oxidation (by sickle cells) engendered by the activities of pro-oxidants and oxidizing substances (Callister, 2003), deficiency or impaired activity of methaemoglobin reductase enzymes (Yubisui and Takashita, 1980), or it may be hereditary (Prchal and Greg, 2005). George *et al.*, (2010), reported that sickle – red blood cells’ – reactive oxygen species (ROS) generation has been attributed to sickle haemoglobin auto – oxidation and Fenton chemistry reaction catalyzed by denatured heme moieties bound to the RBC membrane.



Tamer *et al.*, (2000) also noted that the primary reason for the relatively raised concentration of oxidized haemoglobin (methaemoglobin) in HbSS erythrocyte was the higher production of superoxide ion by this erythrocyte (HbSS) compared to those of HbAA and HbAS erythrocytes. Antioxidant may therefore play a significant role in the reduction of methaemoglobin formation by interfering with any of the mechanism above or stimulating the activity of NADH methaemoglobin reductase. Results of this investigation show that the methanolic leaf extract exhibited a high level conversion of methaemoglobin to haemoglobin at 0.8% w/v of the extract, as indicated by the least concentration of methaemoglobin present (compared to the blood control) in the sickle cell blood after treatment.

In summary, the highest antisickling properties and Fe^{2+}/Fe^{3+} ratio was shown by aqueous leaves extract, while the peak membrane stabilizing effect was seen in methanolic stem extract. In addition to the phytochemical constituents and mineral elements investigated in this study, Nwaoguikpe, (2010), and Fasuyi, (2007) have both reported the presence of amino acids (arginine, phenylalanine, leucine, glutamic acid), anti – oxidant vitamins (vitamin C, A, E, thiamin, riboflavin and nicotinamide) and phenolic compounds (polyphenols such as glycosides and flavonoids) in *T. occidentalis*. It can therefore be inferred, that inherent phytochemicals, minerals, amino acids and vitamins in *T. occidentalis* may synergistically play a role in the reversal of sickled erythrocytes, membrane stabilization and reduction of methaemoglobin levels.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

- i. The result of the present study showed that, aqueous and methanolic extracts of *T.occidentalis* has significant ($P<0.05$) *in vitro* antisickling activity on sickle cell erythrocytes.
- ii. The extracts have shown a great level of protection on sickled erythrocyte membrane with the highest activity seen in methanolic stem extract.
- iii. The extracts increased Fe^{2+}/Fe^{3+} ratio, thereby promoting the availability of oxyhaemoglobin necessary to avert complications associated with SCD.
- iv. All observed pharmacological activities were plant – part and concentration dependent.

6.2 Conclusion

The results of this study scientifically validated the *in vitro* potential of *T. occidentalis* in the management of sickle cell disease. The result showed that, sickled red blood cells under hypoxic condition, in addition to further induction of sickling using sodium metabisulphite, was significantly reversed, *in vitro*, upon administration of leaves, seeds and stem aqueous and methanolic extracts of *T. occidentalis* at 0.1mg/ml, 1mg/ml and 10mg/ml (in a dose dependent manner) The antisickling activity followed the order- Leaves, Stem and Seed. The plant also conferred a high level of protection on red cell membrane and improved the Fe^{2+}/Fe^{3+} ratio. All the various doses of the plant extract used in this study were effective, with the highest doses showing activity that can be compared with the standard drugs used.

6.3 Recommendation

- i. Further investigations are needed to elucidate the exact mechanism(s) of action of *T. occidentalis*.
- ii. Further investigations which will include, isolation, identification and elucidation of the structure of specific plant extract component responsible for the pharmacological effect.
- iii. There is need for replication of this study, *in vivo*, using a knockout transgenic mice having human sickle cell anaemia.

REFERENCES

- Akenami, F.O., Aken'Ova, Y.A., and Osifo, B.O. (1999). Serum zinc, copper and magnesium in sickle cell disease at Ibadan, southwestern Nigeria. *African Journal of Medical Science*. 28(3-4):137-139.
- Akindakun, I.A. (2005). Phytochemical screening of selected tropical green leafy vegetables. *African Journal of Biotechnology*, 4:497-501.
- Akoroda, M.O. (1990a). Ethnobotany of *Telfairia occidentalis* (cucurbitaceae) among Igbos of Nigeria. *Economic Botany*, 44: 29-39.
- Akoroda, M.O. (1990b). Seed production and breeding potential of the Fluted pumpkin, *Telfairia occidentalis*. *Euphytica*, 49: 25-32.
- Akubue, P. I., Kar, A., and Nnchelita, F. N. (1980). Toxicity of extracts of roots and leaves of *Telfairia occidentalis* *Planta Medica*, 38: 339 - 343
- Al-Saqladi, A., Cipolotti, R., and Fijnvandraat, K. (2008). Growth and nutritional status of children with homozygous sickle cell disease. *Annals of Tropical Paediatrics*, 28:165-189.
- Archer, D.R., Stiles, J.K., and Newman, G.W. (2008). C-reactive protein and interleukin- 6 are decreased in transgenic sickle cell mice fed a high protein diet. *Journal of Nutrition*, 138(6):1148-1152.
- Asiegbu, J. E. (1987). Some biochemical evaluation of Fluted pumpkin seed. *Journal of Food Science*, 40, 151- 155.
- Aslan, M., Ryan, T.M., and Adler, B. (2001). Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease. *Proceedings of the National Academy of Science*, 98: 215-220.
- Ataga, K.I., and Orringer, E.P. (2000). Bone marrow necrosis in sickle cell disease: a description of three cases and a review of the literature. *American Journal of the Medical Sciences*, 320, 342-347.
- Atkins, B.L., Price, E.H., Tillyer, L., Novelli, V., and Evans, J. (1997) *Salmonella* osteomyelitis in sickle cell disease children in the east end of London. *Journal of Infection*, 34:133-138.
- Badifu, G.I., and Ogunsina, A.O. (1991). Chemical Composition of kernels from some species of cucurbitaceae grown in Nigeria. *Journal of Plant Foods Human Nutrition*, 41: 35-44.

