STUDIES ON THE INDUCTION OF FOETAL HAEMOGLOBIN AND MOLECULAR MECHANISMS OF THE UP-REGULATION OF THE γ-GLOBIN GENE IN HUMANS USING PLANT INDUCERS OF FOETAL HAEMOGLOBIN

BY

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DECLARATION

I declare that the work in this Dissertation entitled "Studies on the Induction of Foetal Haemoglobin and Molecular Mechanisms for the Up-regulation of the γ-globin gene in Humans using Plant Inducers of Foetal Haemoglobin" 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 work has been presented for another degree or diploma at this or any other institution.

Idowu Asegame AIMOLA _____ _____

CERTIFICATION

This Dissertation entitled "Studies on the Induction of Foetal Haemoglobin and Molecular Mechanisms for the Up-regulation of the γ-globin gene in Humans using Plant Inducers of Foetal Haemoglobin" by Idowu Asegame AIMOLA meets the regulation governing the award of the degree of PhD in Biochemistry of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to God who's inspiration, guidance, support and loving mercies has propelled me this far academically.

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To all my readers' thank you for believing that Science can make a difference in the World and that our research helps make the World a better place.

To God almighty is the glory forever.

ABSTRACT

Five plants used in ethnomedicine to treat sickle cell anemia (SCA) were screened for their ability to induce Foetal Haemoglobin (HbF) synthesis in primary human Erythroid progenitor stem cells (hEPSCs) isolated from SCA individuals. A systematic literature search using MEDLINE, CAB Abstracts, EMBASE and Google Scholar was used to rank plants to be included in this study using the Research Initiative on Traditional Anti-Malaria Method (RITAM) scoring criteria. RITAM score revealed *Telfairia occidentalis* (TO) leaves, *Azadiractha indica* (AI) leaves, *Adansonia digitata* (AD) seeds, *Terminalia catappa* (TC) leaves and *Sorghum bicolor* (SB) leaves with the highest potentials for foetal hemoglobin induction and thus were selected for *in vitro* screening of their proliferative, differentiation, HbF induction and cytotoxicity effects on hEPSCs. The aqueous extracts of TO, AI, TC and SB induced differentiation of peripheral blood mononuclear cells (PBMCs) to erythroid Burst forming erythroid units (BFUe) and higher synthesis of foetal hemoglobin compared to the controls (erythropoietin (EPO) 1U/µl and HU 1µg/ml). Following fractionation using *n*-hexane, ethyl acetate, petroleum ether, acetone, ethanol and distilled water, the solvent fractions revealed varying effects on hEPSCs differentiation and HbF synthesis. TO ethyl acetate fraction and TC distilled water fraction (TCDWF) and *n*-hexane fractions (A and B) showed significantly ($P<0.05$) higher HbF induction of 37.8, 38.5, 28.5 and 20.8% respectively compared to the other plant fractions. TCDWF revealed a 6.8 and 9.2 fold HbF increase in both EPO-dependent and EPO-independent hEPSCs cultures respectively which was higher compared to other active fractions and controls. Caspase-3 activity was significantly ($P \leq 0.05$) by all the active fractions in EPO-dependent hEPSCs, while in EPO-independent hEPSCs, the active fractions appeared to significantly ($P \leq 0.05$) lower caspase-3 activity compared to EPO control. TCDWF fraction however, was differentially cytotoxic causing release of LDH in EPO-dependent hEPSCs while no LDH release was detected when EPO-independent hEPSCs were treated with the active fraction suggesting that TCDWF was apparently cytotoxic to EPO-dependent hEPSCs while being protective to EPO-independent cells. GC-MS of un-derivatized *T. catappa* HbF inducing fraction revealed several potential HbF inducing compounds (9-octadecenoic acid (Oleic acid), 2-hexadecenoic acid (2-HDA), Cis-vaccenic acid (CVA), Apigenin, Docosahexadecenoic acid (DHA), Eicosapentanoic acid (EPA) and Gentisic acid) which were further screened for their γ-globin and HbF inducing capacity on K562, JK-1 and primary transgenic mice bone marrow stem cell (TMbmEPSCs) cultures. Apigenin, DHA, Oleic acid and 2-HDA induced differentiation of erythroleukemic K562 cells and TMbmEPSCs. Apigenin, DHA and 2-HDA also induced γ-globin gene expression in K562 cells. Apigenin was most effective at 75µM while DHA and 2-HDA were effective at 6.5µM and 17µM respectively. Alkaline denaturation assay revealed that 2-HDA increased HbF synthesis significantly ($P<0.05$) higher than the un-induced controls. 2-HDA induction of K562 HbF was detected 24 hours post induction. Cis-vaccenic acid (CVA) similarly induced differentiation of K562 cells in a concentration

dependent fashion but failed to induce γ-globin gene expression in this cell type. CVA (50μM) did not significantly ($P>0.05$) alter JK-1 cell survival as assessed by Trypan blue staining. Likewise, CVA at 50μM induced JK1 and TMbmEPSCs γ-globin gene expression. CVA induced γ-globin expression and increased γ-globin mRNA incrementally as assayed by qRT-PCR. CVA also induced significantly ($P<0.05$) increased JK-1 HbF synthesis. CVA (50μM) did not alter EKLF1 gene expression in JK-1 cells however we observed over a 100 fold increase in Bcl11a gene expression in JK-1 cells. CVA elevated TMbmEPSCs EKLF gene 8 fold relative to the un-induced cells. Erythropoietin (EPO) pre-differentiation of JK-1 cells before CVA induction did not significantly ($P>0.05$) alter CVA induced differentiation of JK-1 cells, although a 2 fold increase in γ-globin gene expression was observed in EPO (2U/ml) pre-differentiated cells compared to un-differentiated JK-1 cells. Paradoxically, a significant inhibition of γ-globin gene expression was observed when CVA (50μM) was added simultaneously with EPO to either EPO pre-differentiated or un-differentiated JK-1 cultures. Inhibition of CVA biosynthesis using 40μM Cycloate suppressed the γ-globin inductive effects of CVA in JK-1 and TMbmEPSCs. In addition, inhibition of fatty acid elongase V (Elovl5) using 10μM Isoxyl also suppressed the γ-globin inductive effects CVA in JK-1 and transgenic mice bone marrow progenitor stem cells. CVA (50μM) failed to completely rescue the inhibitory effects of Cycloate and Isoxyl 48 hours post inhibition in JK-1 cells, suggesting that CVA induced γ-globin expression is mediated through a downstream biosynthetic product of CVA. Isoxyl inhibition significantly ($P<0.05$) enhanced CVA induced BFUe potential of TMbmEPSCs while Cycloate inhibition significantly ($P<0.05$) reduced that potential. The results suggest that CVA directly induce hEPSC differentiation and indirectly modulate β-globin gene expression in humans through mechanisms which involve increased transcription and translation of the γ-globin gene, with minimal cytotoxic effects on human Our findings provide important clues for further evaluations of CVA as a potential HbF therapeutic inducer and a clinical substitute of HU for SCA management.

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LIST OF ABBREVIATIONS USED

SCA	Sickle Cell Anemia
HbF	Foetal Haemoglobin
FDA	Food and Drug Administration
HbAS	Haemoglobin AS
SCD	Sickle Cell Disease
EKLF	Erythroid Kruppel like Finger
LCR	Locus Control Region
HbA	Haemoglobin A
HbA_2	Haemoglobin A_2
HDAC1	Histone Deacetylase 1
SWI/SNF	Switch/Sucrose non-Fermenting Complex
NF-E2/P45	Nuclear Factor E2
COUP-TFII	COUP transcription factor 2
BFUe	Erythroid burst forming unit
DMSO	Dimethyl Sulfoxide
MEL	Murine erythroleukemia
CFU-GM	Granulocyte Macrophages Colony forming unit
CFU-E	Erythroid colony forming unit
CFU-Meg	Megakaryocyte colony forming unit
CFU-Mix	Mixed colony forming unit
HSCs	Haematopoietic stem cells
BM	Bone Marrow
FACs	Flourescence Activated Cell Sorting
CSF	Colony stimulating factor
G-CSF	Granulocyte colony stimulating factor
M-CSF	Macrophages colony stimulating factor

EPO	Erythropoietin
SCF	Stem Cell Factor
SF	Steel Factor
MCGF	Mast Cell Growth factor
IL	Interleukin
cDNA	complementary DNA
IMDM	Iscove Modified Dulbecco Medium
RPMI	Rosewell Park Memorial Institute
MEM	Minimal Essential Medium
FBS	Foetal Bovine Serum
GAPDH	Glyceraldhyde-3-phospahte dehydrogenase
BCL11A	B-cell lymphoma A
RITAM	Research Initiative on Traditional Anti Malaria
MEDLINE	Medical Literature Analysis and Retrieval System Online
CAB	Commonwealth Agricultural Bureaux
EMBASE	Excerpta Medica dataBASE
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
TRI	Trizol
PCR	Polymerase Chain Reaction
IC_{50}	Inhibitory Concentration at 50%
HU	Hydroxyurea
TCDWF	*Terminalia catappa* distilled water fraction
hEPSCs	Human Erythroid Progenitor Stem Cells
LDH	Lactate Dehydrogenase
TCNHB	*Terminal catappa n*-Hexane b
GC	Gas Chromatography
NIST	National Institute of Standards and Technology

CVA	Cis-Vaccenic acid
MGG	May Grumwald Giemsa
2-HDA	2-hexadecenoic acid
OL	Oleic acid
AP	Apigenin
DHA	Docosahexanoic acid
EPA	Eicosapentanoic acid
SA	Stearic acid
GA	Gentisic Acid
mRNA	messenger RNA
TOEAF	*Terminalia catappa* ethyl acetate fraction
Elovl5	Fatty Acid Elongase 5
TMbmEPSCs	Transgenic mouse bone marrow erythroid progenitor stem cells.
Akt	Protein Kinase B
FOXO1	Forkhead box protein O1
mTORC	Mammalian target of Rapamycin
CLA	Cojugated Linoleic acid

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Sickle Cell Anaemia (sometimes referred to as sickle cell disease) refers to the homozygous condition in which an individual inherits the abnormal haemoglobin S gene or its clinically significant variants from both parents (Chowning, 2000). The haemoglobin S genes arise from a mutation in the β-globin gene which leads to a sickle shaped haemoglobin molecule prone to precipitation and clogging of narrow blood vessels in low oxygen tensions and leads to a wide range of pathophysiological manifestations in the patients. The clinical symptoms of Sickle cell anaemia (SCA) is characterized by anaemia, severe pain and potentially life-threatening complications such as malaria, bacterial sepsis, splenic sequestration, acute chest syndrome (pneumonia), stroke and chronic organ damage (Bonds, 2005; Aliyu *et al*., 2008a). All these and other manifestations characterized by; painful crises, stroke, paralysis, avascular necrosis of the femoral head, loss of splenic function and pulmonary hypertension result from chronic hemolysis and intermittent episodes of vascular occlusion that cause both acute and chronic tissue injury and organ dysfunction (Aliyu, *et al*., 2008a). In general, both children and adults with sickle cell anaemia are highly vulnerable to infections (Davies and Oni, 1997; Ware, 2010). This vulnerability is the result of repeated splenic damage by vaso-occlusion. All individuals with the disease, especially young children, are susceptible to malaria and bacterial infections especially by capsulated organisms resulting in sepsis, pneumonia and meningitis, (Booth *et al*., 2010). For most patients the incidence of complications can be reduced by simple protective measures such as prophylactic administration of penicillin, folic acid

supplementation and antimalarials, avoiding climatic extremes, and contact as early as possible with a specialist centre (Booth *et al*., 2010). These precautions are most effective if susceptible infants are identified at birth which is a very scarce (if available) medical practice in Nigeria.

All forms of sickle cell disease are major public health problems which cause over a million deaths each year. Sickle cell disease carries lifelong health considerations (Weatherall, 2010). Generally, 10% of children with sickle cell diseases die before their 5th birthday, most often due to malarial and several other bacterial infections (Williams *et al*., 2005)

To date sickle cell anaemia can be cured by only bone marrow transplant which apart from its attendant risks (Brodsky *et al*., 2008) is also not affordable to most of the affected people who need therapy especially in the developing countries. Recently the United States FDA approved hydroxyurea as a treatment modality for sickle cell anaemia and β-thalassemia (Platt, 2008; Chung *et al*., 2008). It has been found to increase the intra-erythrocytic content of haemoglobin F which has also been previously demonstrated to be beneficial in SCA by reducing episodes of pain crisis and improved quality of life, but its use is limited due to its low specificity and its potential terratogenicity (Quinn *et al*., 2004). Fetal haemoglobin (HbF) has been shown previously to ameliorate the symptoms and improve the quality of life of individuals living with SCA and β-thalasesemia (haemoglobinopathies),(Ware *et al*., 2002; Ataga, 2009; Sankaran, 2011) and hence its induction in individuals with either of the haemoglobinopathy is considered a novel therapeutic approach.(Ataga, 2009.) HbF is the major haemoglobin in human zygotes within the second and third trimesters of gestation and its expression continues until 4-6 months post natal before its synthesis is

down regulated to about 1-2%, and β-haemoglobin becomes the predominant haemoglobin during adult life (Oneal *et al*., 2006; Wang, *et al*., 2011). The mutation responsible for SCA is resident on the β-globin gene, hence infants who have inherited the homozygous for SS gene do not express the SS phenotype at infancy because the faulty β-globin gene is still silent and not being expressed until 4-6 months post natal when the γ-globin gene is switched off and the adult β-globin gene is turned on. Several studies have demonstrated the clinical benefits of sustained fetal haemoglobin (hereditary persistence of fetal haemoglobin) synthesis in which the results of genome wide association studies implicate gene mutations which affect *KLF1* and *BCl11a* gene expressions or drug induced reversal of HbF synthesis from adult haemoglobin in β-thalassemia and SCA individuals (Sankaran, 2011; Yien & Bieker, 2012; Xu *et al*., 2013). Hence current chemotherapeutic approaches to the management of SCA have focused on identifying novel agents that can increase or reactivate the synthesis of HbF in adult β-thalassemia and SCA individuals (Bianchi, *et al*., 2009). Till date, hydroxyurea remains the only FDA approved treatment regimen for SCA despite reports of its low specificity and acceptability among SCA patients and its inherent bone marrow suppression activity.(Sankaran, 2011). Its impacts on organ damage, stroke and carcinogenesis on long term use in SCA patients is also not well known. (Kinney *et al*., 1999; Steinberg *et al*., 2003; Ware, 2010). Hydroxyurea has been shown to induce HbF through the soluble Guanyly cyclase (sGC) mechanism (Cokic *et al*., 2003).

Primary erythroid progenitor stem cells have been demonstrated as the best *in vitro* model for the screening of HbF inducing agents (Bianchi *et al*., 2009; Zein *et al*., 2010). Fibach and others developed a 2 phase liquid culture system which mimics very closely the *in vivo* erythropoietin dependent and independent erythroid progenitors

proliferations and differentiation *in vitro* (Fibach, *et al*., 1989; Bianchi *et al*., 2009). This allows for effective *in vitro* screening of potential HbF inducing agents.

Isolation of novel compounds with therapeutic potential from the plants has been emphasized due to the advantages of these compounds in terms of potency and lesser side effects as reported previously (Willcox *et al*., 2011; Chinsembu and Hedimbi, 2010; Okpuzor *et al*., 2008). Unfortunately, the availability of plant compounds for the induction of fetal haemoglobin synthesis in erythroid progenitor stem cells is very scanty (Bianchi *et al*., 2009). This is despite the abundance of literature on the efficacies of several plant remedies used in the management of SCA (Okpuzor *et al*., 2008; Agyekum *et al*., 2011).

1.2 STATEMENT OF PROBLEM

Sickle cell anaemia is a debilitating disorder that affects over 20 million people globally with over 12 million of them in sub Saharan Africa (Allison, 2007; Fleming, 1989). Nigeria is frequently quoted to have the highest incidence of SCA in the world; over 4 million people are known to live with the disease (Aliyu *et al*., 2008a). In addition global human migration over the years has had significant impacts on the distribution of the haemoglobin S gene and consequently the global burden of the disease (Piel *et al*., 2013). Also sickle cell anaemia has been categorized a public health issue by the world health organization (Grosse, *et al*., 2011). Estimates place its contribution to the number of under 5 deaths as 2% annually in sub-Saharan Africa amounting to nearly 3 million deaths annually (WHO, 2011). Bone marrow transplant remains the only cure for sickle cell disease which provides for new bone marrow cells capable of producing normal β-globin chains; however this procedure is limited due to a lack of compatible donors in addition to its attendant risk of complications. The

procedure is also expensive and out of the reach of many who need it (Siddiqui and Ahmed, 2003; Steinberg *et al*., 2003). Although several inducers of foetal haemoglobin have been identified (Bianchi *et al*., 2009; Agyekum, 2011), most have quite low efficacy and hydroxyurea remains the only FDA approved drug for the management of SCA in spite of its low specificity and potential terratogenicity on long term use (Pace and Zein, 2006). Normal stage specific expression of haemoglobin shifts from foetal haemoglobin to adult haemoglobin shortly after birth which leads to the increased production of the faulty β-globin chains in sickle cell anemic patients. This stage specific switch has been shown to be strongly linked and controlled by the EKLF gene which remains fully turned on and active during expression of the adult β-globin chain (Zhang and Bieker, 1998). Down regulation of the EKLF gene has been shown to reactivate the production of foetal haemoglobin (Stamatoyannopoulos, 2005; Bank, 2006) which has been shown to ameliorate the symptoms of sickle cell anaemia and β-thalassemia (Ataga, 2009; Sankaran *et al*., 2008), which makes EKLF gene a good potential drug target for the therapy of sickle cell anaemia.

1.3 JUSTIFICATION

- Induction of foetal haemoglobin in SCA anaemia patients promises long term therapeutic benefits with less side effects and improved quality of life in these patients as the induction of foetal haemoglobin synthesis has previously been shown to improve the health and reduce frequency of crisis in SCA and β-thalassemia patients (Sankaran *et al*., 2008; Ataga, 2009; Bianchi *et al*., 2009). Hydroxyurea has been shown to induce γ-globin gene expression and consequently HbF in pediatric and adult patients, its long-term effect on organ, damage, stroke, and carcinogenesis remains uncertain in addition to its

suppression of bone marrow function (Kinney *et al*., 1999). Thus, there is a dire need to screen and identify novel safe agents capable of reversing the Foetal to adult haemoglobin switch with a bid to reactivating the synthesis of HbF in SCA and β-thalassemia. Insights gained could lead to a cure for sickle cell anaemia and other related genetic disorders.

The use of plant based therapies for the treatment of SCA through the induction of foetal haemoglobin is rare but holds several potentials (Bianchi *et al*., 2009; Agyekum *et al*., 2011).

This will also enable better understanding of the expression of the γ-globin gene, and also shed more light on the modulation of other similar single nucleotide polymorphisms (SNPs).

1.4 AIM OF STUDY

Primarily the study seeks to identify and assay novel plant component(s) capable of reactivating the production of fetal haemoglobin in humans. The study also seeks:

- To improve the understanding of the control of silencing of the human γ-globin gene.
- To understand the interplay of genes, proteins and other molecules through which these compound(s) elicit their control.

1.5 OBJECTIVES

This study was designed with the following objectives in mind

- Isolate and maintain human erythroid progenitor cultures from human peripheral blood of sickle cell individuals.
- Screen for plant(s) which can induce foetal haemoglobin synthesis in human erythroid progenitor cultures
- Purification and identification of plant fraction(s) responsible for foetal haemoglobin induction.
- Partial characterization and identification of plant component(s) responsible from the active plant fraction(s) for foetal haemoglobin induction.
- Screening of potential foetal haemoglobin inducing plant compounds on K562, JK-1 and transgenic mice bone marrow progenitor stem cells differentiation and foetal haemoglobin synthesis.
- Assay of potential foetal haemoglobin inducing compounds on K562, JK-1 and transgenic mice bone marrow progenitor stem cells γ-globin, β-globin, EKLF and Bcl11a gene expression
- Elucidate globin modulatory activity and mechanisms of foetal haemoglobin inducing plant active compound(s).

CHAPTER 2

LITERATURE REVIEW

2.1 HAEMOGLOBINOPATHIES

Naturally occurring mutations on the globin genes loci lead to improper synthesis of haemoglobin's polypeptides.

Sickle Cell Anaemia and beta thalassemia (sometimes referred to as haemoglobinopathies) refers to the condition in which an individual inherits the abnormal haemoglobin S gene or its clinically significant variants from both parents (Booth *et al*., 2010). Sickle cell anaemia is inherited as an autosomal recessive disease from both parents, whereas individuals heterozygous for sickle cell haemoglobin (HbAS) are generally asymptomatic (Anstey *et al*., 1999; Weinberg, 2005). The β thalassemia syndromes are caused by more than 175 molecular mutations affecting the β globin gene complex, which result in decreased synthetic ratio of β to α globin chains (Perrine, 2005).

While thalassemia patients exhibit a deficiency of α or β globin synthesis, sickle-cell anaemia patients express aberrant haemoglobin. The most severe form of thalassemia is beta-thalassemia major (homozygous) which is characterized by the complete lack of the β-globin chain expression, thus leading to the formation of abnormal haemoglobin which leads severe anaemia. Studies show that over 75% of individuals with sickle cell anaemia or β-thalassemia major if left untreated die within 5 years of age due to complications resulting from anaemia, caused by point-mutations in the globin gene these disorders highlight the complexity and the necessity to understand the key elements governing the formation of the β-globin polypeptide (Piel *et al*., 2013). Hence attempts are being made to use pharmacological agents to treat sickle cell anaemia and thalassemia by increasing foetal haemoglobin content in adults by targeting Gamma (γ)-globin expression. Over-expressed γ-globin chains would equilibrate the deficit in

β-globin and hence correct the shortfall in adult β-globin chains and consequently the anaemia. Toward this goal, different strategies have led to the development of several drugs (Pace & Zein, 2006; Bianchi, *et al*., 2009).