- Bahebeck, J., Ngowe, N.M., Monny, L.M., Sosso, M., and Hoffmeyer, P. (2002). Stress fracture of the femur: a rare complication of sickle cell disease. *Revue de Chirurgie Orthopedique et Reparatrice de l'Appareil Moteur*, 88, 816–818.
- Bao, B., Prasad, A.S., and Beck, F.W. (2008). Zinc supplementation decreases oxidative stress, incidence of infection, and generation of inflammatory cytokines in sickle cell disease patients. *Translational Research*, 152(2):67–80.
- Barden, E.M., Kawchak, D.A., Ohene-Frempong, K., Stallings, V.A., and Zemel, B.S. (2002). Body composition in children with sickle cell disease. *American Journal of Clinical Nutrition*, 76, 218-225.
- Baron, B.W., Mick, R., Baron, J.M. (1994). Hematuria in sickle cell anaemia— not always benign: Evidence for excess frequency of sickle cell anaemia in African Americans with renal cell carcinoma [Review]. *Acta Haematologica*, 92: 119–122.
- Bellet, P.S., Kalinyak, K.A., Shukla, R., Gelfand, M.J., and Rucknagel, D.L. (1995). Incentive spirometry to prevent acute pulmonary complications in sickle cell diseases. *New England Journal of Medicine*, 333:699-703.
- Berney, S.I., Ridler, C.D., Stephens, A.D., Thomas, A.E., and Kovacs, I.B. (1992). Enhanced platelet reactivity and hypercoagulability in the steady state of sickle cell anaemia. *American Journal of Hematology*, 40: 290–294.
- Bertles, J.F., and Milner, P.F. (1968). Irreversibly sickled erythrocytes: A consequence of the heterogeneous distribution of haemoglobin types in sickle cell anaemia. *Journal of Clinical Investigation*, 47:1731- 1741.
- Boham, B.A., and Kocipai, A.C. (1974). Flavonoids and condensed tannins from leaves of *Hawaiian vaccinium vaticulatum* and *V. calycinium*. *Pacific Science*. 48: 458-463.
- Bookchin, R.M., and Lew, V.L. (1980). Progressive inhibition of the Ca-pump and Ca:Ca exchange with normal cytoplasmic Ca buffering in sickle red cells. *Nature* 2:561-562.
- Bookchin, R.M., Ortiz, O.E., and Lew, V.L. (1987). Red cell magnesium content and permeability in sickle cell anaemia. *Clinical Research*, 35:651.
- Bookchin, R.M., Ortiz, O.E., and Lew, V.L. (1989). Mechanisms of red cell dehydration in sickle cell anaemia. Application of an integrated red cell model. In Raess BU, Tunncliffe G (eds): *The Red Cell Membrane: A Model for Solute Transport*. Humana Press, pp 443-461
- Bookchin, R.M., Ortiz, O.E., and Lew, V.L. (1991). Evidence for a direct reticulocyte origin of dense red cells in sickle cell anaemia. *Journal of Clinical Investigation*, 87:113-124.

- Boyko, V.N., and Belskiy, S.N. (1998). The influence of the novel drug Ukrain on hemo- and immunopoiesis at the time of its maximum radio-protective effect. *Drugs Experiment and Clinical Research*, 24(5-6): 335-337.
- Brinker, M., Thomas, K., Meyers, S., Texada, T., Humbert, J., Cook, S., and Gitter, R., (1998) Bone mineral density of the lumbar spine and proximal femur is decreased in children with sickle cell anemia. *American Journal of Orthopedics*, 27, 43–49.
- Brugnara, C., Bunn, H.F., Tosteson, D.C. (1986). Regulation of erythrocyte cation and water content in sickle cell anaemia. *Science*, 23:2388-390.
- Brugnara, C., Gee, B., and Armsby, C.C. (1996). Therapy with oral clotrimazole induces inhibition of the Gardos channel and reduction of erythrocyte dehydration in patients with sickle cell disease. *Journal of Clinical Investigation*, 97:1227-1234.
- Burnett, M.W., Bass, J.W., and Cook, B.A. (1998). Etiology of osteomyelitis complicating sickle cell disease. *Journal of Pediatrics*, 101, 296–297.
- Callister, R. (2003). Methaemoglobin: Its causes and effects on pulmonary function and SPO₂ reading. <http://www.mc.vanderbilt.edu/peds/pidl/hemeone/metheme.htm>
- Canessa, M., Fabry, M.E., and Nagel, R.L. (1987). Deoxygenation inhibits the volume-stimulated, Cl⁻-dependent K⁺ efflux in SS and young AA cells: A cytosolic Mg²⁺ modulation. *Blood*, 70:1861-1866.
- Catherine, B., Baba, I., and Stephen, K. (2010). Infection in sickle cell disease: A review *International Journal of Infectious Diseases*, 327, 449-456.
- Chan, A.C., Chow, C.K., and Chiu, D. (1999). Interaction of antioxidants and their implication in genetic anaemia. *Proceedings of the Society for Experimental Biology and Medicine*, 222:274–82.
- Claster, S., and Vichinsky, E.P. (2003). Managing sickle cell disease. *British Medical Journal*, 327, 1151–1155.
- Dasgupta, T., Robert, P.H., and Dhananjay, K.K. (2006). Protective effect of arginine on oxidative stress in transgenic sickle mouse models. *Free Radical Biology and Medicine*, 41(12):1771–80.
- David, C., Thomas N., and Mark T. (2010). Sickle-cell disease. *Lancet*, 376: 2018–31
- Dawson, V.L., Xia, Y., Dawson, T.M., Snyder, S.H., and Zweier, J.L. (1996). Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proceedings of the National Academy of Sciences of the United States of America USA*, 93: 6770–4.

- De Franceschi, L., Bachir, D., and Galacteros, F. (2000). Oral magnesium pidolate: effects of long-term administration in patients with sickle cell disease. *British Journal of Haematology*, 95: 73–76.
- Demirbas, K.A., Aktener, B.O., and Unsal, C. (2004.) Pulpal necrosis with sickle cell anaemia. *International Endodontic Journal*, 37: 602–606.
- Dennis, D., and Roberts, A. (1990). Trease and Evans *Pharmacognosy*. 2nd Edition. The Alden Press, Oxford, Great Britain, p. 832.
- Driscoll, C.M. (2007). Sickle Cell Disease. *Paediatrics in Review*. 259-268
- Egunyomi, A., Moody, J.O., and Eletu, O.M. (2009) “Antisickling activities of two ethnomedicinal plant recipes used for the management of sickle cell anaemia in Ibadan, Nigeria. *African Journal Biotechnology*, 8:20-25.
- Eliot, R., Davies, M., and Hamse, D.J. (2006). Dermatomyositis-like eruption with long-term hydrourea. *British Journal of Dermatology*, 17: 56-60.
- Emeka, E.J. I. (2009). Preliminary qualitative screening for cancer chemopreventive agents in *Telfairia occidentalis* Hook.f., *Gnetum africanum* Welw., *Gongronema latifolium* Benth. and *Ocimum gratissimum* L. from Nigeria. *Journal of Medicinal Food Plants*. Volume 1. pp. 58 – 63.
- Emodi, J.I., and Okoye, I.J. (2001). Vertebral bone collapse in sickle cell disease: a report of two cases. *East African Medical Journal*, 78, 445– 446.
- Engwerda, C.R., Beattie, L., and Amante, F.H. (2005). The importance of the spleen in malaria. *Trends in Parasitology*. 21:75-80.
- Etzion, Z., Tiffert, T., Bookchin, R., and Lew, V. (1993). Effects of deoxygenation on active and passive Ca²⁺ transport and on the cytoplasmic Ca²⁺ levels of sickle cell anaemia red cells. *Journal of Clinical Investigation*, 92:2489-2498.
- Fasuyi, A.O., and Nonyerem, A.D. (2007). Biochemical, nutritional and hematological implications of *Telfairia Occidentalis* leaf meal as protein supplement in broiler starter diets. *African Journal of Biotechnology*, 6(8): 1055-1063.
- Fasuyi, A.O. (2006). Nutritional potentials of some tropical vegetable leaf meals: chemical characterization and functional properties. *African Journal of Biotechnology*, 15: 49-53
- Flatman, P.W. (1980). The effect of buffer composition and deoxygenation on the concentration of ionized magnesium inside human red blood cells. *Journal of Physiology*, 300:14-30.

- Falade, O., Otemuyiwa, I., Oladipo, A., Oyedapo, O., Akinpelu, B., and Adewusi, S. (2005). The chemical composition and membrane stability activity of some herbs used in local therapy for anaemia. *Journal of Ethnopharmacology*, 102: 15–22
- Folasade, I.S., Odukoya, A.O., and Moody, O.J. (2006). Management of Sick cell Anaemia in Nigeria with Medicinal Plants: Cationic Evaluation of Extracts and Possible Effects on the Efficacy. *Journal of Biological Sciences* 6(1): 100-102
- Forstermann, U., and Munzel, T. (2006). Endothelial nitric oxide synthase in vascular disease: From marvel to menace. *Circulation Research*, 113: 1708–1714.
- Fraker, P., King, L., Leakko, T., and Vollmer, T. (2000). The dynamic link between the integrity of the immune system and zinc status. *Journal of Nutrition*, 130:1399-406.
- Frenette, P.S., and Atweh, G.F. (2007). Sick cell disease: old discoveries, new concepts and future promise. *Journal of Clinical Investigation*. 117:850 - 8.
- Gaston, M.H., Verter, J., and Woods, G. (1986). Prophylaxis with oral penicillin in children with sickle cell anaemia. *New England Journal of Medicine*, 314: 1593–1599.
- Goa, R.L., Xu, C.L., and Jin, J.M. (1992). Effect of total saponins of *Panax ginseng* on haematopoietic progenitor cells in normal human and aplastic anaemia patients. *Zhongguo Zhong Xi Yi Jie He Za Zhi*. 12(5): 285-287.
- Guyton, A.C. and Hall, J.E. (2006). Textbook of medical Physiology, 11th edition. Elsevier Saunders, Philadelphia. pp 598 – 609.
- Geoffrey, T., Amma, O., Freda, O., and Yaw, A. (2009). Complications Associated with Sick Cell Trait: A Brief Narrative Review *The American Journal of Medicine*, 122, 507-512.
- George, A., Sebastian, K., Suvarnamala, P., Narla, M., Yi, Z., Clinton, H., and Theodosia, A. (2010). Elevated Reactive Oxygen Species Production In Sick Erythrocytes Is Modulated by a Pathway Involving Endothelin-1, TGFβ1, PKC, and Rac GTPases. Oral and Poster Abstracts, 53rd *ASH Annual Meeting and Exposition*.
- Gladwin, M.T., and Rodgers, G.P. (2000). Pathogenesis and treatment of acute chest syndrome of sickle-cell anaemia. *Lancet*, 355: 1476-1478.
- Gladwin, M.T., and Vichinsky, E. (2008). Pulmonary complications of sickle cell disease. *New England Journal of Medicine*, 359: 2254–2265.
- Graham, R. S. (1997). Sick cell disease (Seminar). *Lancet*, 350: 725–730.
- Halliwell, B., and Whiteman, M. (2004). Measuring reactive species and oxidative damage *in vivo* and in cell culture: How should you do it and what do the results mean? *British Journal of Pharmacology*, 142: 231–255.