2.2 OCCURRENCE OF SICKLE CELL ANAEMIA

All forms of sickle cell disease are major public health problems which cause over a million deaths each year. Sickle cell disease carries lifelong health considerations (Thein, *et al*., 2009). Generally, 10% of children with sickle cell diseases die before their 5th birthday, most often due to malarial and several other bacterial infections (Williams *et al*., 2005). Sickle cell disease has its greatest frequency in Africa and Nigeria in particular where it is believed to have originated (Piel *et al*., 2013). Over 4 million people are known to be afflicted by Sickle cell disease in Nigeria (Aliyu *et al* 2008a; Aliyu *et al*., 2008b).

Haemoglobin S is widely distributed; it is found most frequently in tropical Africa (Aliyu, *et al*., 2008a), with a gene incidence of up to 40% in some tribes. Sickle-cell anaemia contributes the equivalent of 5% of under-five deaths on the African continent, and up to 16% of under-five deaths in individual West African countries (Aliyu *et al*., 2008a). It occurs among the descendants of the black race in the United States, Central and South America, and the West Indies, with a gene incidence of about 7%. It is also found in Sicily; Greece, southern Turkey, Southern Arabia, and India (Carter and Mendis, 2002).

The higher frequency of the sickle cell trait in Africa is thought to be due to the protective effect of haemoglobin S on malaria as previously demonstrated (Gong *et al*., 2013). Several studies have also revealed that malaria is a major risk factor for individuals living with sickle cell disease (Gong *et al*., 2013).

2.3 MOLECULAR BASIS OF SICKLE CELL ANAEMIA

The haemoglobin S gene arises from the mutation in the β-globin gene which led to the substitution of the 17th nucleotide of the β-globin gene from Adenine to Thymine leading ultimately to the substitution of the 6^{th} amino acid of the β-globin chain from Glutamic acid to Valine which resulted to a sickle shaped haemoglobin molecule prone to precipitation and clogging of narrow blood vessels in low oxygen tensions. The sickle cell gene and all its variants (S, C, D, E, β-thalassemia etc) are recessive and known to follow the Mendelian inheritance pattern. Several mutations are known to be associated with the sickle cell and beta thalassemia mutation as reported previously (Blouin *et al*., 2000; Thein *et al*., 2009).

2.4 CLINICAL PATHOLOGY OF SICKLE CELL ANAEMIA

The clinical phenotype of SCA varies widely, influenced by additional genetic factors, and is characterized by anaemia, severe pain and potentially life-threatening complications such as malaria, bacterial sepsis, splenic sequestration, acute chest syndrome (pneumonia), stroke and chronic organ damage (Bonds, 2005; Aliyu, *et al*., 2008a). All these and other manifestations characterized by; painful crises, stroke, paralysis, avascular necrosis of the femoral head, loss of splenic function and pulmonary hypertension result from chronic hemolysis and intermittent episodes of vascular occlusion that cause both acute and chronic tissue injury and organ dysfunction (Aliyu, *et al*., 2008b). The symptoms of sickle cell anaemia are wide-ranging, affecting many different organs of the body. Haemoglobin S containing red blood cells usually have a shorter life span of between 20-30 days as against 120 days of the haemoglobin A containing red blood cells. The sickle-celled haemoglobin has also been shown to polymerize and cause clots in low oxygen tension which can lead to

periodic episodes of painful crises and could also result in organ damage due to oxygen deprivation (Bonds, 2005; Uzunova *et al*., 2010). A drop in haemoglobin level can cause fatigue, pallor, shortness of breath. The drop is frequently due to the rapid hemolysis of the haemoglobin S which is associated with their reduced lifespan. Hemolysis is associated with a rise in bilirubin clinically seen as jaundice due to its deposition on the skin, sclera and mucous membrane (Stillman, 1982).

In general, both children and adults with sickle cell anaemia are highly vulnerable to infections (Kinney *et al*., 1999; Ware *et al*., 2002). This vulnerability is the result of repeated splenic damage by vaso-occlusion. All individuals with the disease, especially young children, are susceptible to malaria and bacterial infections especially by capsulated organisms resulting in sepsis, pneumonia and meningitis, (Booth *et al*., 2010). For most patients the incidence of complications can be reduced by simple protective measures such as prophylactic administration of penicillin, folic acid supplementation and antimalarials, avoiding climatic extremes, and contact as early as possible with a specialist centre (Booth *et al*., 2010). These precautions are most effective if susceptible infants are identified at birth which is a very rare (if available) medical practice in Nigeria.

2.5 MANAGEMENT OF SICKLE CELL ANAEMIA

To date sickle cell anaemia can be cured by only bone marrow transplant which apart from its attendant risks (Brodsky *et al*., 2008) is also not affordable to most of the affected people who need therapy especially in the developing countries. Other therapeutic measures for the disease are symptomatic and aimed at maintaining the

steady state or treating crisis condition of the patient. The commonly used management drugs are folic acid supplements and anti-malarial prophylactic agents (Chattopadhyay *et al*., 2007). Additional drugs are determined by the physician's diagnosis. An effective management therapy depends on accurate and early diagnosis of the disease. Hydroxyurea is approved by the United States FDA as a treatment modality for sickle cell anaemia and β-thalassemia (Platt, 2008; Chung *et al*., 2008). It has been found to increase the intra-erythrocytic content of haemoglobin F which has also been previously been demonstrated to be beneficial in SCA by reducing episodes of pain crisis and improved quality of life, but its use is limited due to its low specificity and its potential terratogenicity (Quinn *et al*., 2004).

2.6 HUMAN HAEMOGLOBIN GENE STRUCTURE AND EXPRESSION

Human adult haemoglobin is a complex tetramer with a prostethic heme group, it is a multi subunit metalloprotein consisting of two types of globin chain subunits alpha (α) and beta (β) globins (Uzunova *et al*., 2010) see Figure 2.1. The α-globin gene (26kb) which is located on chromosome 16 consists of 5 structurally related and developmentally controlled α-like globin genes; embryonic Zeta (ζ) gene, Psi-alpha 1 (ψα1) and Psi-zeta (ψζ) and two α genes (α2 and α1) (Figure 2.2). The embryonic zeta globin gene is functional and the earliest to be expressed during development while the pseudo genes Psi-alpha 1 and Psi- zeta are non-functional and not normally expressed. The two alpha genes α1 and α2 are expressed from 7 weeks and throughout the individual's life. The α regulatory element (α-MRE) which is located 40 KB upstream of ζ, acts as a distal enhancer. The β-globin gene cluster (Figure 2.3) is located on Chromosome 11 and contains four clustered β-like globin genes arranged in the order

in which they are expressed during growth and development: one embryonic gene Epsilon (ε), two fetal genes Gamma (Gγ and Aγ) and two adult genes Beta (β) and Delta (δ) (Ribeiro and Sonati, 2008). The beta globin gene locus contains an upstream DNA regulatory sequence (Locus Control Region (LCR), which is formed by five DNase I hypersensitive (HS) domains, this region has been shown to be key to the high level of expression of β-globin genes (Liang *et al*., 2009).

Pace and Zein (2006) demonstrated that the Erythroid Kruppel like factor (a red cell specific activator) whose presence is crucial for the establishment of the correct chromatin structure and high-level transcriptional induction of adult β-globin gene. It contains three C2H2 zinc fingers that discriminate between CACCC elements present at erythroid promoters (Siatecka *et al*., 2007). Its preferential binding to the adult β CACCC element over the elements at the murine and human embryonic (ε) or human fetal (γ) globin genes further emphasizes its requirement for normal transcriptional expression of the β-globin gene. Studies have shown that EKLF-knockout sickle cell transgenic mice die at the time of the switch to adult expression because of a profound β-thalassemia, and EKLF-knockout embryonic stem cells do not contribute to the red cell population in chimeric mice(Coghill *et al*., 2001; Liang *et al*., 2009; Yien and Bieker, 2012). However, further analyses of EKLF-knockout mice, revealed not only that human β-globin transcripts were absent from the mouse fetal liver but also that γ-globin transcripts were 5-fold higher and persisted beyond the level seen in the presence of EKLF (Liang *et al*., 2009). Additionally, the absence of EKLF led to a complete lack of DNase-hypersensitive site formation at both the transgenic and endogenous β-globin promoters, and diminution of HS3 at the globin LCR (Coghill *et al*., 2001). These data indicate that EKLF is a major player in activating adult β-globin expression, not only by its transcriptional activation properties but also by its ability to

generate the proper chromatin configuration within part of the β-like globin locus and thus facilitate the silencing of the γ-globin promoter. These results demonstrate that EKLF is critical for normal transcription of the adult β-globin gene within the β-globin gene cluster and suggest a mechanism by which EKLF acts as an integrator of remodeling and transcriptional components to alter chromatin structure and induce adult β-globin expression within the β-like globin cluster and potentially the synthesis of foetal haemoglobin.

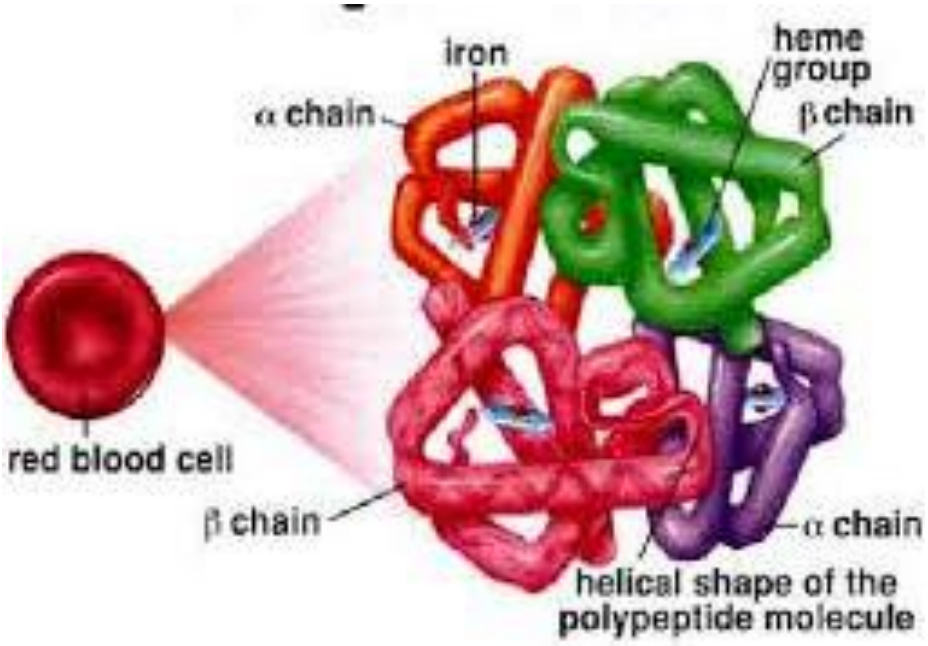


Figure 2.1: Structure of the Haemoglobin Molecule

www.Humanbiology2011.com

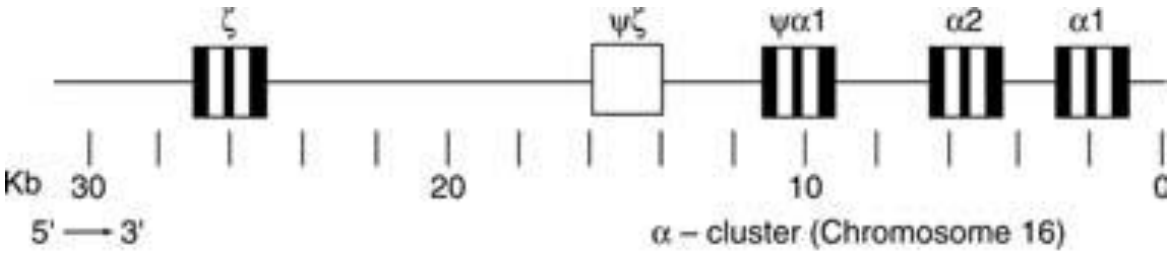


Figure 2.2: Structure of the α-globin gene

http://www.cliffsnotes.com/sciences/biology/biochemistry-ii/eukaryotic-genes/eukaryotic-gene-structure

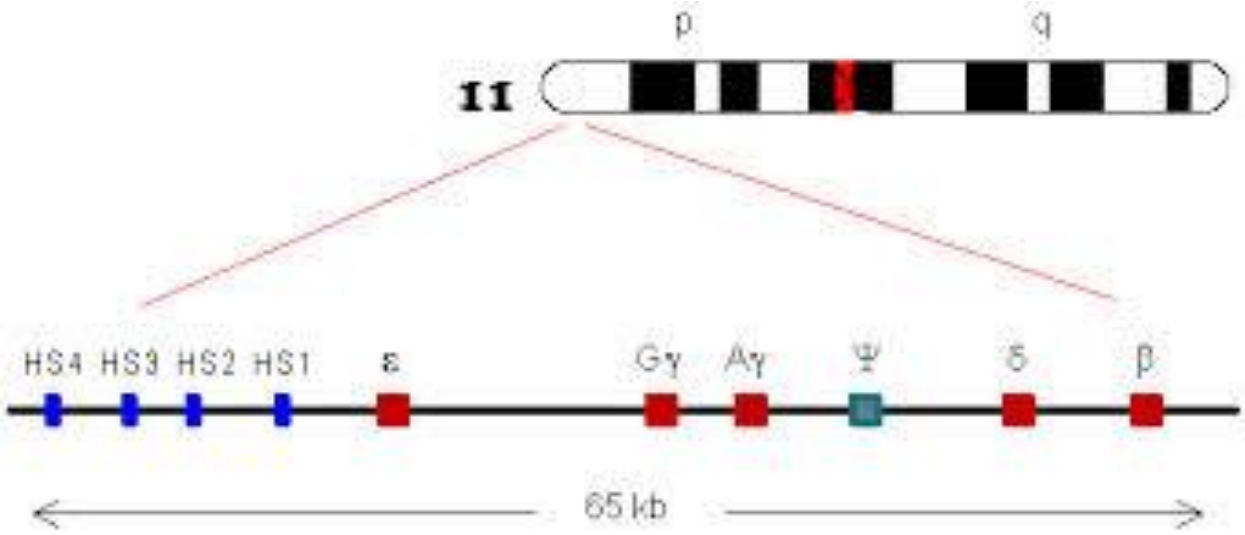


Figure 2.3: Chromosome 11 and the beta-globin gene structure

www.sampadcity.com

2.7 FOETAL HAEMOGLOBIN SWITCH

Humans survive on different types of haemoglobin at different stages of their development. Embroyonic haemoglobin which can be either Gower type 1 (ζ2 ε2) which consist of two embryonic α-chains (ζ) with two embryonic β-globin chains (ε),

Gower type 2 (α2 ε2) which consist of two embryonic alpha chains (α) with two embryonic β-globin chains (ε) or haemoglobin Portland (ζ2 γ2). As the site of hematopoiesis switches to the fetal liver, the fetal globin genes become active and the embryonic globin chains are gradually replaced with fetal haemoglobin HbF (α2 γ2); this marks the first developmental switch. A few months after birth, a second switch takes place and the bone marrow becomes the main site of red blood cells production. In adults, haemoglobin HbA (α2 β2) consists of 98 % of total haemoglobin along with HbA2 (α2 δ2) and HbF, which continues to be produced at low level throughout life (Tavian and Péault, 2005).

Human globin genes switching are tightly regulated by mechanisms not fully understood. At the β-globin locus various trans-acting (erythroid specific and ubiquitous transcription factors and cofactors) specifically interact with the cis-acting elements of the β-globin gene (promoters, enhancers and locus control region) to modulate gene activation or repression through the recruitment and assembly of the basal transcription machinery along with chromatin remodeling complexes like the Histone deacetylase 1(HDAC1) and Switch/Sucrose Non-Fermenting (SWI/SNF) complex, transcriptional activating complexes such as leucine zipper family of transcription factors (NF-E2/p45) complex or transcriptional repressor complexes such as Orphan nuclear transcription factor (COUP-TFII) and the Erythroid-specific Kruppel-like factor (EKLF) (Tanimoto, 2000; Sankaran *et al*., 2010). In spite of numerous advances in mechanisms involved in the human globin gene expression, the critical events that govern the globin genes switching remain obscure. However, the human haemoglobin loci serve as an excellent model for studying developmentally regulated globin expression. Indeed, studies on the transcription regulation of the human globin gene loci have provided information on how lineage specific programs

are turned on during erythroid differentiation (Ginder, 2014; Beauchemin & Trudel, 2009; Patrinos *et al*., 2004).

Although several inducers of foetal haemoglobin have been identified (Bianchi *et al*., 2009), most have quite low efficacy and only Hydroxyurea is the FDA approved drug for the management of SCA in spite of its low specificity and potential terratogenicity on long term use (Kinney *et al*., 1999; Ware, 2010; Baz *et al*., 2012).

Since the γ-globin gene is hypomethylated in the utero, DNA demetthylating agents like Azacitidine (International Nonproprietary Name) or 5-azacytidine have been used to induce γ-globin gene hypomethylation. However, it was observed to be efficient only for short-term administration on patients with Beta-thalassemia (Perrine, 2005; Mabaera & Lowrey, 2008). Cytostatic agents have also been used to alter erythropoiesis kinetics with some level of success. Hydroxycarbamide (International Nonproprietary Name) or Hydroxyurea is a cytostatic drug used for short and long-term treatment of sickle cell anaemia and thalassemia patients. It was shown to increase differentiation of primitive burst-forming unit–erythroid (BFU-E) progenitor cells, and through a nitric oxide signaling pathway led to a modest increase γ-globin level which did not always correlate with an overall increase in HbF as expected.(Platt *et al*., 1984; Fibach *et al*., 1993; Cokic *et al*., 2003; Platt, 2008; Ware, 2010).

Similarly recombinant erythropoietin, a glycoprotein hormone involved in the regulation of erythropoiesis showed no potential γ-globin inductive capacity in patients with sickle cell anaemia and beta thalassemia as Fetal haemoglobin level remained steady as well as moderate to no changes to patient's overall well-being following treatments with recombinant erythropoietin (Sawada *et al*., 1987; Silva *et al*., 1999).

Chromatin histone acetylation has been shown to increase γ-globin gene expression, as such deacetylase inhibitors have been used to promote histone acetylation in erythroid progenitor stem cells. Short chain fatty acid like Butyrate has also been shown to augment Hb F content in various animal model systems as well as in humans (Mankidy *et al*., 2006; Pace & Zein, 2006; Aerbajinai *et al*., 2007). Clinical trials using butyrate resulted in increased γ-globin levels in some patients with no severe toxic side effects at small doses. However neurologic toxic effects sometimes arise in cases of high doses of butyrate.(Weinberg *et al*., 2005; Zein *et al*., 2010). Although most of these therapies showed potential pharmacologic enhancement of fetal haemoglobin in early-phase clinical trials, several associated side-effects have been noted for all of them. Hence, alternative therapeutic strategies involving novel pharmacological agents or novel molecular targets need to be developed to treat sickle cell anaemia and β-thalassemia patients through a better understanding of the regulation of the β globin gene expression during erythropoiesis.

2.8 CELL CULTURES

The use of cell cultures as models for the *in vitro* screening of drugs is widespread and well established in vitro model systems for the study of human biochemical and molecular mechanisms. Several inducible cell lines have been established from humans and used to study erythropoietic systems (Tsiftsoglou, *et al*., 2009). One of the well characterized models for erythropoeisis is MEL (Murine Erythroleukemia) cell line which can be induced into terminal differentiation process in a mouse model. This cell line is blocked at the pro-erythroblast stage of erythroid maturation (Stopka *et al*., 2000). Terminal erythroid differentiation can be induced by adding 2% Dimethyl Sulfoxide (DMSO, Sigma-Aldrich) to the growth medium. These cells have been used

extensively as a model system for erythroid differentiation because they can be consistently induced to express erythroid specific differentiation program characteristic of normal erythropoiesis (Stopka *et al*., 2000; Becker, 2002; Faenza *et al*., 2002; Panigaj *et al*., 2011). K562 cell lines were established from a patient with chronic myeloid leukemia in the acute phase by Lozzio and Lozzio (1975). These cells previously thought to be stem cell precursors of the myeloid lineage are now known to express erythroid specific genes (Huhn *et al*., 1987; Copland *et al*., 2008). Using benzidine its been shown that these cells could be induced to terminally differentiate and accumulate haemoglobin using specific inducers (Hatse *et al*., 1999). Un-induced K562 cells express glycophorin (a protein present in the cell surface of erythroid cells). Several reports have shown that hemin treatment of these cells could induce the synthesis of a variety of embryonic and fetal haemoglobins and not adult Haemoglobin (Okabe-kado, *et al*., 1986; Addya *et al*., 2004; Wen *et al*., 2005). Further studies showed that although K562 cells contain the intact and entire human beta- globin gene loci, it is unable to express the adult haemoglobin due to its lack of an erythroid specific trans acting factor (EKLF) which has been shown to direct the normal expression and synthesis of the adult beta globin (Fordis, *et al*., 1984; Gambari, 2003).

JK-1 cell line was established as a hematopoietic cell line from a patient with chronic myelogenous leukemia in erythroid crisis. Un-induced JK-1 cells usually consists of a mixture of a large number of immature and small number of mature erythroblasts (Okuno *et al*., 1990). JK-1 cells grow in suspension media and carry double Philadelphia chromosomes. Surface marker analysis of the cells revealed them positive for glycophorin A, EP1 and HAe9 (Okuno *et al*., 1990). Erythropoietin has been shown to enhance the growth of these cells in suspension culture and like K562 cells they can be pharmacologically induced to terminally differentiate to the erythroid lineage but

unlike K562 cells they are known to express all forms of human haemoglobin (Okuno *et al*., 1990).

Primary hematopoietic stem cell (Figure 2.4) cultures have also been used extensively as in vitro models for novel pharmacological compounds screening as well as to elucidate new biochemical molecular mechanisms.

Colony forming cultures refer to colonies formed by either myeloid cells or progenitor stem cell cultures on semisolid media. CFU-GM, CFU-E, CFU-Meg, and CFU-Mix, are progenitor stem cells that form *in vitro* colonies of granulocytes and macrophages, erythrocytes, megakryocytes and multilineage cells respectively. These cells have all shown to be derivable or culturable from the blood or bone marrow tissues of vertebrates (Sandstrom *et al*., 1994).

All hematopoietic cells are derived from Hematopoietic Stem Cells (HSCs) which have capacity for self renewal (for the maintenance of a stable HSC pool) and the capacity to differentiate to mature myeloid and lymphoid cells (Salati *et al*., 2013). HSCs are located in the bone marrow BM in a specific microenvironment referred as the hematopoietic stem cell niche, which is critical in regulating their survival, self-renewal and differentiation. They interact with a variety of cells including; stromal, fibroblasts, endothelial mesenchymal and adipocytes.

A variety of techniques have been used to purify these cell populations. The mononuclear cell fraction, obtained by 1.077 g/mL Ficoll/Histopaque density gradient centrifugation, usually contains the most mixed populations of these cells which usually require further selection to target specific cell populations. The fraction of progenitors in the mononuclear cell fraction is frequently enriched by a negative selection process usually by plastic adherence of typically macrophages and monocytes. This leads to

depletion of these cells from the progenitor population. T-lymphocytes however have to be further depleted by adherence to nylon wool (Sandstrom *et al*., 1994). Specific progenitor populations have also been positively selected using magnetic beads, for selection. Additionally, cells are stained with antibodies to specific cell surface markers (e.g. CD34, CD33, CD45RA, HLA-DR, CDllb, CD15) to allow for fluorescence activated cell sorting (FACS) and panning (Toya *et al*., 2011).The CD34 surface antigen is expressed specifically on hematopoietic progenitor cells (Dao *et al*., 2003).

Hematopoietic progenitor stem cells are usually grown as suspension cultures in liquid medium enriched with growth factors to support their growth, development and possible differentiation and erythropoiesis and consequently haematopoiesis is regulated by a cascade of haematopoietic growth factors (interleukins and colony stimulating factors [CSFs]). These growth factors have multiple, and frequently overlapping, functions. Among the CSFs regulating the development and growth of myeloid progenitors; granulocyte-CSF (G-CSF) promotes the development of neutrophilic granulocytes; macrophage-CSF (M-CSF) promotes the development of both granulocytes and macro phage, Erythropoietin (EPO) a related factor regulating the growth of erythrocyte. The Stem cell factor (SCF), also called Steel factor (SF), mast cell growth factor (MCGF), and c-kit ligand, is an important hematopoietic growth factor which by itself possesses little stimulatory activity, but acts in synergy with other hematopoietic growth factors such as interleukin-3 (IL3), interleukin-6 (IL6), etc. to yield large expansions in both total cell and progenitor cell populations. Interleukin L-1α and interleukin L-1β both bind to a common receptor and induce the expression of GM-CSF, G-CSF, M-CSF, and IL-6 by a variety of other cells which act as accessories (Scott *et al*., 1995; Schuh & Morrissey, 1999). Interleukin-2 (IL-2) on the other hand is an essential factor regulating the proliferation and function of T – cell

while Interleukin-3 (IL-3) is another growth factor of major importance to hematopoiesis (Somervaille *et al.*, 2001). In contrast to the relatively lineage-specific actions of Epo, G-CSF, M-CSF and GM-CSF, as well as IL-3 promotes the growth of colonies containing many different cell lineage (Scott *et al.*, 1995). IL-5 a late-acting, T-lymphocyte-derived glycoprotein regulates mainly the proliferation and differentiation of eosinophils and eosinophil precursor (Denburg *et al.*, 1985). IL-6 meanwhile is a non-specific, early acting hematopoietic growth factor. IL-7 regulates early lymphoid progenitors of both the T- and B-cell lineages. IL-9 is a regulator of erythropoiesis while IL-11 regulates the differentiation and proliferation of megakaryocyte progenitors as well as a variety of primitive multipotential hematopoietic progenitor cells (Nagata *et al.*, 1998; Wodnar-filipowicz, 2003).

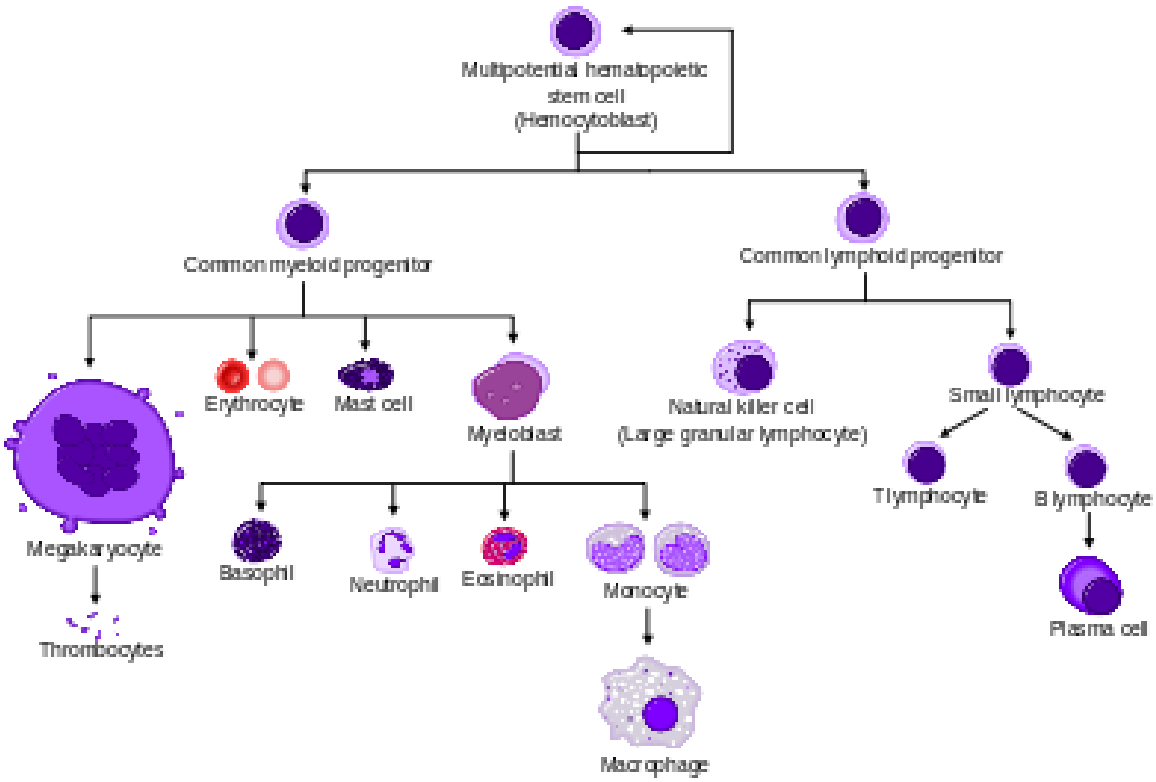


Figure 2.4: structure of haematopoietic system

http://www.en.wikipedia.org

2.9 MEDICINAL PLANTS

Several reports exist for the use of medicinal plants for biomedical purposes and in the management of several diseases (Okpuzor *et al*., 2008; Ameh *et al*., 2012). The use of medicinal plants for disease management and treatment dates back hundreds of years and yet their use is still widespread in Africa because they are cheap, easy to use and have much less reported side effects compared to synthetic drugs (Ameh *et al*., 2012). Several plants have been reportedly used for the management of SCA although their efficacies, safety and mode of actions remain largely unknown. The use of medicinal plants in the management of SCA in developing countries is augmented by the high cost and inaccessibility to these medical technologies of a large population of people affected by the disease especially those in rural areas. The knowledge of the plant to be used in the management of SCA is largely retained by traditional medicine practitioners who in turn most times acquire this knowledge from their ancestors (Okpuzor *et al*., 2008). In Nigeria and other developing countries, a variety of medicinal plants have

been used to manage sickle cell anaemia. Some of the medicinal plants which have been reportedly used include; *Adansonia digitata, Sorghum Bicolor, Azadiractha indica, Telfairia occidentalis, Terminalia catappa, Zanthoxylum xanthoxyloides, Carica papaya, Bryophyllum pinatum, Garcinia kola, Moringa oleifera.*. Different parts of the plants have been reportedly effective for the management of SCA. *A. digitata, C. papaya, T. catappa* leaves and *Z. xanthoxyloides* have been scientifically demonstrated to possess antisickling active principles (Okpuzor *et al*., 2008). *G. kola* which is commonly consumed in Nigeria has been speculated to be effective in the management of SCA as a membrane stabilization agent. Niprisan® a herbal preparation (Wambebe *et al*., 2001) has been reported effective in the reducing the frequency of crisis in individuals with SCA, further studies have also demonstrated that Niprisan increases the survival rate of sickle cell transgenic mice (Iyamu *et al*., 2003). However, very little information exists for the use of medicinal plants as foetal haemoglobin inducers (Bianchi *et al*., 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Glassware/Plasticware

Culture plates (Falcon)

92 well microtitre plates (Falcon)

0.4µ cell filter

3.1.2 Reagents

All reagents used were molecular grade

Phosphate buffered saline (pH 7.2) (Sigma)

Giemsa stain (Sigma)

May Grumwald stain (Sigma)

O-dianisidine chloride (Sigma)

Hydrogen peroxide (Sigma)

Benzidine chloride (Sigma)

Tri reagent® (Qiagen)

Qiagen® micro-elution kit

Promega Reverse Transcription System (A3500)®

High-Capacity cDNA Reverse Transcription Kit® (Applied biosystem)

Rosewell park memorial institute (RPMI)® 1640 media

Eagles Minimal essential medium (MEM)®

Iscove modified Dulbecco medium (IMDM)®

Ficoll® (Stem cell technologies)

Glycerol (Sigma)

Ethanol (Sigma)

Methanol (Sigma)

Acetic acid (Sigma)

β-mercaptoethanol (Promega)

Foetal bovine serum (FBS) (Sigma)

Nicotinamide Adenine Dinucleotide (NAD), (Sigma)

Lactic Acid (Sigma),

Caspase 3 substrate; Ac-Val-Gln-Val-Asp-pNA (ANASPEC)

3.1.3 **Cells**

Primary Human erythroid progenitor stem cells isolated from peripheral blood of sickle cell individuals

Erythroleukemia K562 cells lines kindly provided by Biekerlab, Mount Sinai School of Medicine New York)

JK-1 cell lines kindly provided by Biekerlab, Mount Sinai School of Medicine New York)

Mice bone marrow erythroid progenitor stem cells expressing the full complement of the human beta globin gene.

3.1.4 **Plants**

Adansonia digitata fruit was obtained from Zaria, Nigeria in the month of June

Sorghum bicolor leaves was obtained from Institute of Agricultural Research farm site in the month of June.

Azadirachta indica was obtained from the premises of National Research Institute of Chemical Technology, Zaria, Nigeria in the month of June

Telfairia occidentalis was obtained from Zaria, Nigeria in the month of June

Terminalia catappa was obtained from Zaria, Nigeria in the month of June. All the plants were identified at the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria and voucher numbers deposited.

3.1.5 **Primers**

All primers were obtained from Eurofins®

Human Glyceraldehyde 3-phosphate dehydrogenase primers (GAPDH)

5’-CAATGACCCCTTCATTGACC-3’

3’-GATCTCGCTCCTGGAAGATG-5’

Human γ-globin primers

H-gammaGfor: ACCGTTTTGGCAATCCATTTC

H-gammaGrev: TATTGCTTGCAGAATAAGCC

Human β-globin primers

H-betaGfor: TACATTTGCTTCTGACA

H-betaGrev: ACAGATCCCCAAAGGAC

Human Erythroid Kruppel like factor (EKLF) primers

hEKLF 5’-CGGACACACAGGATGACTTC-3’

hEKLF 3’-GTTGGTGAGGAGGAGATCCA-5’

Human B-cell lymphoma 11 A (BCL11A) primers

Hbcl11A-for 5’-AACCCCAGCACTTAAGCAAA-3’

Hbcl11A-rev 5’-GGAGGTCATGATCCCCTTCT-3’

3.2 METHODS

3.2.1 Research Initiative on Traditional Anti-malarial Method

Potential ethnomedicinal plants used to treat sickle cell anaemia were identified using the Research Initiative on Traditional Anti-malarial Method (RITAM) technique (Willcox *et al*., 2011). The first aim of the study was to design a standardized score that could be used to prioritize traditionally used herbal sickle cell treatment remedies.

Design of the score

A "remedy" was defined as a specific preparation from a specific portion of a plant specie.

A systematic literature search using MEDLINE, CAB Abstracts, EMBASE and Google Scholar was used to rank plants to be included in this study using specified criteria (see Table 1); an overall score, composed of three components:

- Frequency of citation in ethnobotanical studies
- Erythropoietic and antisickling Efficacy in vitro and in vivo (Table 3.1)
- Safety (Table 3.1)

A total RITAM score was computed for each of the identified plants and the results are shown in Table 4.2.

Table 3.1: RITAM Score Computation Criteria

Aspect (max score)	Criterion	score	Actual score	reference
Type of study	Primary ethnobotanical study (original study consulted as source of information)	3		
	OR Primary ethnobotanical study quoted in a review but data not available	2		
	use to improve RBCs /hemoglobin	2		
Botanical Identification	Voucher specimen in herbarium	1		
	two or more informants confirm the use of the plant	2		
	informants have experience in managing sickle cell anemia	1		
	laboratory efficacy score for in vitro erythropoietic/Antisickling activity of plants			
Antisickling activity		score		
inhibits sickling in vitro		0.5		
reverses sickled cells		0.5		
total				
	laboratory efficacy score for in vivo erythropoietic activity of plants			
		score		
2% increase in Hb		1		
2-10% increase in Hb		2		
10% increase in Hb		3		
2% increase in RBC/PCV		0.5		
2-10% increase in RBC/PCV		1		
10% increase in RBC/PCV		1.5		
Total (11.5)				
	Safety score			
LC_{50}		score		
1 µg/ml		-2		
1-1000µg/ml		-1		
1-100mg/ml		0.5		
100mg		0.2		
LD_{50}				
≤ 5mg/kg body weight		-10		
5-50mg/kg bodyweight		-8		
51-500mg/kg bodyweight		-5		
2000mg/kg and above		6		
Total				

3.2.2 **Extraction of plant**

T. catappa, A. indica, S. bicolor and *T. occidentalis* leaves and *A. digitata* seeds were oven dried at 27^{O}C to a constant weight and pulverized using a mechanical grinder.

Exactly 50g of the powdered leaves and seeds were extracted for 24hrs with 200mls of distilled water at 27^{O}C. Extracts were filtered and then concentrated on a water bath at 37^{O}C as previously described (Iweala, et al., 2010).

3.2.3 **Partition Chromatography.**

Plant aqueous extracts capable of inducing hEPSCs to BFUe formation were partially purified using a combination of partition chromatography and column chromatography technique as previously described (Graham, 1991; Morrison & Stotz, 1955).

Crude aqueous extracts were firstly partitioned using *n*-Hexane to deplete extracts of non-polar components in a separating funnel.

Partitioning was carried out by adding 50mls of *n*-Hexane to 200mls of aqueous extract in a separating funnel and the mixture agitated vigorously for 2 minutes.

The mixture was then allowed to stand (by securing the separating funnel on a retort stand using a clamp) for 4 hours or until two clear liquid phases were formed.

The lower aqueous phase was then carefully collected and labeled as plant residue

The residue and *n*-Hexane fraction were then concentrated on a water bath at 27^{O}C.

The residue was re-suspended in absolute methanol, loaded on a silica gel column and allowed to bind over night

3.2.4 **Column Chromatography**

Plant residue was then further purified by column chromatography using solvents with increasing polarity. A 50cm x 1cm silica gel column was prepared by suspending 50g silica gel in distilled water.

The silica gel slurry was applied to the column and the gel was allowed to pack overnight

The column was then equilibrated using absolute methanol.

Plant solution in methanol was applied to the pre-equilibrated silica gel column and allowed to bind. Flow-through was re-applied to the column over and over again for 5 cycles to ensure maximal binding of plant components. The 6^{th} cycle of flow through (which was almost colorless) was subsequently discarded.

The column was eluted using 200mls of the following solvents in the given sequence; petroleum ether, ethylacetate, acetone, ethanol and distilled H_2O.

Eluates were concentrated on a water bath at $27^{O}C$

3.2.5 **Determination of Inhibitory Concentration at 50% (IC_{50}) of crude extracts using Clonogenic Assay**

IC_{50} was determined using peripheral blood mononuclear cells (PBMCs) isolated from peripheral blood as previously described (Stamatoyannopoulos, 2005) in order to be able to estimate safe concentrations of plant extracts to use for further screening.

5×10^3 PBMCs were seeded on culture plates in semi-solid Eagles minimal essential medium (MEM) supplemented with 10% FBS, 0.9% nutrient agar.

Plant crude extracts was added to the culture at varying concentrations of 10mg/ml, 25mg/ml, 50mg/ml, 250mg/ml and 1000mg/ml.

Cells were cultured at 37^OC, 5% CO_2 in a fully humidified environment for 48hrs

Erythroid Burst forming unit colonies were enumerated at the end of 48hrs

3.2.6 **Isolation of human Hematopoietic Progenitor Stem Cells (hHPSCs) from peripheral blood**

Haematopoietic progenitor stem cells were isolated from human peripheral blood using a slight modification of previously reported protocols using Ficoll (Zein *et al*., 2010) or Glycerol gradient.

- Ethical clearance for this study was sought and obtained from the Ahmadu Bello University Teaching Hospital Research ethics board.
- Human peripheral blood was obtained from SCA individuals in steady state (patients were on folic acid and paludrine management regimen) by venipuncture using a 23 gauge needle.

- A glycerol gradient (1.077g/ml) was prepared by dilution of 1.68ml of stock glycerol to 25ml using sterile dH_2O, and its osmolality adjusted using 1.2mm/kg H_2O using 1.2M NaCl.
- Blood was diluted two fold using PBS, and carefully layered on Ficoll-paque or Glycerol gradient (1.077g/ml) in a centrifuge tube.
- The centrifuge tube containing the blood layered on the gradient was centrifuged at 2500 rpm for 30 minutes.
- The upper phase consisting of plasma and platelets was discarded and the interphase containing peripheral blood mononuclear cells (PBMCs) was then carefully collected into a sterile tube and the PBMCs were pelleted at 6500rpm for 2 minutes cells were washed twice with 10mM PBS pH 7.4.

3.2.7 **Isolation of mouse Hematopoietic Progenitor Stem Cells (mHEPSCs) from sickle cell transgenic mouse bone marrow**

Mouse haematopoietic progenitor stem cells were isolated as previously described (Bloiun *et al*., 2000).

Transgenic mouse expressing the complete human β-globin gene locus in steady state was euthanized under a stream of nitrogen gas

Femur bones of mouse was obtained from mouse by dissection of the mouse

Bone marrow was flushed out using 1X PBS with a 22G needle

Cell suspension was filtered using a sterile 100µ filter to remove bone fragments and cell clumps.

3.2.8 **Purification and Culture of Hematopoietic Stem Cells (HSC)**

PBMCs and mouse bone marrow cells were cultured on a plastic culture dish as described previously by Fibach *et al*., (1989) in Iscove modified Dulbecco medium (IMDM) supplemented with 20% foetal bovine serum (FBS) at $37^{O}C$ and 5% CO_2 for 24hrs.

Floating cells rich in haematopoietic progenitor stem cells were carefully harvested avoiding adherent macrophages, monocytes and lymphoid cells.

Progenitor stem cells were seeded in IMDM supplemented with 20% FBS, 2mM β-mercaptoethanol, 100 U/mL penicillin, and 0.1mg/mL streptomycin.

Cells were cultured at $37^{O}C$, 5% CO_2 in a fully humidified environment

3.2.9 **Induction of hEPSCs differentiation and BFUe formation using aqueous plant extracts.**

Cells were seeded at a density 2×10^{6} cells/ml in culture plates containing semisolid IMDM containing 0.9% methylcellulose, 100U/ml penicillin and 0.1mg/ml streptomycin.

Cells were cultured for 48 hours at $37^{O}C$, 5%CO_2 and BFUe colonies were enumerated under an inverted microscope.

3.2.10 Assay for effects of plant Solvent fractions on hEPSCs Foetal Haemoglobin synthesis and Differentiation

hEPSCs was seeded at a density of 1×10^6 cells/ml in IMDM supplemented with 20% FBS, β-mercaptoethanol (0.01%), 100U/ml penicillin and 0.1mg/ml streptomycin in 96 well culture plates.