- Harborne, J.B. (1973). *Phytochemical Methods. A Guide to Modern Techniques of Plant Analysis*. 1st Edition., Chapman and Hall, London, pp 149-188.
- Harris, J.W., Brewster, H.H., Ham, T.H., and Castle, W.B. (1956). Studies on the destruction of red blood cells X: The biophysics and biology of sickle-cell disease. *Archives of Internal Medicine*. pp. 145-168.
- Haynes, J., and Obiako, B. (2002). Activated polymorphonuclear cells increase sickle red blood cell retention in lung: Role of phospholipids. *American Journal of Physiology, Heart Circulation Physiology*, 282: 22–30.
- Hebbel, R.P., Morgan, W.T., Eaton, J.W., and Hedlund, B.E. (1988). Accelerated autoxidation and heme loss due to instability of sickle haemoglobin. *Proceedings National Academy of Science, USA* 85:237-241.
- Hebbel, R.P., Leung, A., and Mohandas, N. (1990). Oxidation-induced changes in micro-rheologic properties of the red blood cell membrane. *Blood*, 76: 1015–1020.
- Herrick, J.B. (1910). Peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anaemia. *Archives of Internal Medicine*, 6: 517–521.
- Heyman, M.B., Katz, R., and Hurst, D. (1985). Growth retardation in sickle-cell disease treated by nutritional support. *The Lancet*. 325(8434):903–906.
- Hibbert, J.M., Hsu, L.L., and Bhathena, S.J. (2005). Pro-inflammatory cytokines and the hyper-metabolism of children with sickle cell disease. *Experimental Biology and Medicine* 230(1):68–74.
- Ihekoronye, A., and Ngoddy, P. (1985). Integrated Food Science and Technology for the Tropics. Macmillan Education Ltd. *Pakistan Journal of Nutrition*, 7(1): 130-132.
- Iyamu, E., Turner, E., and Asakura, T. (2002). *In Vitro* Effects Of Niprisan Nix – 0699): A Naturally Occurring, Potent Antisickling Agent. *British Journal of Hematology*, 118: 337-343.
- Jaliwala, Y., Pand, P., Parto, V., Chourosia, N., Bhatt, N., Amit, P., and Mohanty, P., (2011). Pharmacognostic and Priliminary Phytochemical secreening of *Ficus arnottiana* miq. *Journal of Current Pharmaceutical Research*, 6(1):21-27.
- James E. B., Joe, W. K., and Chadwick, C. H. (2001). Organizational Research: Determining Appropriate Sample Size in Survey. *Research Information Technology, Learning, and Performance Journal*, 19 (1): 43-50
- Janeway, C., and Travers, P. (1997). *Immunology. The immune system in health and disease*. 3rd Edition, London: Churchill Livingstone. pp 324-331

- Jison, M.L., Munson, P.J., and Barb, J.J. (2004). Blood mononuclear cell gene expression profiles characterize the oxidant, hemolytic, and inflammatory stress of sickle cell disease. *Blood* 104: 270–280.
- Joiner, C.H., Platt, O.S., and Lux, S.E. (1986). Cation depletion by the sodium pump in red cells with pathological cation leaks. Sickle cells and xerocytes. *Journal of Clinical Investigation*, 78:1487-1496.
- Joiner, C.H., Dew, A., and Ge, D.L (1988). Deoxygenation – induced cation fluxes in sickle cell disease: Relationship between potassium net efflux and net sodium influx. *Blood cells*, 13:339-354
- Katherine, C. W., and Granger, D. N. (2007). Sickle Cell Disease: Role of reactive oxygen and nitrogen metabolites. *Clinical and Experimental Pharmacology and Physiology*, 34, 926–932
- Kaul, D.K., Zang, X., and Dasgupta, T. (2002). Arginine therapy of transgenic-knockout sickle mice improves microvascular function by reducing non-nitric oxide vasodilators, hemolysis, and oxidative stress. *American Journal of Pediatric*, 295(1):39–47.
- Kaul, D.K., Liu, X.D., Choong, S., Belcher, J.D., Vercellotti, G.M., and Hebbel, R.P. (2004). Anti-inflammatory therapy ameliorates leukocyte adhesion and microvascular flow abnormalities in transgenic sickle mice. *American Journal of Physiology and Heart Circulation Physiology*, 287: 293–301.
- Kenner, D.L., and Yves, R.M. (1996). A European Professional Perspective. *Botanical Medicine*, pp. 482-487.
- Kim, S.K., and Miller, J.H. (2002). Natural history and distribution of bone and bone marrow infarction in sickle haemoglobinopathies. *Journal of Nuclear Medicine*, 43, 896–900.
- King, S.M., Donangelo, C.M., and Knutson, M.D. (2008). Daily supplementation with iron increases lipid peroxidation in young women with low iron stores. *Journal of Experimental Biology and Medicine*, 233(6):701–707.
- Kirby, S.L., and Bentley, S.A. (1991). Xyloside effects on *in vitro* haematopoiesis: functional and bio-chemical studies. *Journal of Cell Physiology*, 148(1):116-123.
- Kumar, G., Jayaveera, K., Ashok, C., Bharathi, T., Umachigi, S., Vrushabendra, S. (2008). Evaluation of antioxidant and antiacne properties of terpenoidal Fraction of *Hemidesmus indicus* (Indian sarsaparilla) *International Journal of Aesthesia and Anti-aging Medicine*, 1:1–8.
- Kuypers, F.A., Scott, M.D., Schott, M.A., Lubin, B., and Chiu, D.T. (1990). Use of ektacytometry to determine red cell susceptibility to oxidative stress. *Journal of Laboratory and Clinical Medicine*, 116: 535–545.

- Lakshman, R. B. (2012). Phytochemical Screening, Quantitative Estimation Total Phenolics and Total Flavonoids, Anti Microbial Evaluation of *Cyamopsis tetragonoloba*. *International Journal of Research in Pharmaceutical and Biomedical Sciences*, 3 (3) :1139-1142.
- Larcher, V.F., and Wyke, R.J. (1982). Defective yeast opsonisation and functional deficiency of complement in sickle cell disease. *Archives of Disease in Childhood*, 57:343-346.
- Lauf, P., Bauer, J., and Adragna, N. (1992). Erythrocyte K-Cl cotransport: Properties and regulation. *American Journal of Cell Physiology*, 263(9):174-182.
- Lew, V.L., Hockaday, A., and Sepulveda, M.I. (1985). Compartmentalization of sickle cell calcium in endocytic inside-out vesicles. *Nature*, 315:58-59.
- Li, Y., Cao, W., Guo, J., Miao, S., Ding, G., Li, K., Wang, J., and Guo, G. (2011). Comparative investigations on the protective effects of rho-dioside, ciwujianoside-B and astragaloside IV on radiation injuries of the haematopoietic system in mice. *Phytotherapy Research*, 25(5): 644- 653.
- Liu, S., Derick, L., Zhai, S., and Palek, J. (1991). Uncoupling of the spectrin-based skeleton from the lipid bilayer in sickled red cells. *Science*, 252:574-576.
- Lucas, S. (2004). The morbid anatomy of sickle cell disease and sickle cell trait. In: Practical management of haemoglobinopathies. Oxford: Blackwell; pp. 145-148.
- Makani, J., Williams, T., and Marsh, K. (2007). Sickle cell disease in Africa: burden and research priorities. *Annals of Tropical Medicine and Parasitology*, 101:3-14.
- Martin, C., Johnson, C., Cobb, C., Tatter, D., and Haywood, L. (1996). Myocardial infarction in sickle cell disease. *Journal of the National Medical Association*, 88: 428-32.
- McCullough, K.D., and Wally, J.B. (2007). The dose-dependent effects of chronic iron overload on the production of oxygen free radicals and vitamin e concentrations in the liver of a murine model. *Biological Research*, 8(4):300-4.
- Mehanna, A.S. (2001). Sickle cell anaemia and antisickling Agents Then and Now. *Current Medicinal Chemistry*, 8(2): 79-88.
- Mgbemene, C.N., and Ohiri, F.C. (1999). Antisickling Potential of *Terminalia catappa* leaf extracts. *Journal of Pharmaceutical Biology*, 37: 152-154.
- Miliauskas, G., Venskutonis, P., and van Beek, T.A. (2004). Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chemistry*, 85:231-7.