T. occidentalis, A. digitata and *T. catappa* solvent fractions were added at a final concentration of 0.6, 114 and 220µg/ml while *A. indica* solvent fractions was added at a final concentration of 0.2, 38 and 74µg/ml at the start of culture, and cells were incubated at 37^OC 5% CO_2 in a humidified environment.

Foetal haemoglobin synthesis was assayed for in hEPSCs cell lysate after 14 days of cell culture (Fibach *et al*., 1989).

Plant fractions active in inducing hEPSCs foetal Haemoglobin synthesis were further assayed on their ability to induce EPO dependent and independent hEPSCs differentiation and commitment to the erythroid lineage using benzidine brown staining.

hEPSCs was seeded at a density of 1×10^6 cells/ml in IMDM supplemented with 20% FBS, β-mercaptoethanol (2mM) 100U/ml penicillin and 0.1mg/ml streptomycin with 2U/ml EPO added to the culture medium (EPO-dependent cultures) or without EPO added to culture medium (EPO-independent cultures).

Cells were induced with of either 0.6, 114 and 220µg/ml of *T. occidentalis*, *A. digitata* and *T. catappa* solvent fractions or 0.2, 38 and 74µg/ml of *A. indica* solvent fractions for 14 days and foetal haemoglobin synthesis was assayed for using alkaline denaturation assay

Total Haemoglobin synthesis was also determined in both EPO-dependent and independent committed hEPSCs using Benzidine colorimetric assay (Hafid-Medheb *et al*., 2003).

Fold increase in foetal haemoglobin synthesis was estimated relative to un-induced Cells.

3.2.11 Assay for Cytotoxicity Effects of Plant Active Solvent Fractions

Cytotoxity was assayed as a measure of intracellular Caspase 3 activity and Lactate Dehydrogenase release from hEPSCs in both EPO-dependent and independent cultures (Weyermann *et al.*, 2005).

hEPSCs were seeded at a density of 1×10^6 cells/ml Cells were induced with 220 μg/ml of TCDWF for 14 days, and the level of intracellular caspase 3 activity was

assayed colorimetrically at 405nm using the ANASPEC® chromogenic substrate

Ac-Val-Gln-Val-Asp-pNA according to the manufacturers protocol.

Lactate dehydrogenase (LDH) was monitored in the cell-free medium after 14-day

culture as previously described (Weyermann *et al*., 2005) using 30mM lactate in 0.2M Tris-HCl pH 7.3, 6.6mM NADH was added to the reaction mixture and the setup was incubated at 37^OC for 30 minutes LDH activity was calculated as increase in absorbance of NADH at 340nm (Weyermann *et al*., 2005)

3.2.12 UV-Visible Spectral and GCMS Studies of *Terminalia catappa* distilled water fraction

T. catappa distilled water fraction was subjected to ultraviolet spectral scan from 200 to 800nm and gas chromatography–mass spectrometry (GCMS) analysis using a Shimadzu GCMS-QP2010plus machine. Eluates from the column were subjected to mass spectra analysis, and the Mass spectra of TCDWF GC eluates was then analysed using NIST 2005 library. Potential candidate compounds for further screening were then selected based on similarity index of NIST 2005 and 2011 library hits.

3.2.13 Culture of erythroleukemic K562 and JK-1 cell lines

K562 and JK-1 cell lines were kept frozen in RPMI 1640 medium supplemented with 20% FBS, 100 U/mL penicillin, and 0.1mg/mL streptomycin and 10% glycerol in liquid nitrogen at -192^{O}C until needed (Siatecka *et al*., 2007).

Cells were slowly thawed at 27^{O}C and quickly resuspended in RPMI 1640 medium supplemented with 20% FBS, 100 U/mL penicillin, and 0.1mg/mL streptomycin.

Cells were cultured at 37^{O}C 5% CO_2 in a fully humidified environment (Gambari, 2003).

3.2.14 Cytospin Preparations

Stained cytospin cell preparations were used to qualitatively ascertain differentiation levels and morphology of cells. Approximately 2×10^4 cells were washed with PBS 1X and centrifuged onto clean glass slides for 5 minutes at 800rpm (Cytospin 3; Thermo Shandon, Pittsburgh, PA) (Gabianelli *et al*., 2003).

3.2.15 **Screening for Potential γ-globin inducing active plant compounds using K562 and JK1 Cell Induction**

K562 cells were induced using either Apigenin, 2-hexadecenoic acid, Cis vaccenic acid (CVA), Docosahexanoic acid (DHA), Eicosapentanoic acid (EPA), Gentisic acid, Oleic acid or Stearic acid. K562 cells were seeded at a density of 5×10^4 cells/ml. Firstly the effects of these select compounds on K562 cell viability was assayed for using two different concentrations (at subtoxic level and twice the subtoxic level of previous human or animal cell studies with the compounds) of these compounds. K562 cells were seeded at a density of 5×10^5cells/ml in RPMI 1640 supplemented with 20% FBS, 100U/ml penicillin and 0.1mg/ml streptomycin. A control was set up with just 0.5% DMSO added to K562 cells. Specified concentrations of the compounds were added to cell culture medium at the start of culture and cells were incubated at 37^OC, 5% CO_2 for 96 hours and cell viability as percentage of Cells not picking the Trypan blue stain. To assess the effect of these compounds on K562 cell differentiation, we used three different concentrations of each compound based on concentrations which were not toxic from the preceding study. Apigenin was added at a final concentration of 25, 50 or 75µM, 2-hexadecenoic acid was added at a final concentration of 3.5, 8.5 and 17 µM, Cis vaccenic acid was added at a final concentration of 50, 70 and 100µM, DHA was added at a final concentration of 6.5, 12.5 and 25µM, EPA was added at a final concentration of 6.5, 12.5 and 25µM, Gentisic acid was added at a final concentration of 200, 400 and 800µM, Oleic acid was added at a final concentration of 3.5, 8.5 and 17µM and Stearic acid was added at a final concentration of 25, 35 and 70µM. A control was set-up with solvent added to cells.

K562 cells were induced for 5 days and percentage of differentiated cells was assessed using benzidine blue staining at 48 and 120 hours post induction, and γ-globin gene expression was assessed 120 hours post induction. This enabled the selection of γ-globin inducing active plant compounds and their effective concentrations. A control was set-up with just solvent added.

K562 γ-globin inducing active compounds (Apigenin, 2-HDA, Oleic acid and Cis-vaccenic acid (CVA)) were further screened on their ability to induce differentiation, globin synthesis, KLF1and Bcl11a gene expression in JK-1 cells. JK-1 cells were seeded at a density of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FBS and 100U/ml penicillin and 0.1mg/ml streptomycin. Apigenin was added at a final concentration of 25, 50 and 75µM, 2-HDA and Oleic acid were added at final concentration of 3.5, 8.5 and17µM and CVA was added at a final concentration of 50, 70 and 100µM. JK-1 induction was carried out for 96 hours, differentiation was assessed using benzidine blue staining 24 and 72 hours post induction and γ-globin gene induction and foetal haemoglobin synthesis was assessed 96 hours post induction.

3.2.16 **Assessment of the Effects of Different Concentration of Cis Vaccenic Acid (CVA) on JK-1 globin gene Induction**

CVA was used to induce JK-1 γ-globin gene expression at 3 different concentrations 50, 70 and 100µM and solvent control to identify the most effective γ-globin gene inducing CVA concentration. JK-1 cell induction was carried out for 72 hours, and γ-globin and β-globin gene induction was monitored using semi-quantitative PCR.

3.2.17 Assay for the Effect of CVA on JK-1 Developmental Globin Gene Expression

CVA was used to induce JK-1 globin gene expression for 72 hours.

JK-1 cells were seeded at a density of 1×10^6 cells/ml, and CVA was added at a final concentration of 50µM at the start of culture.

Cells were cultured at 37^OC and 5%CO_2 and γ-globin and β-globin gene expression was assessed at 24, 48 and 72 hours post induction using semi-quantitative PCR.

A repeat 96 hours developmental induction of JK-1 γ-globin gene expression using 50µM CVA was carried out and γ-globin gene expression was quantitatively assayed 24, 48, 72 and 96 hours post induction using quantitative real time PCR.

3.2.18 Assay for the Effect of Erythropoietin (EPO) on CVA-induced JK-1 differentiation and γ-globin gene expression

EPO has been shown to be required for erythroid development and synthesis (Nagata *et al*., 1998). Hence the effect of EPO on CVA induction of JK-1 cell differentiation and γ-globin expression.

JK-1 cells were seeded at a density of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FB, 100U/ml penicillin and 0.1mg/ml streptomycin. Cells were either pre-differentiated using EPO (2U/ml) for 48 hours or left undifferentiated before induction with CVA (50µM), or CVA (50µM) + EPO (2U/ml). EPO (2U/ml)

and un-induced controls were also set up. Induction was carried out for 96 hours at $37^{O}C$ and 5% CO_2.

JK-1 cell differentiation and commitment to the erythroid lineage was assessed 96 hours post induction using benzidine giemsa staining, γ-globin expression was also assessed quantitatively using quantitative real time PCR.

3.2.19 Assessment of the Effects of Fatty Acid Elongase 5 (Elovl5) and Δ^9-Desaturase inhibition on CVA-Induced JK-1 γ-globin expression (CVA rescue experiments)

Elovl5 and Δ^9 desaturase are enzymes critical in the biosynthesis of CVA (Tripathy and Jump, 2013).

Inhibition of Elovl5 and Δ^9-desaturase was carried out with Isoxyl and Cycloate respectively.

JK-1 cells were seeded at a density of 1×10^6 in RPMI 1640 medium supplemented with 20% FBS, 100U/ml penicillin and 0.1mg/ml streptomycin, and transgenic mouse bone marrow progenitor stem cells were seeded at a density of and 2×10^6 cells/ml in IMDM supplemented with 20% FBS, 2U/ml EPO, 100U/ml penicillin and 0.1mg/ml streptomycin. Isoxyl or Cycloate was added to each culture at a final concentration of 10μM and 40μM respectively at the start of culture.

CVA (50μM) was used to induce cells previously the cells at 0, 24 and 48 hours post inhibition. Cells were incubated at $37^{O}C$, 5% CO_2 in a humidified environment. Controls consisted of un-inhibited un-induced JK-1 cells, un-induced Isoxyl inhibited JK-1 Cells and un-induced Cycloate inhibited JK-1 cells.

γ-globin and EKLF gene expression was assayed quantitatively relative to the un-induced control 24 hours post CVA induction.

3.2.20 Assay for the Effects of CVA on Differentiation and γ-globin gene expression of Transgenic mice Early Progenitor stem cells and Committed Erythroid Progenitors

To ascertain if CVA acts on both early stem cells or committed progenitors;

TMbmEPSCs were seeded at 5000 cells/ml in semisolid IMDM supplemented with 0.9% methylcellulose 100U/ml penicillin and 0.1mg/ml streptomycin. CVA (50μM) was added to the culture dish at 0hrs or 48 hours after the start of the culture and BFUe was enumerated under an inverted microscope 24 hours post CVA induction.

3.2.21 Trypan Blue Exclusion Staining

Cell viability was accessed by Trypan blue exclusion staining as previously described (Okabe-kado *et al*., 1986). Approximately 5×10^4 Cells were washed with 1X cold PBS, and re-suspended in 40μl 1X PBS.

Ten microlitres of 0.4% Trypan blue dye was added to the cell suspension and mixed thoroughly. The mixture was incubated at room temperature for 2 minutes.

Ten microlitres of the stained cells was carefully loaded onto a haemocytometer, and the blue stained cells were counted under an inverted microscope and presented as a percentage of total number of cells present.

3.2.22 **Giemsa Staining**

Giemsa Staining was carried out to qualitatively determine morphology of cells. cyto spin slide preparations was used as previously described (Gabianelli *et al*., 2003). Giemsa stain was done as previously described (Fibach *et al*., 1993).

Slides were fixed in absolute methanol for 1 minute.

Slides were air dried.

Slides were stained with diluted Giemsa stain (1:20, vol/vol) for 20 min.

Slides were washed twice with distilled water 15 seconds each.

Slides were air dried

Cells were viewed using a compound light microscope at 1000X magnification.

Cell photos were documented using a Nikon digital camera coupled to the microscope.

3.2.23 **Benzidine Staining (Brown)**

Benzidine (brown) staining was carried out to quantitatively estimate progenitor stem cells which have differentiated and committed to the erythroid lineage (Fibach, 1989). Slides were prepared by smearing 10µl of cell culture on a clean sterile glass slide

Slides were stained for 30 minutes using a 3% benzidine solution in 90% glacial acetic acid and 10% hydrogen peroxide solution (Fibach, 1989).

Slides were air dried

Benzidine positive (brown colored) cells were enumerated using a compound light microscope at X 400 magnification.

3.2.24 **Benzidine Staining (Blue)**

Benzidine Stain was prepared by combining on ice 2mg/ml benzidine in 0.5M acetic acid with 30% H_2O_2.

Fifty microlitres of cells were stained with 10µl freshly prepared benzidine stain. Cells were viewed within 5 minutes of staining.

3.2.25 **Benzidine-Giemsa Staining**

Benzidine-Giemsa staining of JK-1 cells was carried out to effectively qualitatively monitor differentiation and commitment to the erythroid lineage of JK-1 cells (Ji *et al*., 2010).

Cells were stained in 1% o-dianisidine in methanol for 1 minute, and washed twice in distilled water.

Cells were then destained in 1% H_2O_2 for 90 seconds and washed once with distilled water.

Cells were counter stained in giemsa stain for 30 minutes, and washed in distilled water for 30 minutes.

Pinkish (orange) coloured cells were identified as benzidine positive cells under a 1000X magnification.

3.2.26 **Alkaline Denaturation Assay for Foetal Haemoglobin**

Cells were harvested and lysed using 3 repeated cycles of freezing and thawing.

Fetal haemoglobin was estimated in the cell lysate using alkaline denaturation assay as previously described (Jonxis and Huisman, 1956).

To 6µl of cell lysate 100µl Drabkin's solution is added (Hb is converted to cyanmet form). Haemoglobin A was denatured by addition of 2µl of 0.2M NaOH to 28µl of the Hb solution, and the mixture was well agitated, and incubated for 2 minutes at $27^{O}C$

Saturated ammonium sulfate was added to the mixture after 2 minutes of incubation.

After vigorous mixing the denatured material was allowed to precipitate for 5-10 minutes.

The precipitate was removed by centrifuging the mixture at 10000rpm for 10 minutes.

The supernatant was used to quantify foetal haemoglobin at 415nm using the equation given below.

$$\%\text{of HbF} = \frac{OD^{415}\ \text{test sample} \times 100}{OD^{415}\ \text{control} \times 20}$$

A control solution was prepared by mixing 1.4 ml of Cyammet-Hb, 1.6 ml of distilled H_2O and 2ml of saturated ammonium sulphate.

3.2.27 **Total Haemoglobin Assay**

Total intracellular haemoglobin was determined using a previously described technique (Hafid-Medheb *et al*., 2003). Cells were washed twice with phosphate-buffered saline

(PBS), resuspended in 0.5 mL distilled water, and lysed under hypotonic conditions for 10 minutes

The cell lysate was centrifuged at 10 000 rpm in a microfuge for 15 minutes at 37℃ to remove cellular debris. 200µl of the supernatant was used for the assay.

The assay mixture was prepared in duplicate by adding the reagents in the following order: 100 µL supernatant, 900µL distilled water, 100µL freshly prepared benzidine-HCl (10 mg/mL), 40µL of 30% H_2O_2. The contents were mixed well to allow for color development, and after exactly 90 seconds the absorbance at 604 nm was measured in a spectrophotometer.

Haemoglobin concentration was determined by comparing absorbance values to a standard curve prepared using the above reaction mixture and conditions with standard pure human haemoglobin.

3.2.28 **RNA Extraction**

Total RNA was isolated from cultured cells as previously described (Rio *et al*, 2010). Approximately $2x10^6$ cells were pelleted at 1000rpm for 5 minutes at 4^OC

Supernatant rich in culture media was discarded

Cell pellet was resuspended in 250µl cold TRI Reagent and then lysed by pipetting up and down

Cell extract was kept at -80^OC for storage

Fifty microlitres chloroform was added to precipitate and solubilize protein and vortexed for 15 sec

The mixture was then incubated at room temperature for 15 minutes

The mixture was centrifuged at13000rpm $4^{O}C$ for 15 minutes

Upper aqueous phase rich in RNA was collected for RNA and transfered to a fresh tube

RNA was precipitated by the addition of 125µl Isopropanol for 10 minutes at $27^{O}C$

RNA precipitate was centrifuged at 13000rpm $4^{O}C$ for 10 minutes to pellet RNA

The supernatant was discarded and the RNA pellet washed with 250µl 70% EtOH

The tubes were centrifuged at 13000rpm $4^{O}C$ for 5 minutes

RNA pellet was air dried for 7 minutes

RNA pellet was dissolved in 87.5ul DDH_2O + 10µl RDD Buffer + 2.5µl DNase I

The solution was incubated at $27^{O}C$ for 25 minutes to allow for complete removal of contaminating DNA.

3.2.29 **RNA Clean-up**

Contaminating proteins in RNA solution may inhibit cDNA synthesis, hence the RNA solution was cleaned further using Qiagen® RNAeasy mini kit according to manufacturers specifications.

To RNA solution was added 350µl RLT Buffer (without β-mercaptoethanol or dithiothretol added to RLT) and mixed

To the mixture above was added 250µl 100% EtOH and mixed

The entire mixture was then transferred to micro-elution spin column, with the lids closed, and the solution was incubated at room temperature to allow maximum RNA binding to column.

The column was centrifuged at 13000rpm at 27^{O}C for 15 seconds, and the eluate discarded.

Spin column was then transferred to a new collection tube and 500µl RPE Buffer was added, with the lid closed

The column was centrifuged at 13000rpm at 27^{O}C for 15 seconds

The eluate was discarded and the collection tubes re-used, 500µl of 80% EtOH was then added and the lid of the column closed.

The column was centrifuged at 13000rpm at 27^{O}C for 2 minutes

Spin columns were transferred to new collection tube and the lids left open

The columns were centrifuged at 13000rpm at 27^{O}C for 5 minutes to dry RNA. The eluate was discarded and the columns were allowed to stand with the lids open for 7 minutes to ensure complete drying of RNA.

Spin columns were transferred to Eppendorf tube with lid, and add 14ul DDH_2O was added to the column to elute RNA.

The column was centrifuged at 13000rpm at 27^{O}C for 1 minute

RNA concentration and purity was documented using a Nanodrop spectrophotometer

RNA was stored at -80^{O}C if not utilized immediately

3.2.30 **cDNA Synthesis**

A cDNA master mix was prepared as previously described by Siatecka *et al.*, (2007), by adding the following reagents in the given proportion 2.5µl 10X Buffer, 10µl

$MgCl_2$, 2.5µl dNTP, 0.75µl Random Primers, 0.25µl Oligo dT, 0.6µl RNasin, 0.6µl Reverse Transcriptase.

1µg RNA was diluted to 7.7µl with ddH_2O RNAse free water

To the RNA was added 15µl master mix, and the mixture was mixed by pipetting up and down

Samples were placed in a PCR machine, and cDNA was synthesized using the program below

- 10 minutes at 25^OC
- 60 minutes at 45^OC
- 5 minutes at 95^OC
- 0 minutes at 4^OC

cDNA was diluted to 150µl with DDH_2O RNAse free and transferred to a new Eppendorf tube

3.2.31 **Semi quantitative PCR**

Semi quantitative PCR was used to qualitatively screen for γ, β and KLF1 expression in K562 and JK1 cells as previously described (Yien and Bieker, 2012).

A PCR master mix was prepared by adding the following reagents in the given proportion 1µl 10mM dNTP (1:1:1:1 of 40mM NTP stocks), 1µl Forward Primer (γ, β, KLF1 or Bcl11a) (100ng/µl), 1µl Reverse Primer (γ, β, KLF1 or Bcl11a) (100ng/µl), 0.25µl Taq DNA polymerase (5U/µl) and 49µl w/ DDH_2O. An internal control reaction was setup using primers specific for human GAPDH gene.

To 1µl cDNA was added 49µl of PCR master mix

PCR was carried out in a BioRad MJ Research thermal cycler machine as follows:

- 3 minutes at 95^{O}C

- Set for 27 cycles
  - 1 minute at 95^{O}C
  - 1 minute at 60^{O}C
  - 1 minute 72^{O}C
  - 0 minutes at 4^{O}C

Amplicons were run on agarose gel for visualization

3.2.32 **Quantitative Real time PCR**

Quantitative Real time PCR was carried out to measure gene expression of γ, β, KLF1 and Bcl11a genes using Glyceral 3-phosphate dehydrogenase (GAPDH) gene as a reference gene as previously described (Siatecka *et al*., 2007).

A PCR master mix was prepared by adding the following reagents in the given proportion proportion 1µl 10mM dNTP (1:1:1:1 of 40mM NTP stocks), 1µl Forward Primer (γ, β, KLF1 or Bcl11a) (100ng/µl), 1µl Reverse Primer (γ, β, KLF1 or Bcl11a) (100ng/µl), 0.25ul Taq DNA polymerase (5U/ul)

- 49ul w/ DDH_2O

To 1µl cDNA was added 49µl of PCR master mix

PCR was carried out in an Applied Biosystems 7900HT Fast Real-Time PCR System as follows:

- 3 minutes at 95^{O}C

- Set for 40 cycles
  - 1 minute at 95^{O}C
  - 1 minute at 60^{O}C
  - 1 minute 72^{O}C

3.2.33 Statistical Analysis

All values are expressed as the mean ± SD. The statistical comparisons were made by means of one way ANOVA, and the difference between various treatments was compared using Duncan multiple range test Values are considered statistically significant at $P<0.05$.