- Mohammad, N., Sajid, A., and Muhammad, Q. (2011). Preliminary Phytochemical Screening of Flowers, Leaves, Bark, Stem and Roots of *Rhododendron arboretum*. *Middle-East Journal of Scientific Research*, 10 (4): 472-476.
- Mohanty, D., Mukherjee, M., and Colah, R.B. (2008). Iron deficiency anaemia in sickle cell disorders in India. *Indian Journal of Medical Research*, 127(4):366–9.
- Mota, M., Thomas, G., and Barbosa-Filho, J. (1985) Anti-inflammatory actions of tannins isolated from the bark of *Anacardium occidentale* L. *Journal of Ethnopharmacology*, 13:289–300.
- Mpiana, P., Mudogo, V., Ngbolua, K., Tshibangu, D., Shetonde, O., and Mbala, M. (2007). *In vitro* antisickling activity of anthocyanins from *Ocimum basilicum* L. (*Lamiaceae*). *International Journal of Pharmacology*, 3: 371-374.
- Mpiana, T., Kotote, N., Matthieu, T., Teddy, K., Emmanuel, K., Daniel, S., and Virima M. (2010). *In vitro* effects of anthocyanin extracts from *Justicia secunda* Vahl on the solubility of haemoglobin S and membrane stability of sickle erythrocytes. *Blood Transfusion*, 8(4): 248–254.
- Murray, R.K., Granner, D.K., and Rodwell, V.W. (2006). *Harper's Illustrated Biochemistry*. 27th Edition. McGraw Hill Companies. Asia. pp 409-411
- Nagel, R.L., Fabry, M.E., and Steinberg, M.H. (2003). The paradox of haemoglobin SC disease. *Blood Reviews* 17: 167–178.
- Naran, A., and Fontana, L. (2001) Sickle cell disease with orbital infarction and epidural hematoma. *Pediatric Radiology*, 31, 257–259.
- Nelson, D., Rizvi, S., Bhattacharyya, T., Ortega, J., Lachant, N., and Swerdlow, P. (2003). Trabecular and integral bone density in adults with sickle cell disease. *Journal of Clinical Densitometry*, 6, 125–129.
- Neonato, M., Guilloud-Bataille, M., Beauvais, P., Begue, P., Belloy, M., Benkerrou, M., Ducrocq, R., Maier-Redelsperger, M., De Montalembert, M., Quinet, B., Elion, J., Feingold, J., and Girot, R. (2000) Acute clinical events in 299 homozygous sickle cell patients living in France. French Study Group on Sickle Cell Disease. *European Journal of Haematology*, 65, 155–164.
- Nwaoguikpe, R. N., and Ejele, E.A. (2010). Amino Acid Profile of Some Anti-sickling Plant Extracts and their Haemoglobin Polymerization Inhibition. *Nigerian Journal of Biochemistry and Molecular Biology*, 25 (2): 53 - 59.
- Nwogu, L.A., Igwe, C.U., and Emejulu, A.A. (2008). Effects of *Landolphia owariensis* leaf extract on the liver function profile and haemoglobin concentration of albino rats. *African Journal of Biotechnology*, 2(12): 240-242.

- Obadoni, B.O., and Ochuko, P.O. (2001). Phytochemical studies and comparative efficacy of the crude extracts of some Homostatic plants in Edo and Delta States of Nigeria. *Global Journal of Pure and Applied Science*, 8b:203-208.
- Odoemena, C.S., and Onyeneke E.C. (1998). Lipids of Fluted pumpkin (*Telfairia occidentalis*) seeds. *Proceedings of the 1st African Conference on Biochemistry of Lipids*, (ACBL'98), Ambik Press, Benin City, Nigeria, pp: 147-151.
- Oduola, T., Adeniyi, F., Ogunyemi, E., Bello, I., and Idowu, T. (2006). Antisickling agent in an extract of unripe pawpaw (*Carica papaya*): Is it real? *African Journal Biotechnology*, 5(20): 1947-1949.
- Okeahialam, T.C., and Obi, G.O. (1982). Iron deficiency in sickle cell anaemia in Nigerian children. *Annals of Tropical Pediatrics*, 2:89–92.
- Okoli, B.E., and Mgbeogu C.M., (1983) “Fluted Pumpkin, *Telfairia occidentalis*: West African vegetable crop”. *Economic Botany*, 37: 145-149.
- Okoli, B.E., and Nyanayo, B.L. (1988). Polynology of *Telfairia* L.(Cucurbitaceae). *Folia Geobotanica Phytotaxonomica*, 23: 281-286.
- Okpala, I.E. (2004). Assessment of severity and hydroxyurea therapy in sickle cell disease. Practical management of haemoglobinopathies. Oxford: *Blackwell*; pp. 103-110
- Okwu, D.E., and Okwu, M.E. (2004). Chemical composition of *Spondias mombin* Linn. Plant parts. *J. Sustain. Agric Environ.*, 6(2): 140 – 147.
- Okwu, D.E. (2007). Phytochemicals, Vitamins and Mineral contents of two Nigeria medicinal plants. *International Journal of Molecular Medicine*, 1 (4): 375-381.
- Oladipo, O.O., Temiye, E.O., and Ezeaka, V.C. (2005). Serum magnesium, phosphate and calcium in Nigerian children with sickle cell disease. *West African Journal of Medicine*, 24(2):120-123.
- Oniyangi, O., and Omari, A. (2006). Malaria prophylaxis in sickle cell disease. *Cochrane Database System Reviews*, 4:CD003489.
- Onwubalili, J.K. (1983). Sickle cell disease and infection. *Journal of Infectious Diseases*, 7:2-20.
- Oyewole, O.A., and Abalaka, M.E. (2012). Antimicrobial Activities of *Telfairia occidentalis* (fluted pumpkins) Leaf Extract against Selected Intestinal Pathogens. *Journal of Health Science*, 10:119–27. pp 1–4.
- Oyolu, C. (1980). Maximizing the contribution of Okro (*Hibiscus esculentus*) to the national diet. Papers presented at the 3rd conference of Nutritional Society, Nigeria at University of Ife, November 30 – December 3.