CHAPTER 4

RESULTS

4.1 RITAM SCORES OF SCREENED PLANT EXTRACTS

The RITAM scores of each of the 10 selected plants for the studies were computed for the aqueous extracts only since they are more similar to traditional preparations. Computation of total RITAM score for each of the plant was the sum of the individual scores for their ethnomedicinal use for SCA, laboratory erythropoietic efficacy and safety components. The total RITAM scores for each plant is shown in Table 4.1. Using this computation method and criteria, *T. occidentalis* gave the highest RITAM score of 20, while *Garcinia kola* revealed a RITAM score of 8. The five plants *T. occidentalis, A. indica, A. digitata, T. catappa* and *S. bicolor* with RITAM scores of 20, 17.5, 17, 15 and 14 respectively (Table 4.1) were selected and used for further studies.

Table 4.1: RITAM scores of selected plants

S/No	Plant	RITAM score	status
1	*Telfairia occidentalis*	20	Selected
2	*Azadirachta indica*	17.5	Selected
3	*Adansonia digitata*	17	Selected
4	*Terminalia catappa*	15	Selected
5	*Sorghum bicolor*	14	Selected
6	*Zanthoxylum xanthoxyloides*	12	Not selected
7	*Carica papaya*	11	Not selected
8	*Bryophyllum pinatum*	10.5	Not selected
9	*Moringa oleifera*	8.5	Not selected
10	*Garcinia kola*	8	Not selected

4.2 INHIBITORY CONCENTRATION (IC_{50}) OF *T.CATAPPA, T. OCCIDENTALIS, A. DIGITATA, S.BICOLOR* AND *A.INDICA* AQUEOUS FRACTIONS ON HUMAN PBMCS CLONOGENIC POTENTIALS

Table 4.2 shows the inhibitory potential of *T.catappa, T. occidentalis, A. digitata, S.bicolor* and *A.indica* on human peripheral blood mononuclear cells (PBMCs) growth

on semisolid IMDM. *T. occidentalis* showed the highest IC_{50} value of 15.77mg/ml and thus the lowest inhibitory potential followed by *A. digitata* which revealed an IC50 of 2.98mg/ml. *T. catappa* and *S. bicolor* revealed IC50 values of 2.02mg/ml and 2.58mg/ml respectively (Table 4.2).

Table 4.2: IC_{50} of *T.catappa, T. occidentalis, A. digitata, S.bicolor* and *A.indica* on growth inhibition of hEPSCs

Plant	**IC_{50}(mg/ml)**
Telfairia occidentalis	15.77mg/ml ±2.11
Adansonia digitata	2.98mg/ml ±0.11
Sorghum bicolor	2.5mg/ml ± 0.85
Terminalia catappa	2.02mg/ml ±0.21
Azadirachta indica	0.8mg/ml ±0.06

4.3 EFFECT OF *T.CATAPPA, T. OCCIDENTALIS, A. DIGITATA, S.BICOLOR* AND *A.INDICA* AQUEOUS EXTRACTS ON HUMAN ERYTHROID PROGENITOR STEM CELL DIFFERENTIATION

Aqueous plant extracts were assayed for their ability to induce differentiation of hEPSCs to colonies of committed erythroid burst forming units on semi-solid IMDM. Extracts were used at a concentration 17mg/ml, 34mg/ml and 0.67mg/ml.

Plates 4.1-4.5 show the BFUe inducing potential of the crude aqueous extracts of *T.catappa, T. occidentalis, A. digitata, S.bicolor* and *A.indica. A. digitata* did not induce BFUe formation of hEPSCs in semisolid IMDM cultures (Plate 4.1). Similarly *A. indica* did not induce the formation BFUe colonies of hHSC on semisolid IMDM (Plate 4.2). However it was observed that the aqueous extract of *T. occidentalis, T. catappa* and *S. bicolor* induced BFUe colony formation of hEPSCs (Plates 4.3, 4.4 and 4.5 respectively). BFUe colonies induced by *T. occidentalis, T. catappa* and *S.bicolor* were similar to the control EPO (1U/ml). No BFUe colonies was observed when hEPSCs were induced with HU (1µg/ml). *T occidentalis* aqueous extract was effective at 0.67mg/ml while *T.catappa* was effective at 0.17mg/ml and 0.34mg/ml. *S. bicolor* was effective at 0.34mg/ml

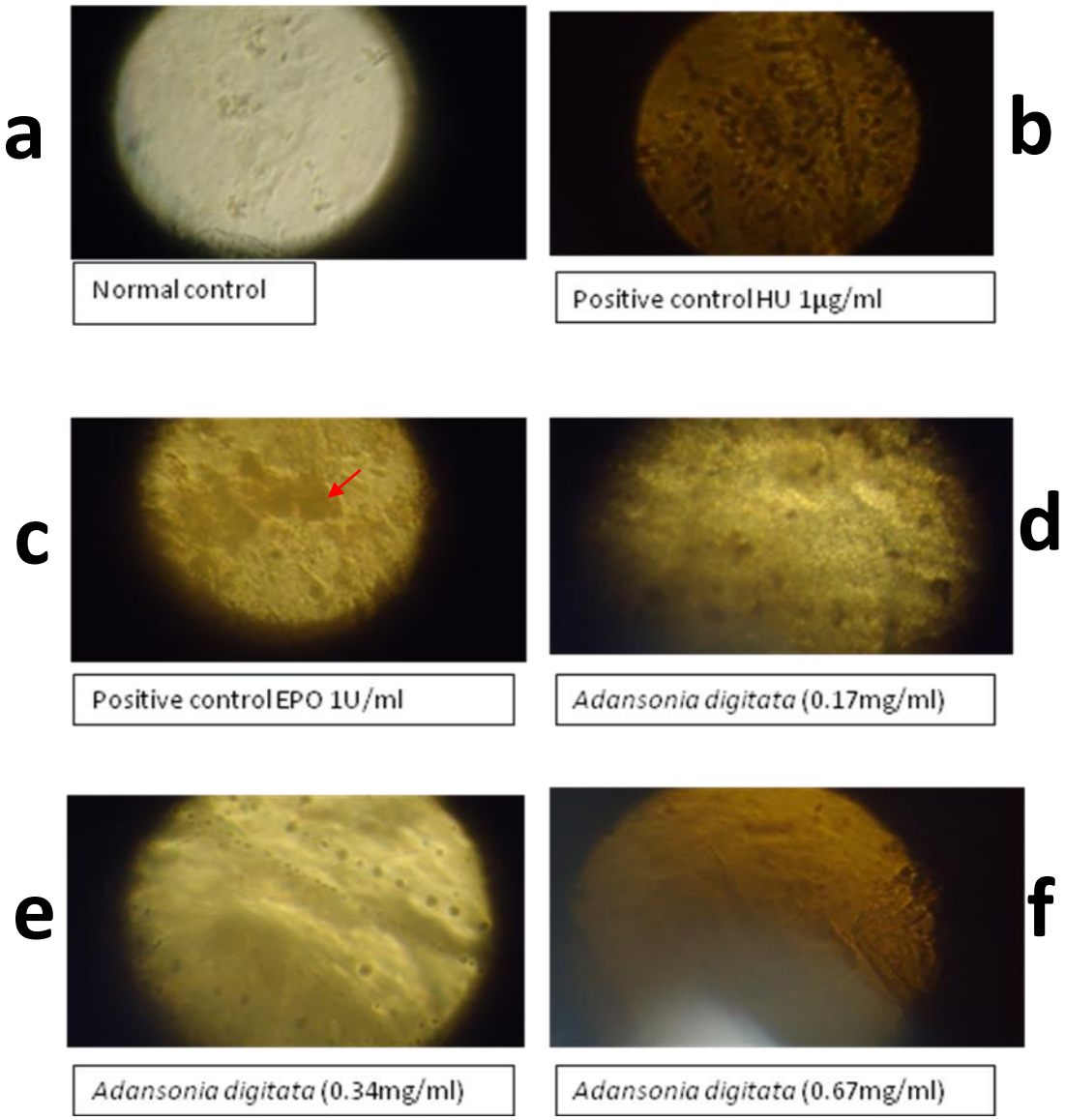


Plate 4.1: BFUe colonies from 72 hour cultures of erythroid progenitor stem cell induced with **a**. un-induced control **b**. Hydroxyurea (1µg/ml) **c**. Erythropoietin 1U/ml **d**. *Adansonia digitata* aqueous seed extract (0.17mg/ml) **e**. *Adansonia digitata* aqueous seed extract (0.34mg/ml) **f**. *Adansonia digitata* aqueous seed extract (0.67mg/ml) differentiation Magnification X 400. Cultures were carried out in fully humidified atmosphere with 5 % CO_2, arrows indicate BFUe colonies

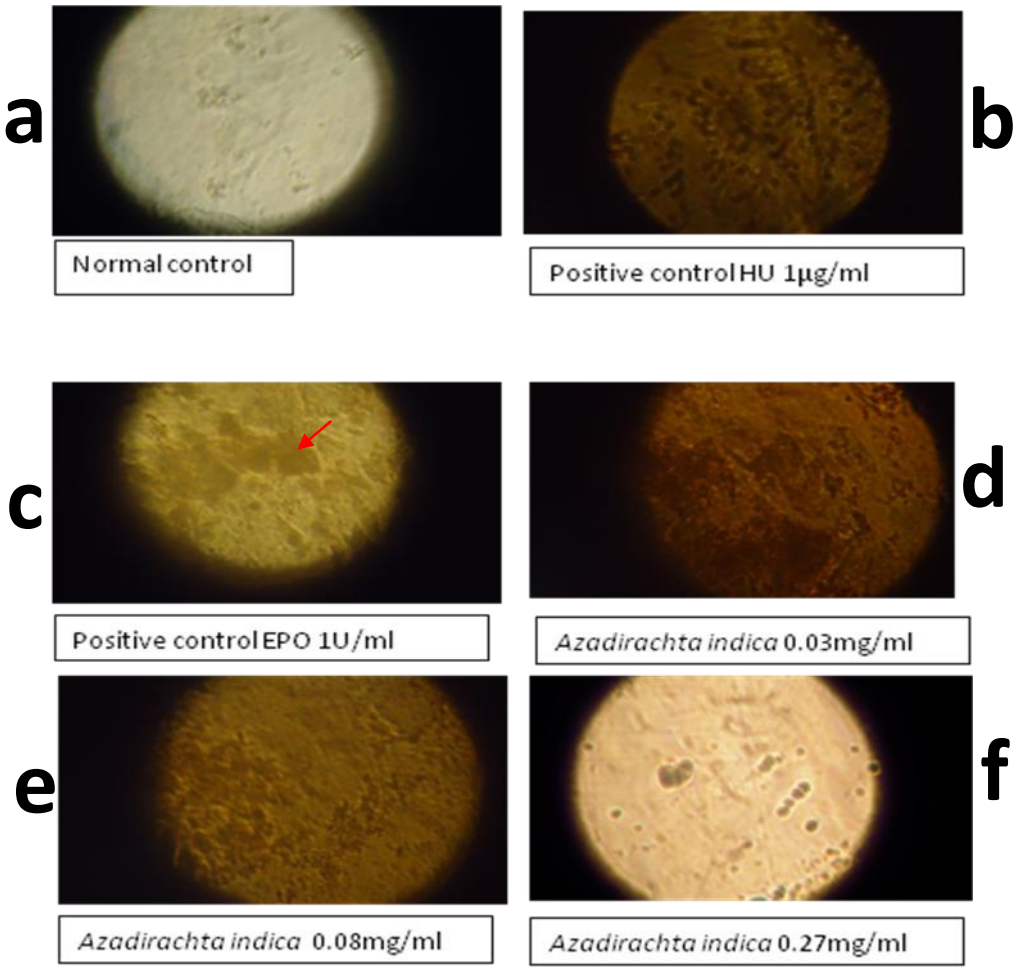


Plate 4.2: BFUe colonies from 72 hour cultures of erythroid progenitor stem cell induced with **a**. un-induced control **b**. Hydroxyurea (1µg/ml) **c**. Erythropoietin 1U/ml **d**. *Azadiractha indica* aqueous leaf extract (0.03mg/ml) **e**. *Azadiractha indica* aqueous leaf extract (0.08mg/ml) **f**. *Adansonia digitata* aqueous seed extract (0.27mg/ml) differentiation Magnification X 400. Cultures were carried out in fully humidified atmosphere with 5 % CO_2, arrows indicate BFUe colonies

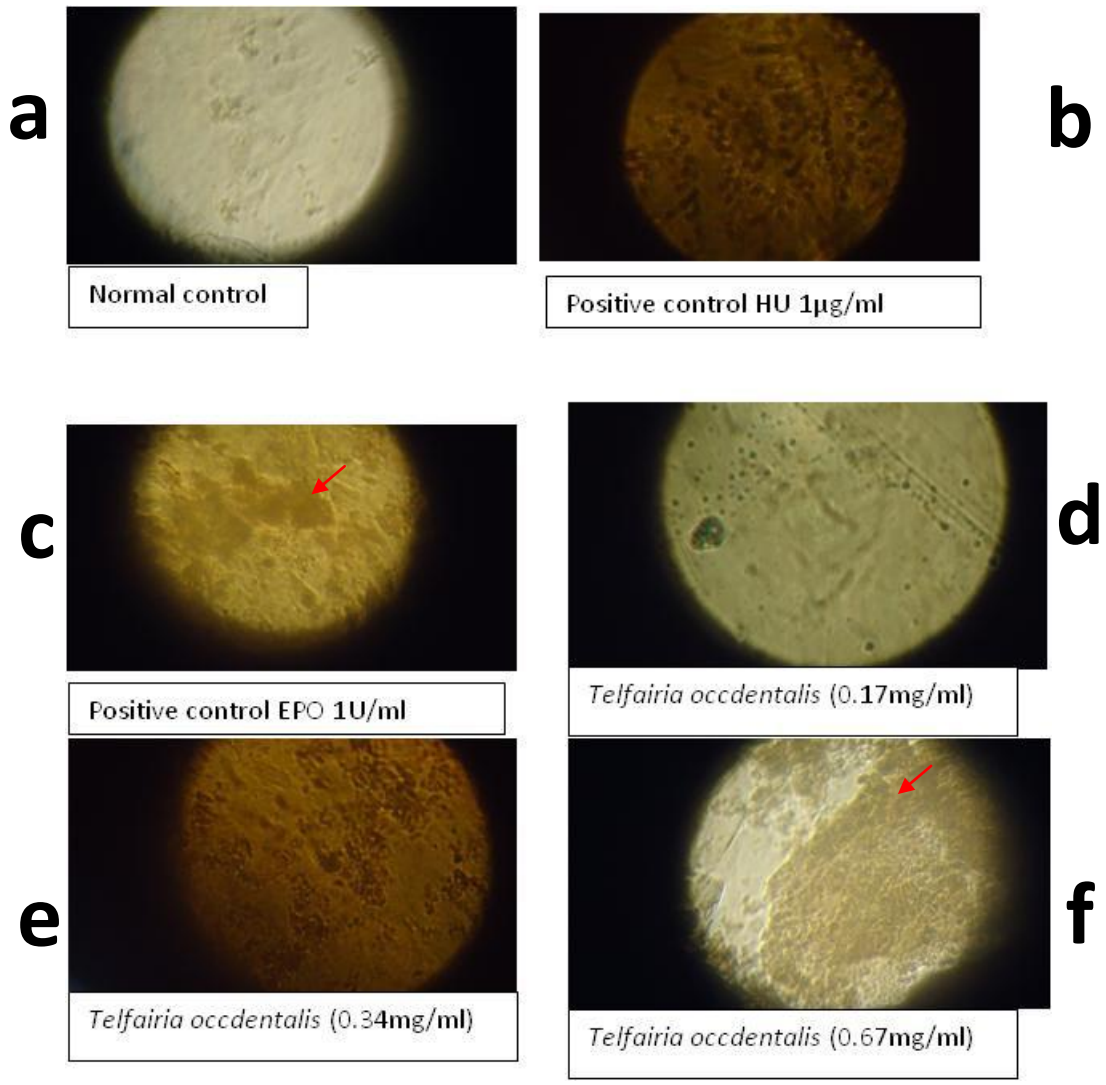


Plate 4.3: BFUe colonies from 72 hour cultures of erythroid progenitor stem cell induced with **a**. un-induced control **b**. Hydroxyurea (1µg/ml) **c**. Erythropoietin 1U/ml **d**. *Telfairia occidentalis* aqueous leaf extract (0.17mg/ml) **e**. *Telfairia occidentalis* aqueous leaf extract (0.34mg/ml) **f**. *Telfairia occidentalis* aqueous leaf extract (0.67mg/ml) differentiation Magnification X 400. Cultures were carried out in fully humidified atmosphere with 5 % CO_2, arrows indicate BFUe colonies

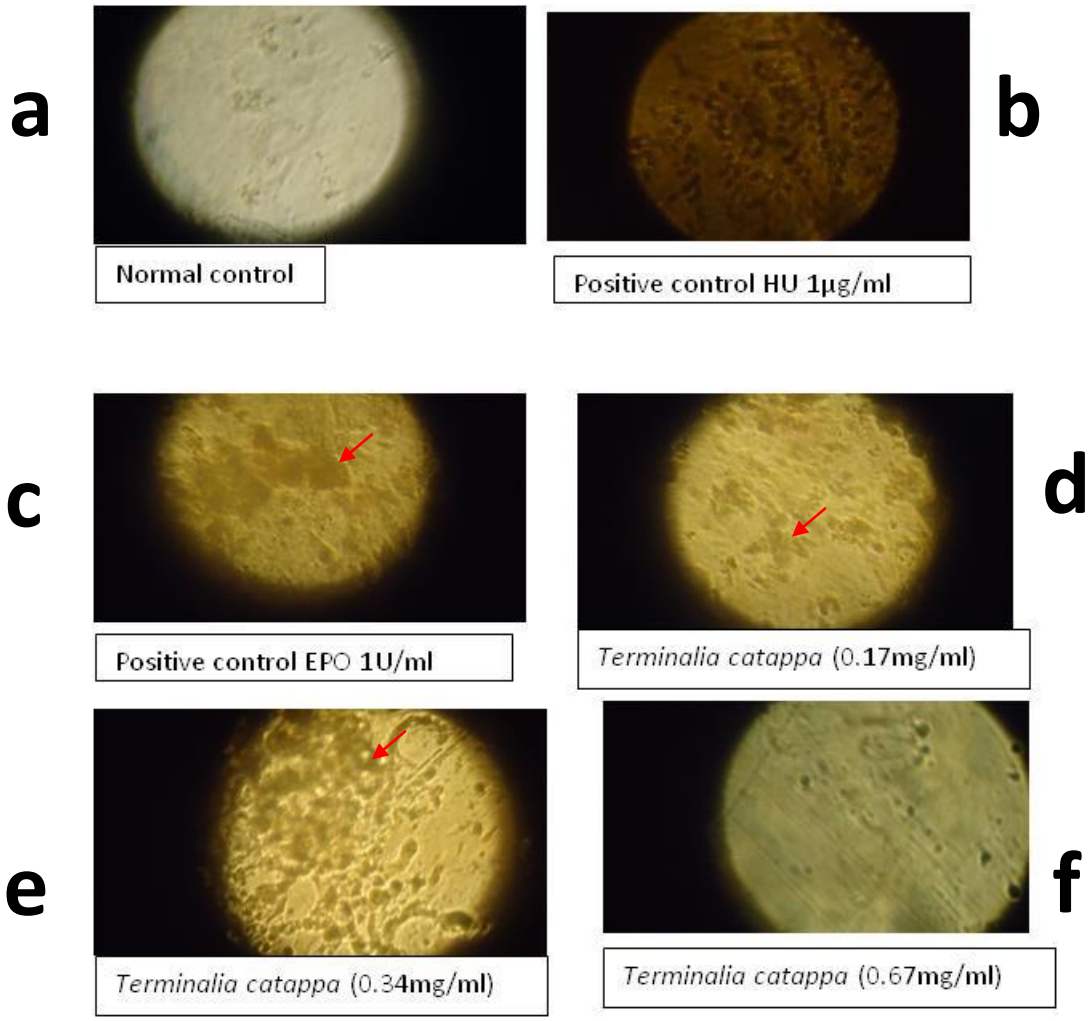


Plate 4.4: BFUe colonies from 72 hour cultures of erythroid progenitor stem cell induced with **a**. un-induced control **b**. Hydroxyurea (1µg/ml) **c**. Erythropoietin 1U/ml **d**. *Terminalia catappa* aqueous leaf extract (0.17mg/ml) **e**. *Terminalia catappa* aqueous leaf extract (0.34mg/ml) **f**. *Terminalia catappa* aqueous leaf extract (0.67mg/ml) differentiation Magnification X 400. Cultures were carried out in fully humidified atmosphere with 5 % CO_2, arrows indicate BFUe colonies

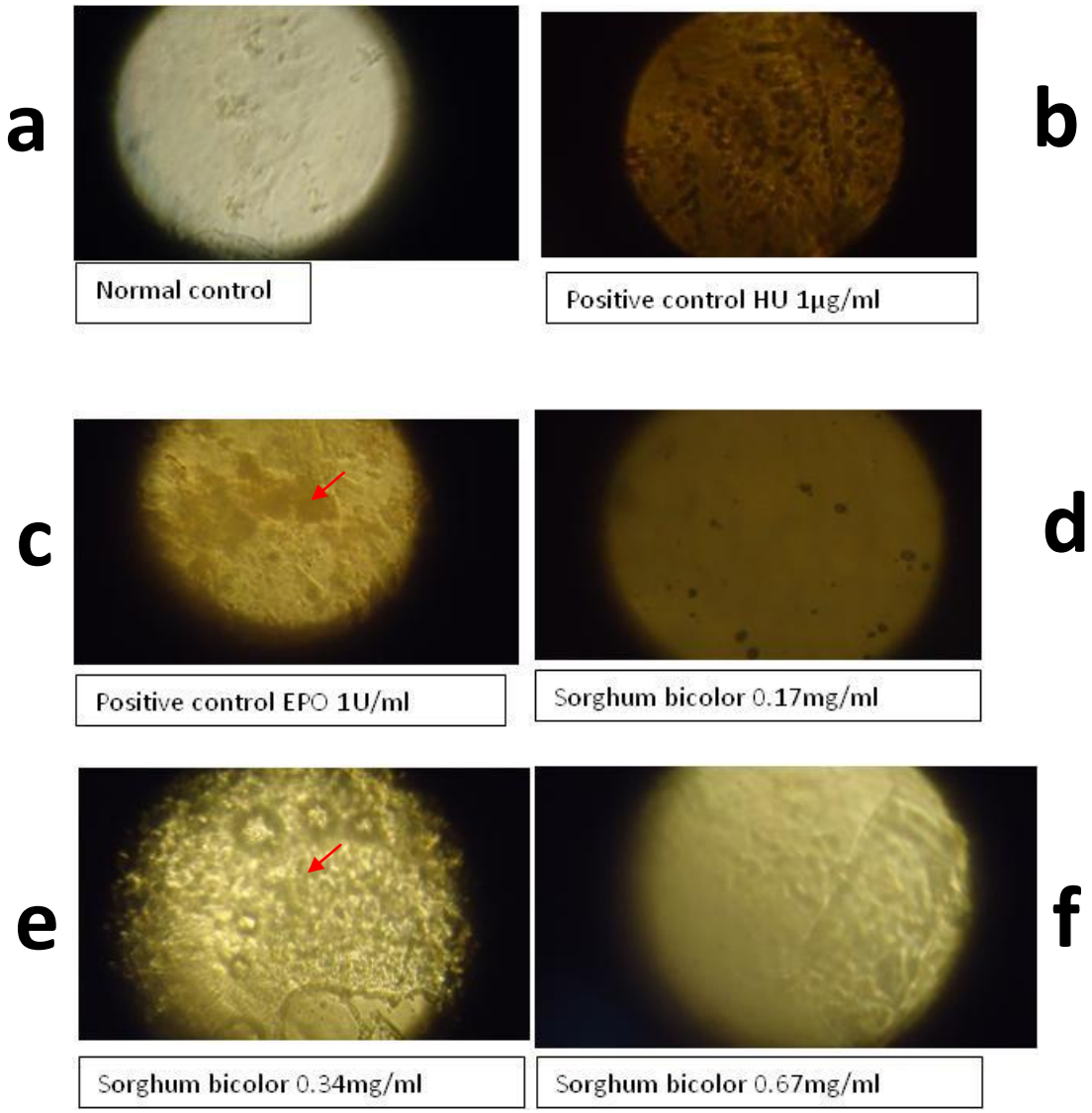


Plate 4.5: BFUe colonies from 72 hour cultures of erythroid progenitor stem cell induced with **a**. un-induced control **b**. Hydroxyurea (1µg/ml) **c**. Erythropoietin 1U/ml **d**. *Sorghum bicolor* aqueous leaf extract (0.17mg/ml) **e**. *Sorghum bicolor* aqueous leaf extract (0.34mg/ml) **f**. *Sorghum bicolor* aqueous leaf extract (0.67mg/ml) differentiation Magnification X 400. Cultures were carried out in fully humidified atmosphere with 5 % CO_2, arrows indicate BFUe colonies

4.4 EFFECT OF *T. CATAPPA, T. OCCIDENTALIS, A. DIGITATA, S. BICOLOR* AND *A. INDICA* AQUEOUS EXTRACTS ON HUMAN ERYTHROID PROGENITOR STEM CELL FOETAL HAEMOGLOBIN SYNTHESIS

The aqueous extracts of the plants were assayed for their ability to induce foetal Hb synthesis in human erythroid progenitor stem cells, using increasing concentrations of the plant extracts, the data showed that the plants had varying inductive capacities on human erythroid progenitor stem cell foetal haemoglobin synthesis; *A. indica* crude aqueous extract showed significantly higher ($P<0.05$) foetal Hb inducing activity at 0.27mg/ml followed by *T. catappa* at 0.17mg/ml followed by *S. bicolor* at 0.67mg/ml and *T. occidentalis* at 0.34mg/ml see Figure 4.1. The foetal Hb inducing effect of *A. digitata* crude aqueous extract was most effective at a concentration of 0.17mg/ml..

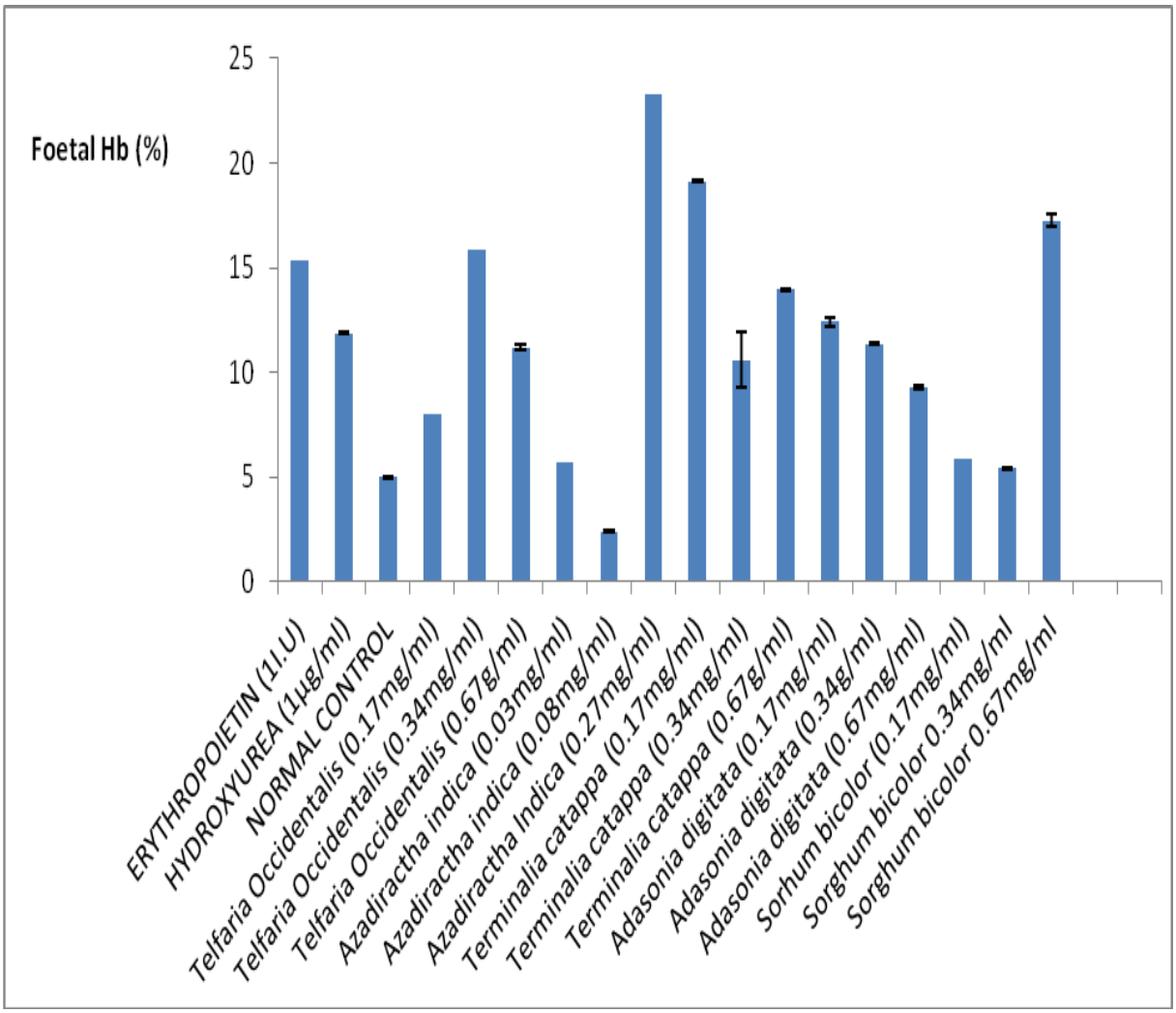


Figure 4.1: Percentage Foetal Hb content of erythroid progenitor stem cells treated with crude aqueous extracts of selected plants

4.5 EFFECT OF *T.CATAPPA, T. OCCIDENTALIS, A. DIGITATA, S.BICOLOR* AND *A.INDICA* AQUEOUS EXTRACTS ON HUMAN ERYTHROID PROGENITOR STEM CELL TOTAL HAEMOGLOBIN SYNTHESIS

All the crude aqueous extracts used significantly ($P<0.05$) lowered total haemoglobin synthesis compared to the un-induced control (Figure 4.2). The effect of *T. occidentalis* was concentration dependent with the highest concentration of *T. occidentalis* (0.37mg/ml) being the most effective in lowering haemoglobin synthesis. The effect of *S. bicolor* was also concentration dependent but unlike *T. occidentalis*, a lower concentration of *S. bicolor* lowered haemoglobin synthesis more compared to higher concentrations (0.34mg/ml and 0.67mg/ml) of *S. bicolor*. All the other plant aqueous extracts showed no specific total haemoglobin lowering concentration dependent pattern. In addition the positive controls used (HU (1µg/ml) and EPO (1U/µl) also significantly ($P<0.05$) lowered total Hb in erythroid progenitor stem cells.

4.6 EFFECT OF *TELFAIRIA OCCIDENTALIS* SOLVENT FRACTIONS ON FOETAL HAEMOGLOBIN SYNTHESIS IN HUMAN ERYTHROID PROGENITOR STEM CELLS

Telfairia occidentalis aqueous extract was partitioned using solvents of varying polarity; All the *T. occidentalis* solvent fractions induced foetal Hb synthesis significantly higher than the DMSO solvent control (Figure 6). The results revealed that the ethyl acetate fraction was most effective in inducing foetal Hb synthesis significantly higher than the positive controls (EPO (1I.U/ml) and HU 1µg/ml) in human erythroid progenitor stem cells compared to other fractions. The *T. occidentalis* induced foetal Hb increase was found to be concentration dependent with *T. occidentalis* ethyl acetate fraction being most effective at 220µg/ml (Figure 4.3)

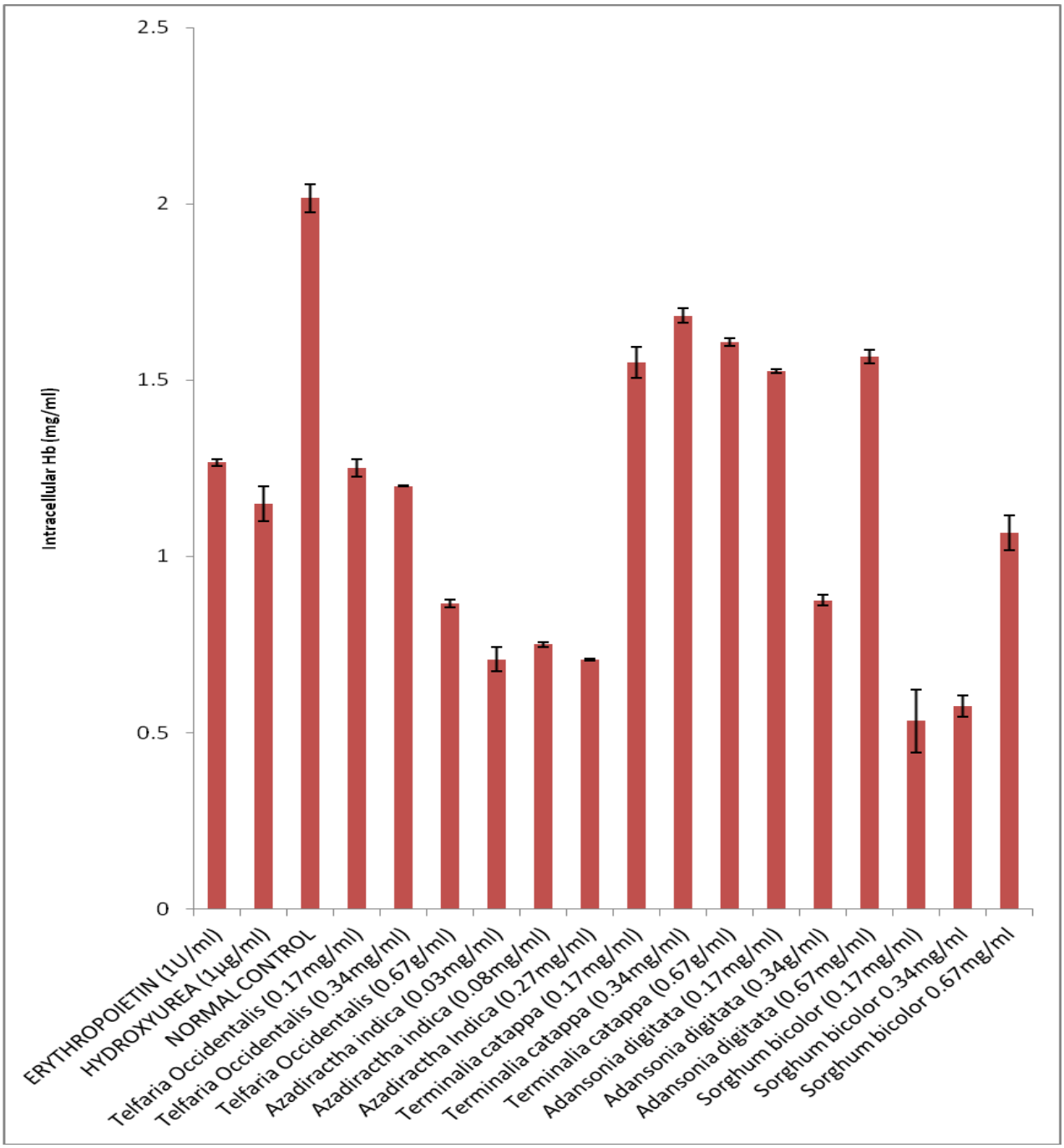


Figure 4.2: Total haemoglobin content of erythroid progenitor stem cells treated with different concentrations of crude aqueous extracts of selected plants

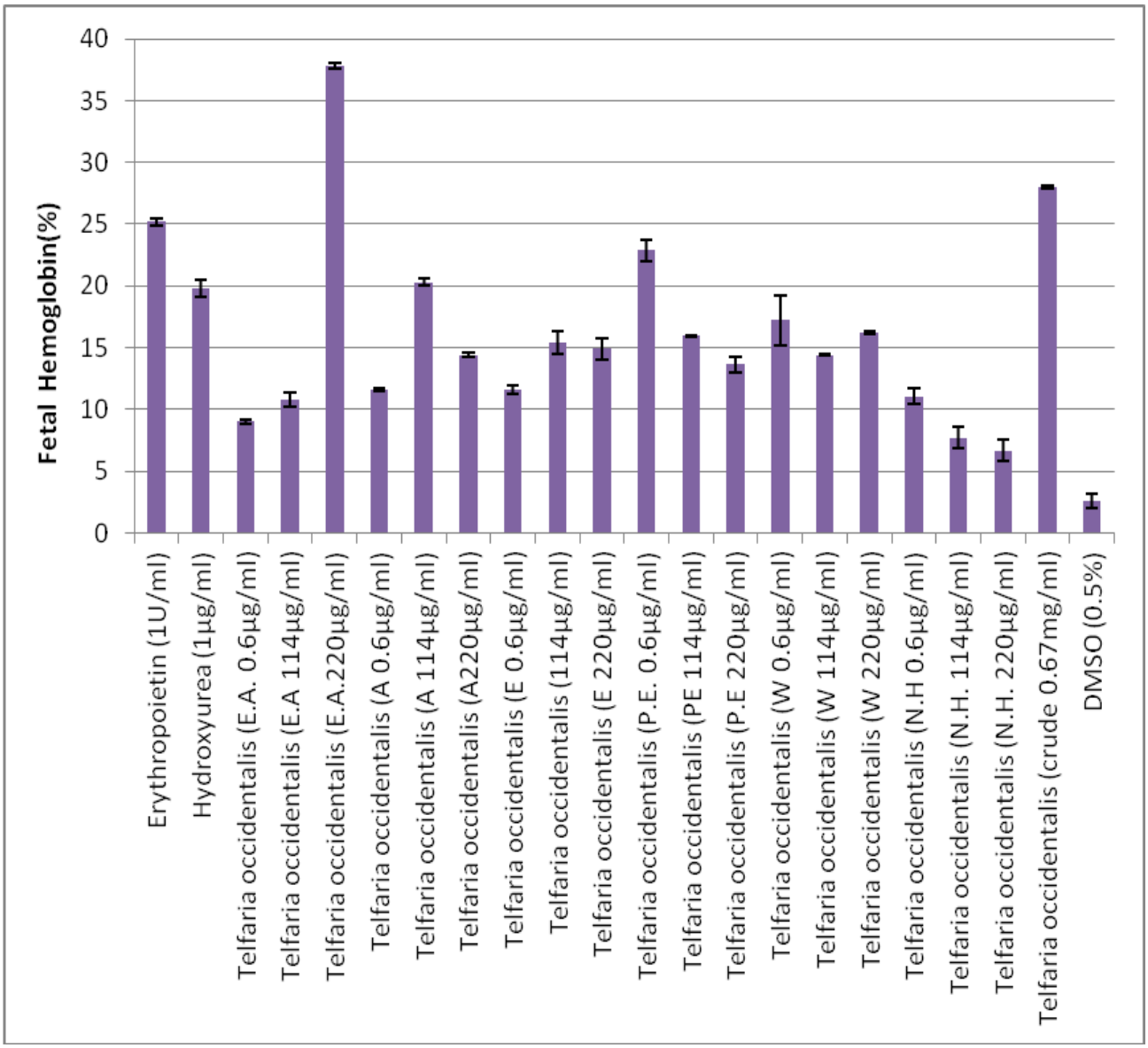


Figure 4.3: Effect of *Telfairia occidentalis* solvent fractions on Foetal Haemoglobin content in cultured erythroid progenitor stem cells

KEY

E.A.= ethyl acetate fraction

A = acetone fraction

E = ethanol fraction

P.E. = petroleum ether fraction

W = distilled water fraction

N.H. = *n*-hexane fraction

4.7 EFFECT OF *SORGHUM BICOLOR* SOLVENT FRACTION ON FOETAL HAEMOGLOBIN SYNTHESIS IN ERYTHROID PROGENITOR STEM CELLS

All the S .*bicolor* solvent fractions induced foetal Hb synthesis significantly higher than the DMSO solvent control (Figure 4.4). All of the *S. bicolor* solvent extracts failed to induce foetal Hb synthesis significantly ($P<0.05$) comparable to both positive controls (EPO (1U/ml) and HU 1μg/ml) (Figure 4.4).

4.8 EFFECT OF *AZADIRACTHA INDICA* SOLVENT FRACTION ON FOETAL HAEMOGLOBIN SYNTHESIS IN ERYTHROID PROGENITOR STEM CELLS

A. indica aqueous extract was partitioned using solvents of varying polarity; All the *A. indica* solvent fractions induced foetal Hb synthesis was significantly ($P<0.05$) higher than the DMSO solvent control (Figure 4.5). All of the *A. indica* solvent extracts did not induce foetal Hb synthesis significantly comparable to both positive controls (EPO (1U/ml) and HU 1μg/ml) (figure 4.5).