- Parks, D.A., Williams, T.K., and Beckman, J.S. (1988). Conversion of xanthine dehydrogenase to oxidase in ischemic rat intestine: A re-evaluation. *American Journal of Physiology*, 254: 768–74.
- Pauling, L., Itano, H.A., Singer, S., and Wells, I.C. (1949). Sickle cell anaemia, a molecular disease. *Science*, 110: 543–48.
- Pellegrini, B., Kerbaui, J., and Fisberg, M. (1995). Zinc, copper and iron and their interrelations in the growth of sickle cell patients. *Archivos Latinoamericanos de Nutricion*, 45(3):198–203.
- Powars, D., Hiti, A., Ramicone, E., Johnson, C., and Chan, L. (2002) Outcome in haemoglobin SC disease: a four-decade observational study of clinical, hematologic, and genetic factors. *American Journal of Hematology*, 70, 206–215.
- Prasad, A.S., Schoomaker, E.B., and Ortega, J. (1975). Zinc deficiency in sickle cell disease. *Clinical Chemistry*, 21(4):582–587.
- Prasad, A.S., Brewer, G.J., and Schoomaker, E.B. (1978). Hypocupremia induced by zinc therapy in adults. *Journal of the American Medical Association*, 240:2166–2168.
- Prasad, A., Abbasi, A., and Rabbani, P. (1981). Effect of zinc supplementation on serum testosterone level in adult male sickle cell anaemia subjects. *American Journal Hematology*, 10:119–127.
- Prasad, A.S., Meftah, S., and Abdullah, J. (1988). Serum thymulin in human zinc deficiency. *Journal of Clinical Investigation*, 82(4):1202–1210.
- Prasad, A. (1998). Zinc and Immunity. *Molecular and Cellular Biochemistry*, 188(1):63–9.
- Prasad, A., Beck, F., Kaplan, J., Chandrasekar, P.H., Ortega, J., and Fitzgerald, J.T. (1999). Effect of zinc supplementation on incidence of infections and hospital admissions in sickle cell disease. *American Journal Hematology*, 61:194—202.
- Prasad, A.S. (2002). Zinc deficiency in patients with sickle cell disease. *American Journal of Clinical Nutrition*, 75(2):181-182.
- Prasad, A.S. (2008). Clinical, immunological, anti-inflammatory and antioxidant roles of zinc. *Experimental Gerontology*, 43(5):370–377.
- Prchal, J.F., and Gregg, X.T. (2005). *Red cell enzymes*. American Society of Haematology Education Program Book. pp 19-23.
- Reed, J.D., Reddin-Lallinger, R., and Orringer, E.P. (1987). Nutrition and sickle cell disease. *American Journal of Hematology*, 24:441–455.

- Richard, C. C., Robert, E., and Scully, M.D. (1997). Case Records of the Massachusetts General Hospital - Case 34-1997. *New England Journal of Medicine*, 337:1293-301.
- Robert, J., Behrens, M., and Tyler, C. (2000). Sickle Cell Disorders: Evaluation, Treatment, and Natural History. *Archives of Internal Medicine*, pp 53 - 59.
- Robert, M., Bookchin, M., and Virgilio, L. (1996). Pathophysiology of Sickle Cell Anaemia. *Hematology/Oncology Clinics of North America*, 10(6): 112-120
- Shah, J.S., Shah, M.B., Goswami, S.S., and Santani, D.D. (2006). Mechanism of action of antiulcer activity of bark extracts of *Manikara hexandra* against experimentally induced gastric ulcers in rats. *Pharmacology Magazine*, 2: 40-45
- Sally C. D., and Lola O. (1997). clinical review: Management of patients with sickle cell disease. *British Medical Journal*, 315:656–660
- Serjeant, G.R. and Serjeant, B.E. (2001). *Sickle cell disease*. Oxford, UK. Oxford University Press, pp 207-208.
- Serjeant, G.R. (1997). Sickle-cell disease. *Lancet*, 350: 725–730.
- Serjeant, G.R., Serjeant B.E., and Milner, P.F. (1969). The irreversibly sickled cell: A determinant of haemolysis in sickle cell anaemia. *British Journal of Haematology*, 17:527-533.
- Sofowora, A. (1996). *Medicinal Plant and Traditional Medicine in Africa*. 2nd Edition. Spectrum Books, Ibadan, Nigeria, pp: 112.
- Sofowora, E.A., and Isaac-Sodeye, W.A. (1971). Reversal of sickling and crenation in erythrocytes by the root of *Fagara xanthoxyloides*. *Lloydia*, 34: 383.
- Stary, F. (1998). *The Natural Guide to Medicinal Herbs and Plants*. Tiger Books International, London. pp. 12-16.
- Stuart, M.J., and Nigel, R.L. (2004). Sickle-cell disease. *Lancet*, 364:1343-60.
- Sultana, C., Shen, Y., Rattan, V., Johnson, C., and Kalra, V.K. (1998). Interaction of sickle erythrocytes with endothelial cells in the presence of endothelial cell conditioned medium induces oxidant stress leading to transendothelial migration of monocytes. *Blood*, 92: 3924–3935.
- Taylor, O. O., Fetuga, B. L., and Oyenuga, V.A. (1983). Accumulation of mineral elements in five tropical leafy vegetables as influenced by nitrogen fertilization and age. *Scientia Horticulturae*, 18:313-322.

- Tamer, L., Gurbuz, P., Guzide, Y., Birol, G., and Fikri, B. (2000). Erythrocyte membrane Na^+ - K^+ / Mg^{++} and Ca^{++} / Mg^{++} Adenosine 5' Triphosphate in patients with sickle cell anaemia. *Turkish Journal of Haematology*, 17(1):23-26.
- Tietz, N. W. (1976). *Fundamentals of Clinical Chemistry*. 2nd Edition. Philadelphia. Published by W. B. Saunders Company. pp.873-944.
- Tindall, H.D. (1968). *Commercial vegetable growing*. Oxford press. London, p. 69
- Tomer, A., Harker, L., Kasey, S., and Eckman, J. (2001a). Thrombogenesis in sickle cell disease. *Journal of Laboratory and Clinical Medicine*, 137: 398–407.
- Tomer, A., Kasey, S., and Connor, W. (2001b). Reduction of pain episodes and prothrombotic activity in sickle cell disease by dietary n-3 fatty acids. *Thrombosis and Haemostasis*, 85(6):966–974.
- Toyin, M.S., Luqman, A.O., and Wahab, A.O. (2008). Aqueous extract of *Telfairia occidentalis* leaves reduces blood sugar and increases haematological and reproductive indices in male rats. *African Journal of Biotechnology*, Vol. 7 (14), pp. 2299-2303.
- Tyler, V.E. (1999). Back to the future. *Journal of National Production*, 62: 1589-1592.
- Ugbor, C. (2006). The effect of vegetable extracts on the antisickling potential of *Aloe vera* www.biochemistry.org/meetings/abstracts/BS2006/BS20060567.pdf. Retrieved on the 14/08/2012
- Usha.P., and Devanand, P. (2011), Efficiency of Different Plant Foliar Extracts on Grain Protection and seed Germination in Maize. *Research Journal of seed Science*, pp 4.
- Underwood, E.J. (1977). *Trace elements in human and animal nutrition*. 4th Edition, New York, NY: Academic Press Inc. 1977; 56–108.
- Van-Burden, T.P., and Robinson, W. C. (1981). Formation of complexes between protein and Tannin acid. *Journal of Agricultural and Food Chemistry*, 1: 77.
- Vichinsky, E., Kleman, K., and Embury, S. (1981). The diagnosis of iron deficiency anaemia in sickle cell disease. *Blood*, 58(5):963–968.
- Vichinsky, E.P., Neumayr, L.D., and Earles, A.N. (2000). Causes and outcomes of the acute chest syndrome in sickle cell disease. *New England Journal of Medicine*, 342: 1855–1865.
- [Wambebe, C.](#), [Khamofu, H.](#), [Momoh, J.](#), [Ekpeyong, M.](#), [Audu, B.](#), [Njoku, O.](#), [Bamgboye, E.](#), [Nasipuri, R.](#), [Kunle, O.](#), [Okogun, J.](#), [Enwerem, M.](#), [Audam, J.](#), [Gamaniel, K.](#), [Obodozie, O.](#), [Samuel, B.](#), [Fojule, G.](#), and [Ogunyale, O.](#) (2001). Double-blind, placebo-controlled, randomised cross-over clinical trial of NIPRISAN in patients

with Sickle Cell Disorder. *International Journal of Phytotherapy and Phytopharmacology* 8(4):252-261

- Weatherall, D., Hofman, K., Rodgers, G., Ruffin, J., and Hrynkow, S. (2005). A case for developing North-South partnerships for research in sickle cell disease. *Blood*, 105: 921–923.
- William, B.M., and Corazza, G.R. (2007). *Hyposplenism: a comprehensive review*. Part I: Basic concepts and causes. *Haematology*, 12:1-13.
- Williams, H., Davies, A., and Chapman, S. (2004). Bone within a bone. *Clinical Radiology*, 59, 132–144.
- Wood, W.G. (1976). Hemoglobin synthesis during human fetal development. *British Medical Bulletin*, pp. 282.
- Wood, K.C., Hebbel, R.P., and Granger, D.N. (2004). Endothelial cell P-selectin mediates a proinflammatory and prothrombotic phenotype in cerebral venules of sickle cell transgenic mice. *American Journal of Physiology and Heart Circulation Physiology*, 286: 1608–1614.
- Wood, K.C., Hebbel, R.P., and Granger, D.N. (2005). Endothelial cell NADPH oxidase mediates the cerebral microvascular dysfunction in sickle cell transgenic mice. *Federation of American Societies for Experimental Biology*, 19: 989–991.
- World Health Organisation, (2001). *Geneva Legal Status of Traditional Medicine and Complementary/Alternative Medicine: A Worldwide Review*. World Health Organisation, Geneva, pp: 129-143.
- World Health Organization, (2006). Sickle cell anaemia. *Report by the secretariat. 59th World Health Assembly*, pp 16.
- World Health Organisation, (2008) *Fact Sheet No 134: Traditional Medicine*
- World Health Organisation, (2011). *Produced by the Centre for Genetics Education*. <http://www.genetics.edu.au>. pp 295-300
- World Health Organisation, (2012). Source: <http://www.vanguardngr.com/2012/06/pain-and-penury-of-sickle-cell-disease-not-beyond-science/>
- Wun, T., Paglieroni, T., Tablin, F., Welborn, J., Nelson, K., and Cheung, A. (1997). Platelet activation and platelet–erythrocyte aggregates in patients with sickle cell anaemia. *Journal of Laboratory and Clinical Medicine*, 129: 507–516.
- Yubisui, T., and Takeshita, M. (1980). Reduction of methaemoglobin through flavin at the physiological concentration by NADPH-flavin reductase of human erythrocytes. *Journal of Biochemistry*, 87(6): 1715-1720.

Zehtabchi, S., Sinert, R., and Rinnert, S. (2004). Serum ionized magnesium levels and ionized calcium-to-magnesium ratios in adult patients with sickle cell anaemia. *American Journal of Hematology*, 77(3):215–222.