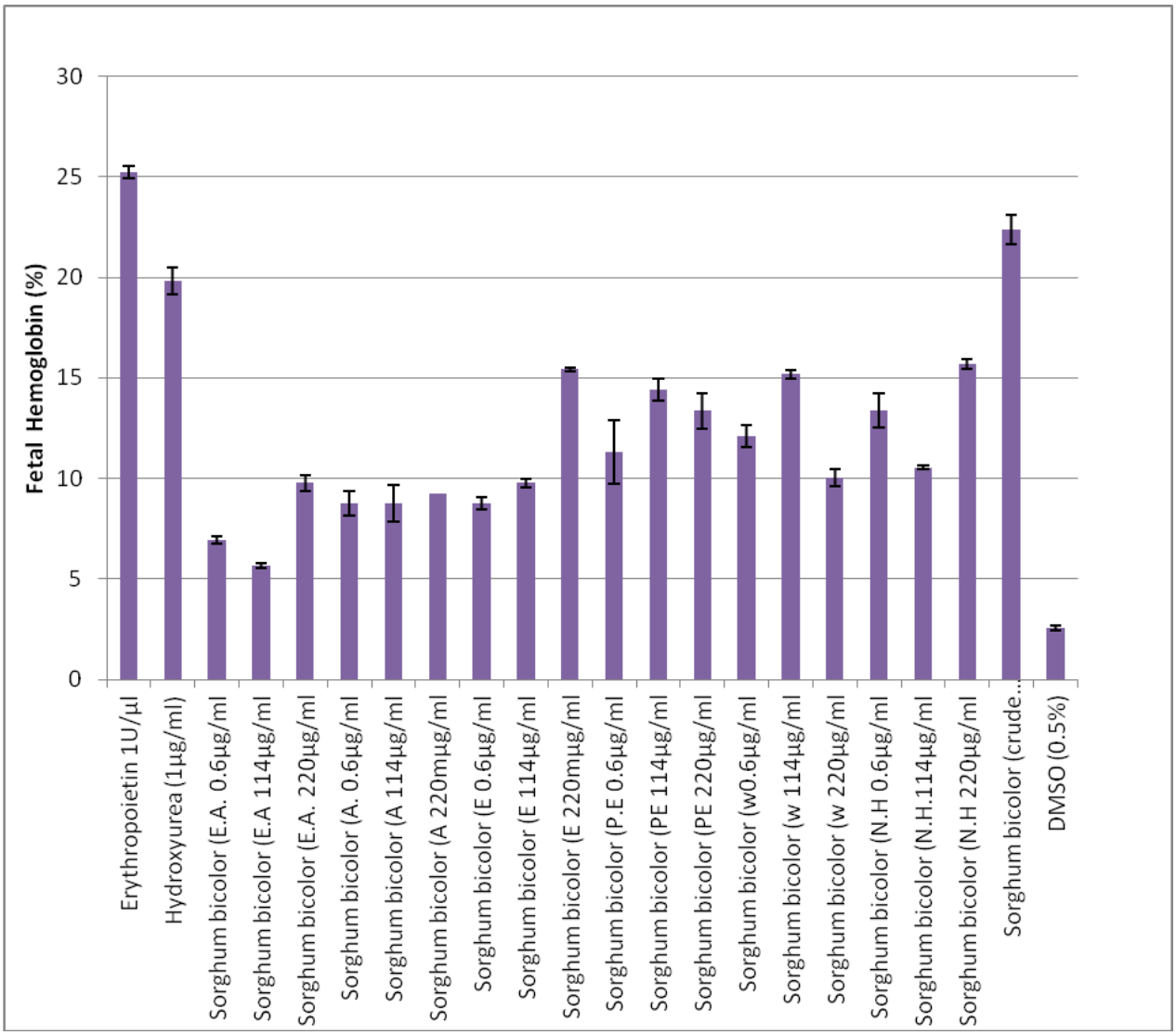


Figure 4.4: Effect of *Sorghum bicolor* solvent fractions on foetal haemoglobin synthesis in erythroid progenitor stem cells

KEY

E.A.= ethyl acetate fraction

A = acetone fraction

E = ethanol fraction

P.E. = petroleum ether fraction

W = distilled water fraction

N.H. = *n*-hexane fraction

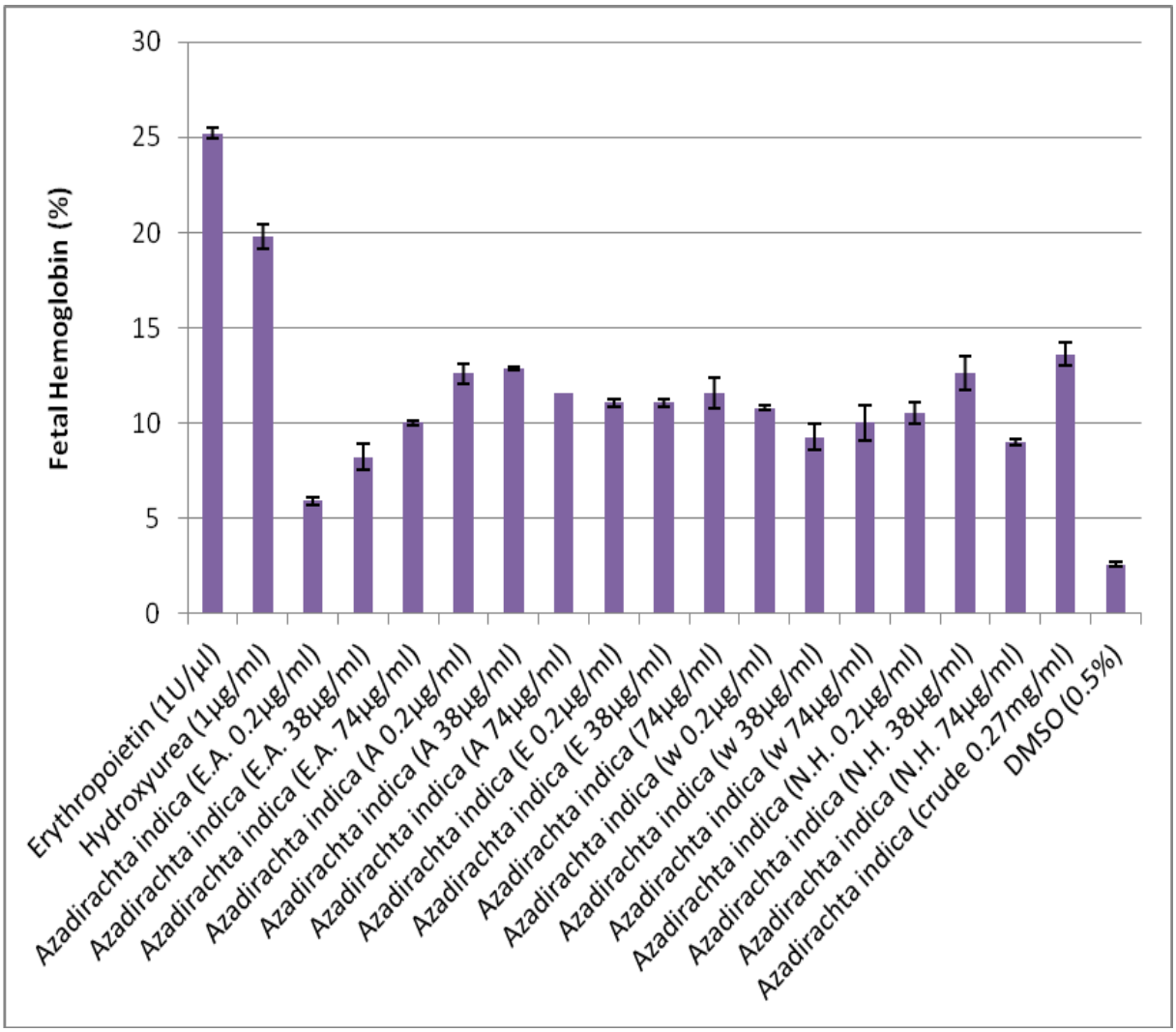


Figure 4.5: Effect of *Azadiractha indica* solvent fraction on foetal haemoglobin synthesis in erythroid progenitor stem cells

KEY

E.A.= ethyl acetate fraction

A = acetone fraction

E = ethanol fraction

P.E. = petroleum ether fraction

W = distilled water fraction

N.H. = n-hexane fraction

4.9 EFFECT OF *TERMINALIA CATAPPA* SOLVENT FACTIONS ON FOETAL HAEMOGLOBIN SYNTHESIS IN HUMAN ERYTHROID PROGENITOR STEM CELLS

Terminalia catappa aqueous extract was partitioned using solvents of varying polarity; both ethyl acetate and *n*-Hexane produced 2 differently colored fractions which was separately collected and labeled a and b. All the *T. catappa* solvent fractions induced foetal Hb synthesis, significantly ($P<0.05$) higher than the DMSO solvent control (Figure 4.6). The results showed that the distilled water and *n*-hexane fraction was most effective in inducing foetal Hb synthesis significantly higher than the positive controls (EPO (1U/ml) and HU 1µg/ml) in human erythroid progenitor stem cells compared to other fractions. The *T. catappa* distilled water fraction (TCDWF) induced foetal Hb increase was found to be concentration dependent with *T. catappa* distilled water fraction being most effective at 220µg/ml. *T. catappa n*-Hexane fraction was effective in inducing foetal Hb synthesis above the positive controls at 0.6µg/ml (Figure 4.6).

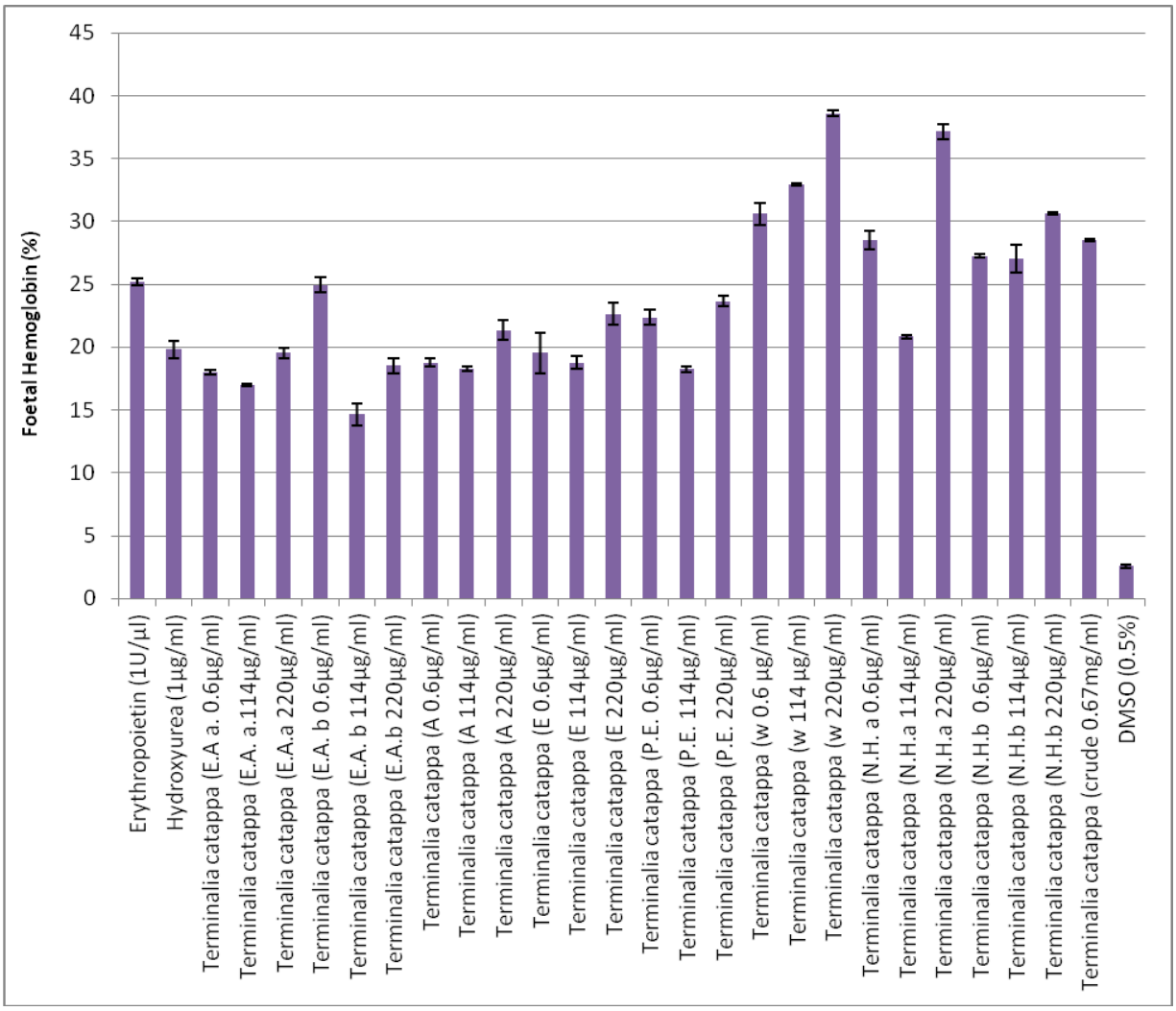


Figure 4.6: Effect of *Terminalia catappa* solvent fraction on foetal haemoglobin synthesis in erythroid progenitor stem cells

KEY

E.A.= ethyl acetate fraction

A = acetone fraction

E = ethanol fraction

P.E. = petroleum ether fraction

W = distilled water fraction

N.H. = n-hexane fraction

4.10 EFFECT OF FOETAL HAEMOGLOBIN INDUCING SOLVENT FRACTIONS ON DIFFERENTIATION AND COMMITMENT TO ERYTHROID PROGENY OF HUMAN ERYTHROID PROGENITOR STEM CELLS

Cellular growth and differentiation were analysed by Benzidine staining of smeared slides on days 5, 10 and 14. TCDWF appeared to selectively induce differentiation of human erythroid progenitor stem cells in both EPO dependent and independent culture systems. TCDWF induced differentiation and commitment to the erythroid lineage of human erythroid progenitor stem cell was most prominent on day 3 of EPO-independent cultures Figure 4.7, compared to EPO-dependent cultures which became significantly high on day 12 of cultures Figure 4.8.

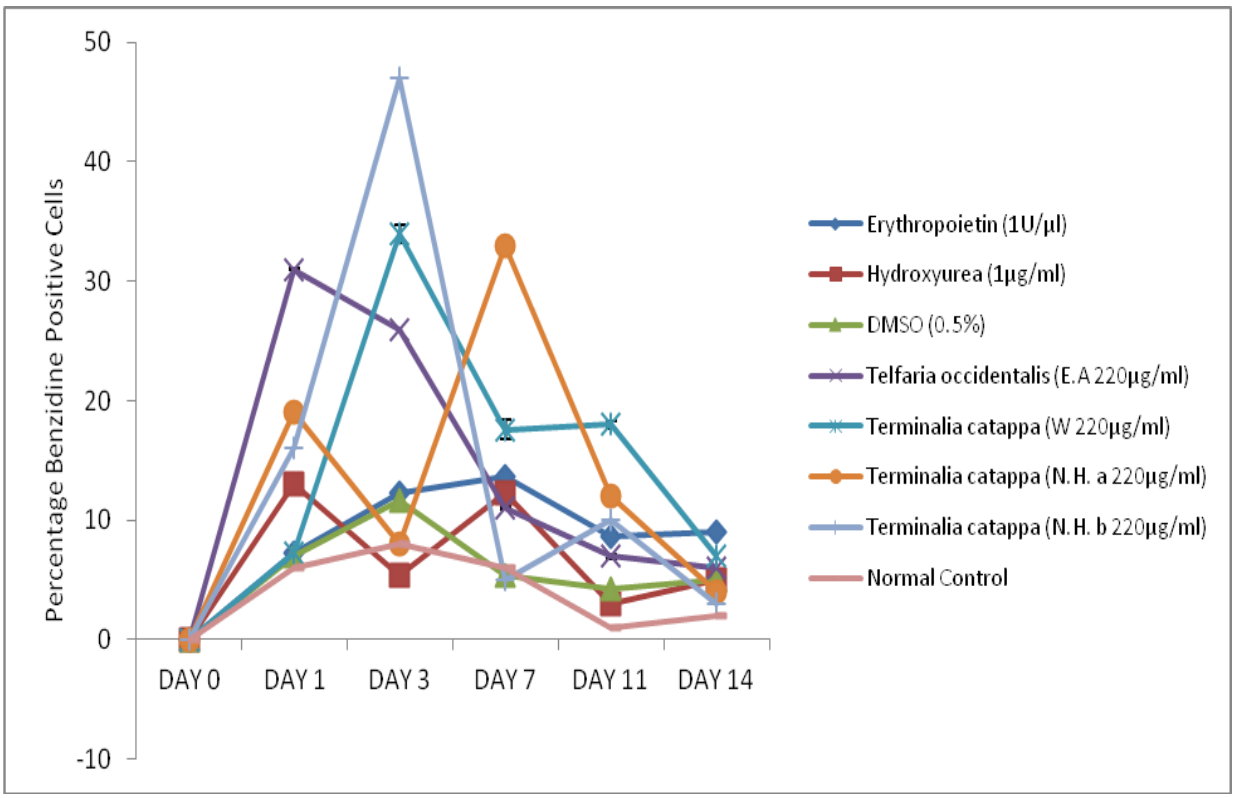


Figure 4.7: Effect of active Foetal Haemoglobin inducing solvent fractions on commitment to erythroid progeny of erythroipoietin independent Erythroid progenitor stem cells

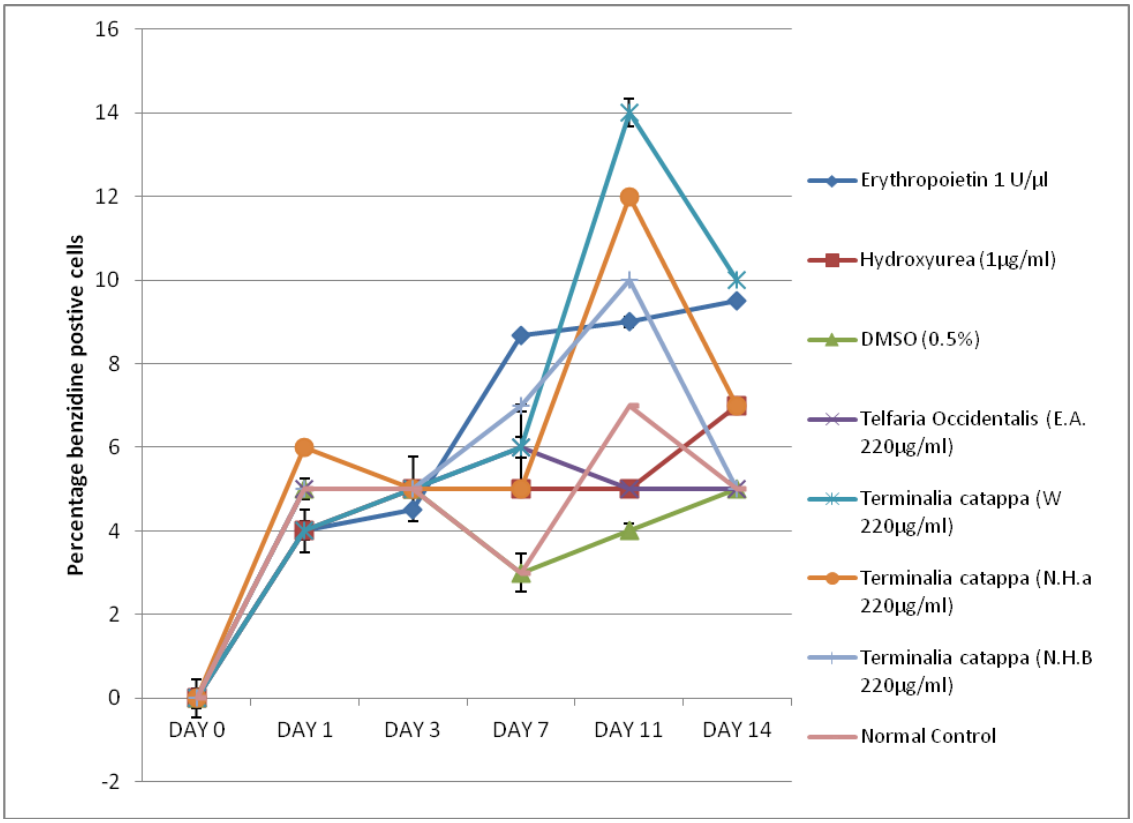


Figure 4.8: Effect of active Foetal Haemoglobin inducing solvent fractions on commitment to erythroid progeny of erythroipoietin dependent Erythroid progenitor stem cells

4.11 EFFECT OF *TERMINALIA CATAPPA* AND *TELFAIRA OCCIDENTALIS* FOETAL HAEMOGLOBIN INDUCING ACTIVE FRACTIONS ON RELATIVE FOETAL HAEMOGLOBIN SYNTHESIS IN ERYTHROPOIETIN INDEPENDENT AND DEPENDENT HUMAN ERYTHROID PROGENITOR STEM CELLS

TCDWF increased significantly ($P<0.05$) total haemoglobin synthesis compared to the un-induced control cells. However total haemoglobin increased synthesis induced by TCDWF was lower when compared to HU (1µg/ml) induced cells. TCDWF induced foetal Hb synthesis was significantly higher than HU(1µg/m), EPO (1U/ml) and un-induced controls in EPO independent human erythroid progenitor stem cells (Figure 4.9). This difference however was not evident in EPO-dependent hEPSCs (Figure 4.10).

4.12 EFFECT OF *TERMINALIA CATAPPA* AND *TELFAIRIA OCCIDENTALIS* FOETAL HAEMOGLOBIN INDUCING ACTIVE FRACTIONS ON FOLD INCREASE OF FOETAL HAEMOGLOBIN IN HUMAN ERYTHROID PROGENITOR STEM CELLS

Induction of foetal haemoglobin in EPO-independent and dependent progenitor stem cell cultures (agents were added at the specified concentration on the first day of culture, and cells were harvested and total and foetal haemoglobin were quantified on the 14^{th} day of culture). TCDWF induced significantly higher foetal Hb fold increase compared to positive controls of EPO 1U/ml and HU 1µg/ml and the 0.5% DMSO control. *Terminalia catappa* n-Hexane fractions A and B foetal Hb fold induction were significantly higher than 0.5% DMSO control but not significantly higher than HU (1µg/ml). *Telfairia occidentalis* ethyl acetate fraction foetal Hb fold induction was also not significantly higher than the HU (1µg/ml). TCDWF induced foetal Hb fold increase was more pronounced in EPO-independent cultures compared to EPO dependent cultures (Figure 4.11).