Zemel, B.S., Kawxhak, D.A., Fund, E.B., Ohene-Frempong, K., and Stallings, V.A. (2002). Effect of zinc supplementation on growth and body composition in children with sickle cell disease. *American Journal of Clinical Nutrition*, 75:300-7.

**SICKLE – CELL MORPHOLOGY FOLLOWING DIFERENT TREATMENT
APPENDIX**

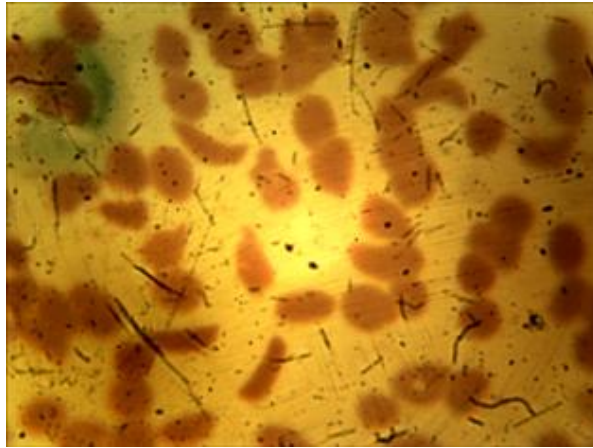


Plate I: Sodium metabisulphite - induced sickling

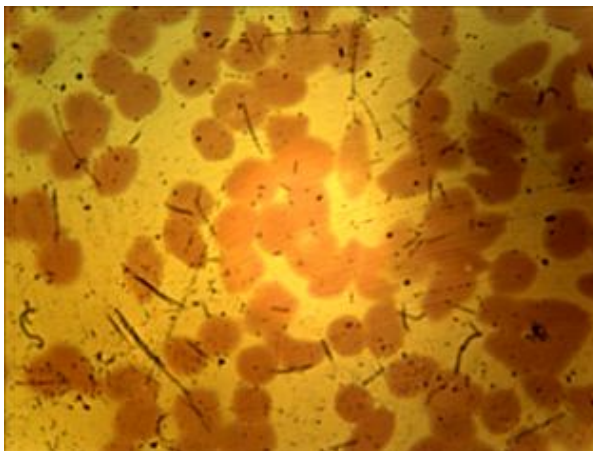


Plate II: PABA-treated Sickled Cells

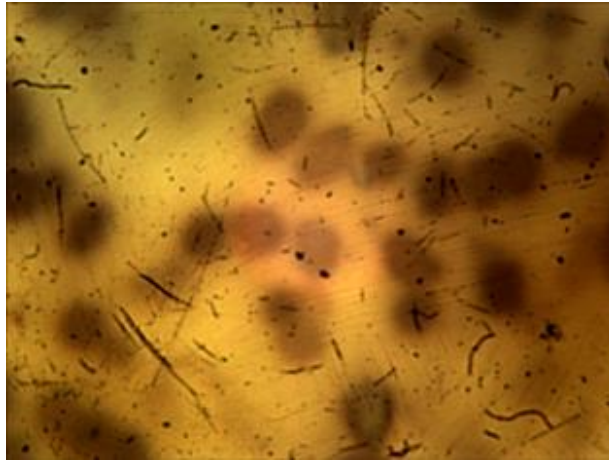


Plate III: Aqueous Leaves extract-treated Sickled Cells

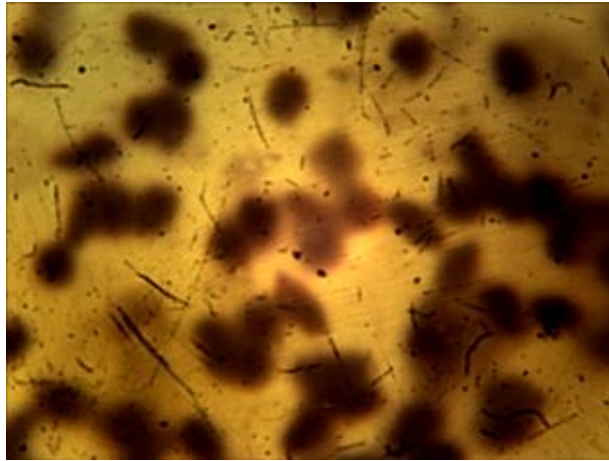


Plate IV: Methanolic Leaves extract-treated Sickled Cells

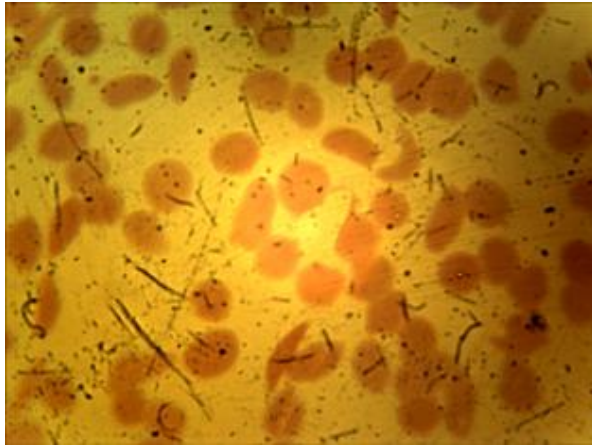


Plate V: Normal Saline Treated Sickled Cells (Negative control)

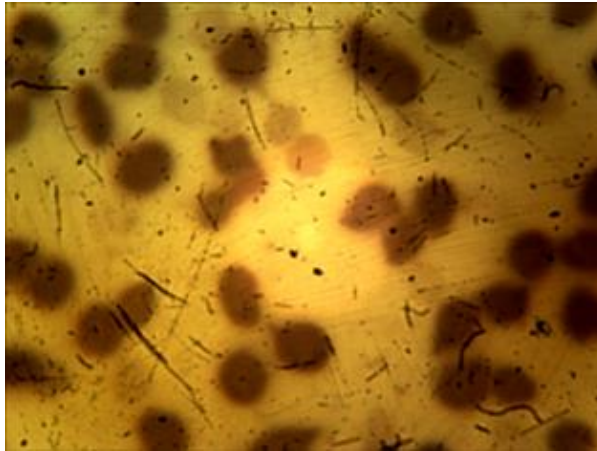


Plate VI: Partially Purified fraction-treated sickled cells