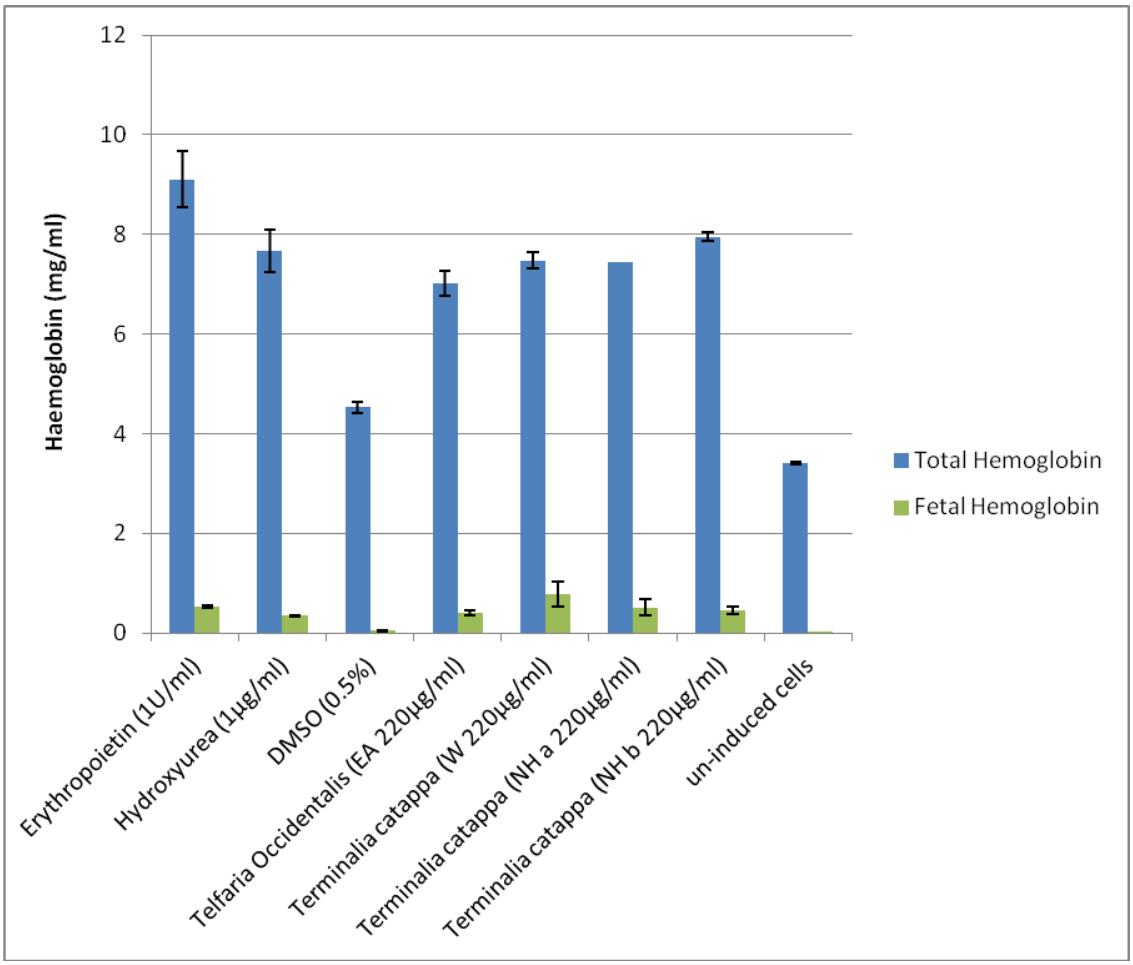


Figure 4.9: Relative induction of Foetal Haemoglobin synthesis in erythropoietin independent hEPSCs by Foetal Haemoglobin inducing active solvent fractions

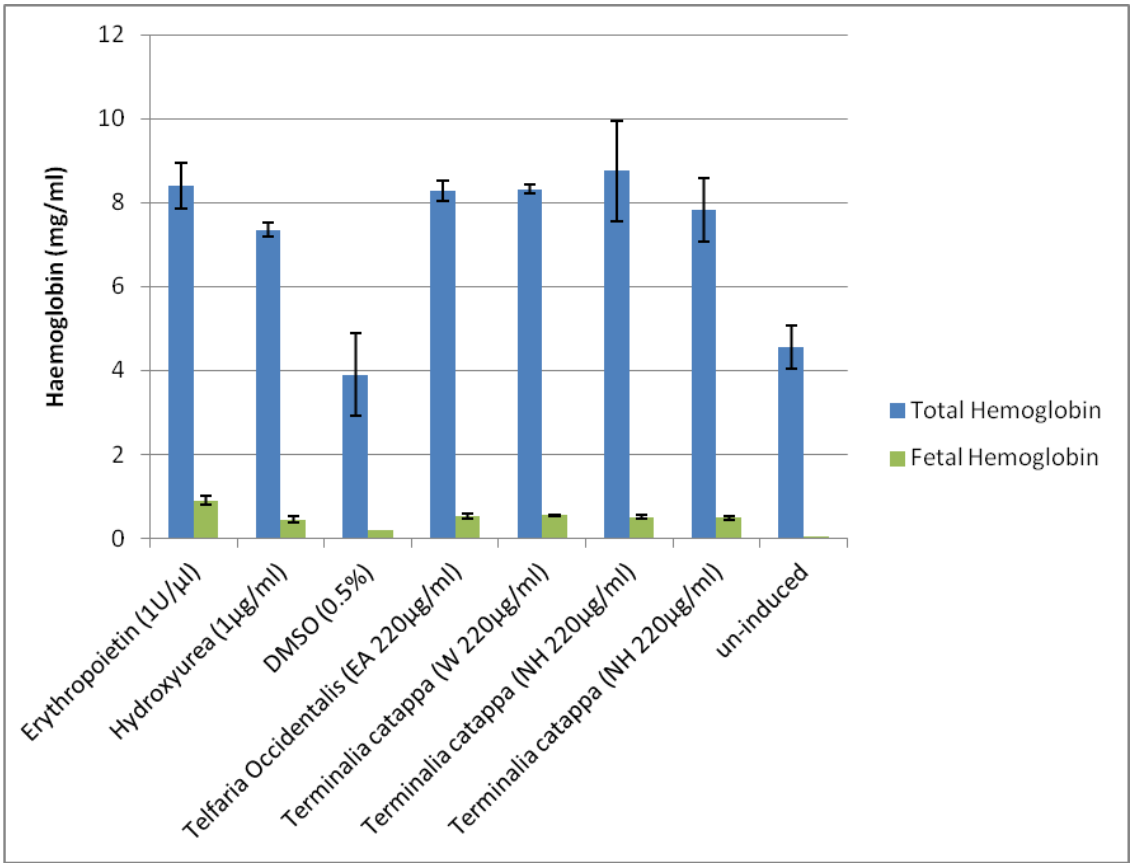


Figure 4.10: Relative induction of Foetal Haemoglobin synthesis in erythropoietin dependent hEPSCs by Foetal Haemoglobin inducing active solvent fractions

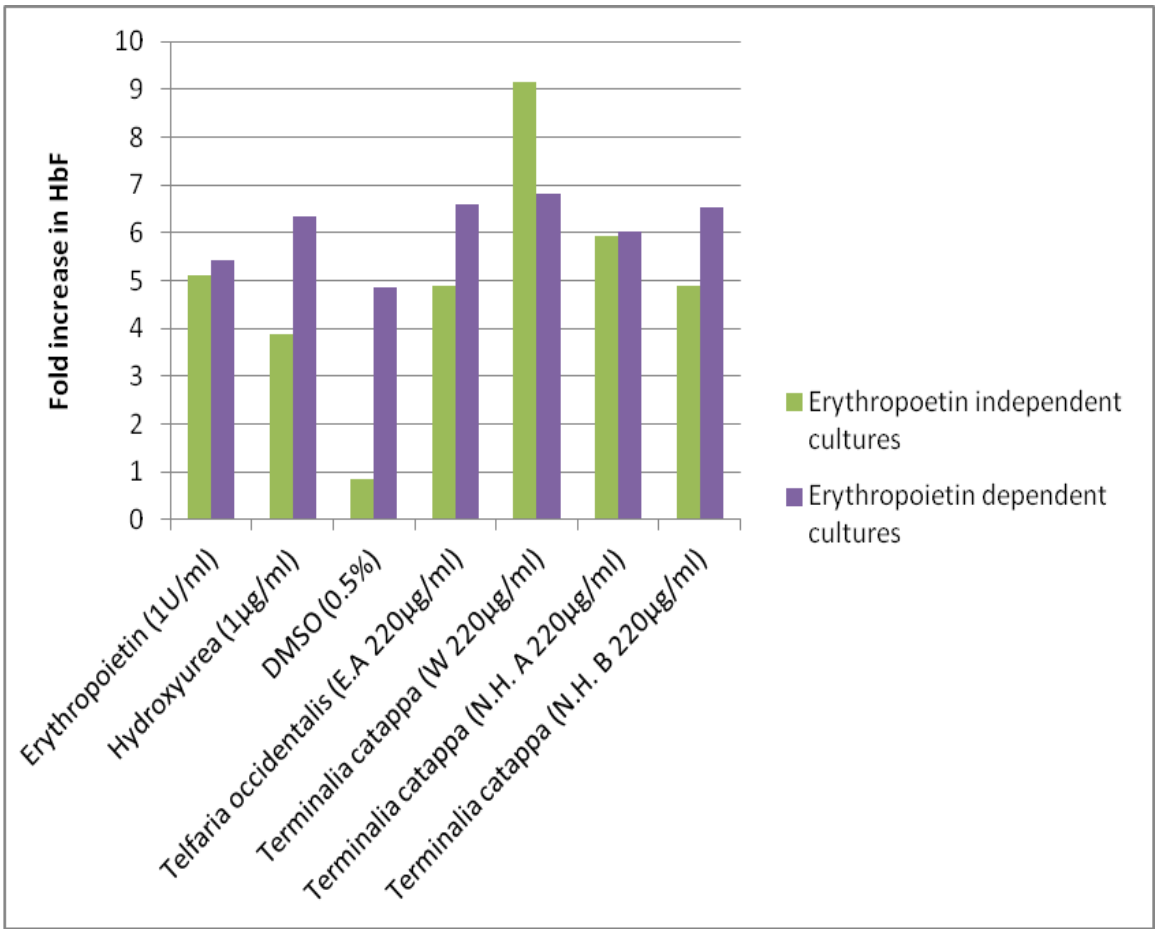


Figure 4.11: Relative induction of Foetal Haemoglobin synthesis in cultured erythropoietin dependent and independent HSC by Foetal Haemoglobin inducing active solvent fractions

4.13 CYTOTOXICITY OF FOETAL HAEMOGLOBIN INDUCING ACTIVE FRACTIONS

All the foetal Hb inducing active fractions were assayed for their cytotoxic potential in cultured hEPSCs. Cytotoxity is presented as a measure of LDH release and intracellular Caspase 3 activity of hEPSCs (Figures 4.12-4.15). LDH release was significantly higher in TCDWF and *Terminalia catappa* n-Hexane fraction (TCNHF) treated cells compared to un-induced EPO-independent hEPSCs (Figure 4.12). On the other hand this trend was completely reversed in EPO-dependent hEPSCs (Figure 4.13), where no LDH release was detected in TCDWF and TCNHB fractions treated cultures. Caspase 3 activity was significantly lowered in *Terminalia catappa* foetal Hb inducing solvent fractions treated EPO- independent and dependent hEPSCs compared to EPO (1IU/ml), HU (1µg/ml) and un-induced cells (Figures 4.14 and 4.15 respectively). On the other hand *Telfairia occidentalis* ethyl acetate fraction induced significant Caspase 3 activity in both EPO- independent and dependent cells comparable to HU(1µg/ml) and un-induced hEPSCs (Figures 4.14 and 4.15 respectively).

4.14 ABSORBANCE SPECTRA OF *TERMINALIA CATAPPA* DISTILLED WATER FRACTION (TCDWF)

The UV-Visible absorbance spectra was determined to ascertain the structural homogeneity of the component compounds of the most active foetal haemoglobin inducing fraction (TCDWF). The UV-Visible spectra of TCDWF revealed a single clear and distinctive peak at 400-402nm in the visible spectra range see Figure 4.16.

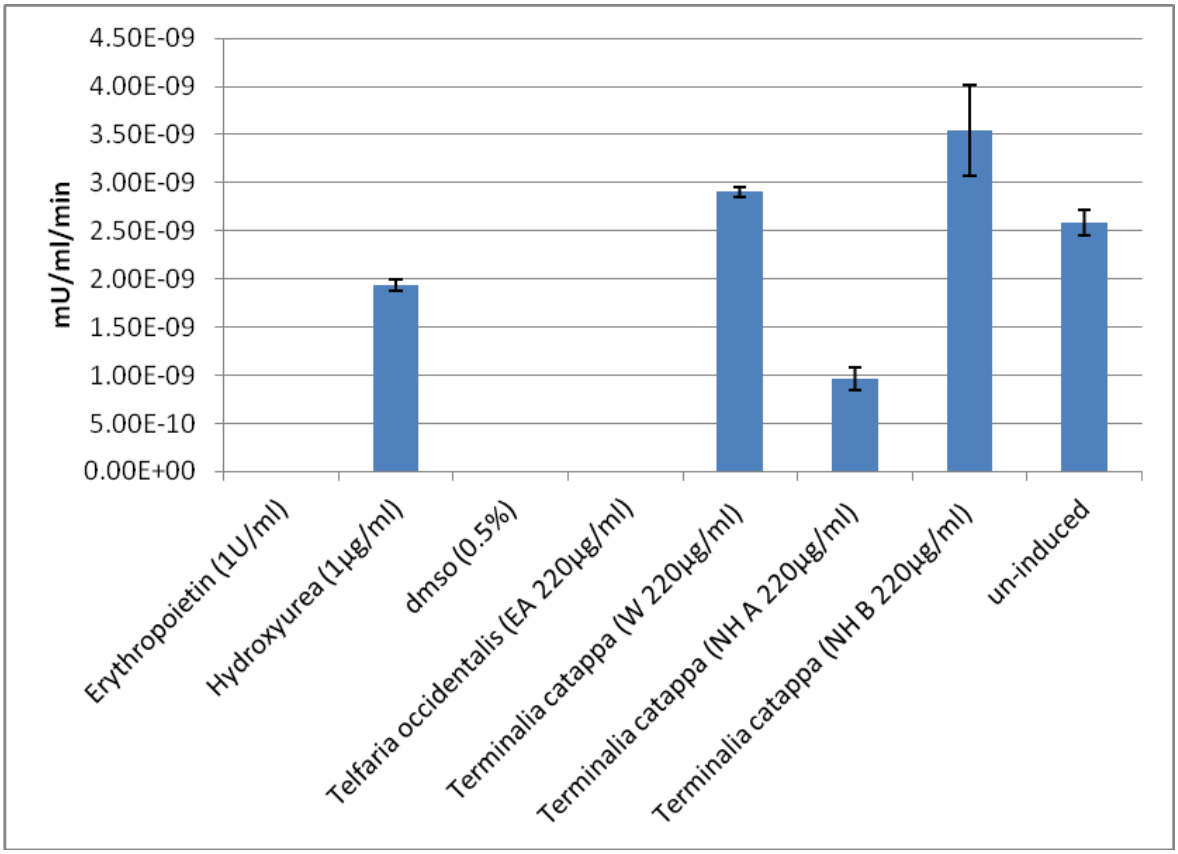


Figure 4.12: Effect of Foetal Hb inducing active fractions on erythropoietin independent hEPSCs Lactate dehydrogenase release.

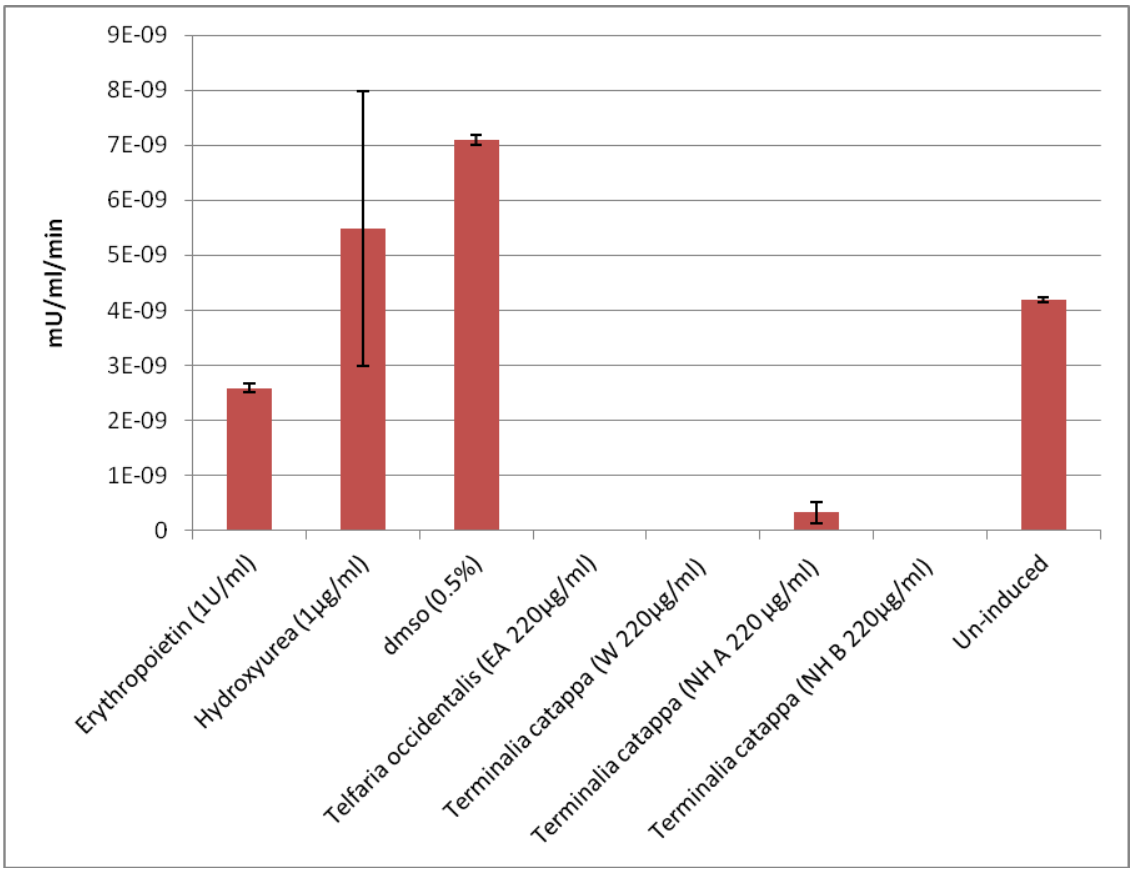


Figure 4.13: Effect of Foetal Hb inducing active fractions on erythropoietin dependent hEPSCs Lactate dehydrogenase release.

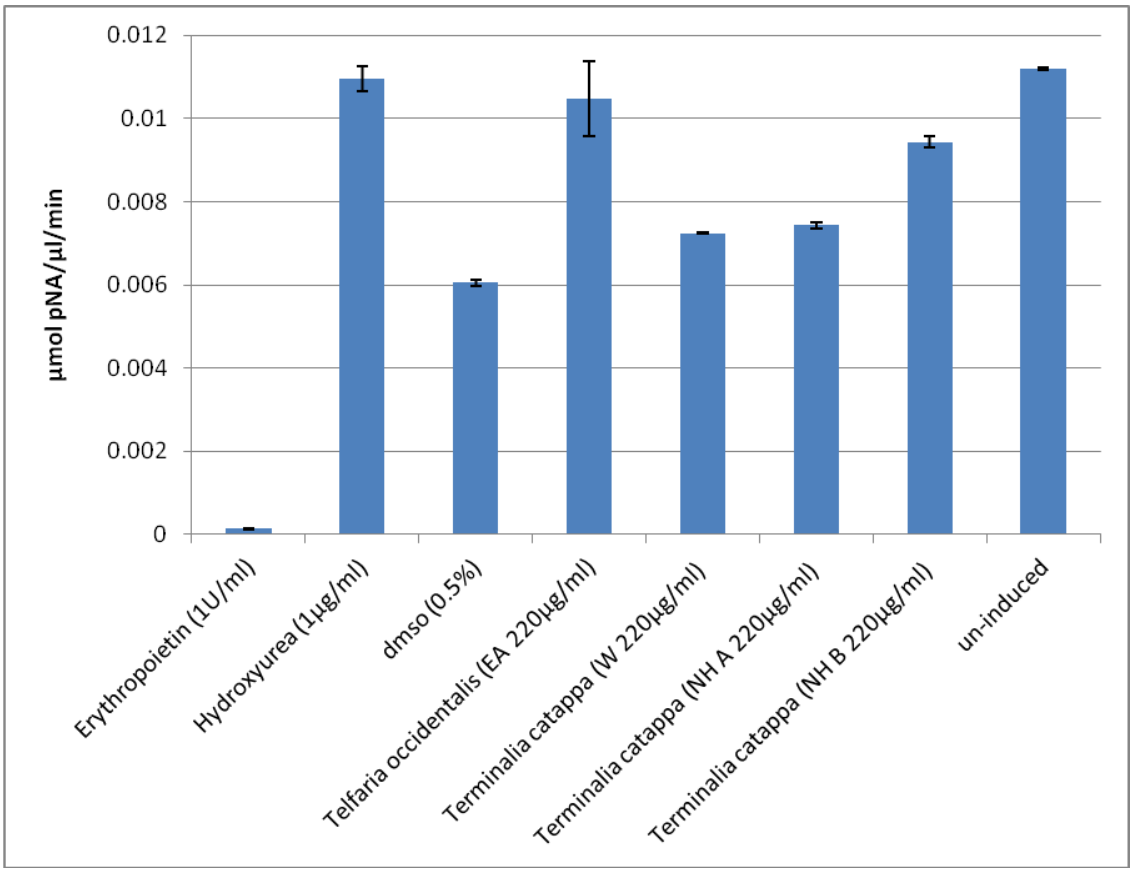


Figure 4.14: Effect of Foetal Haemoglobin inducing active solvent fractions on Caspase 3 activity of erythropoietin independent hEPSCs

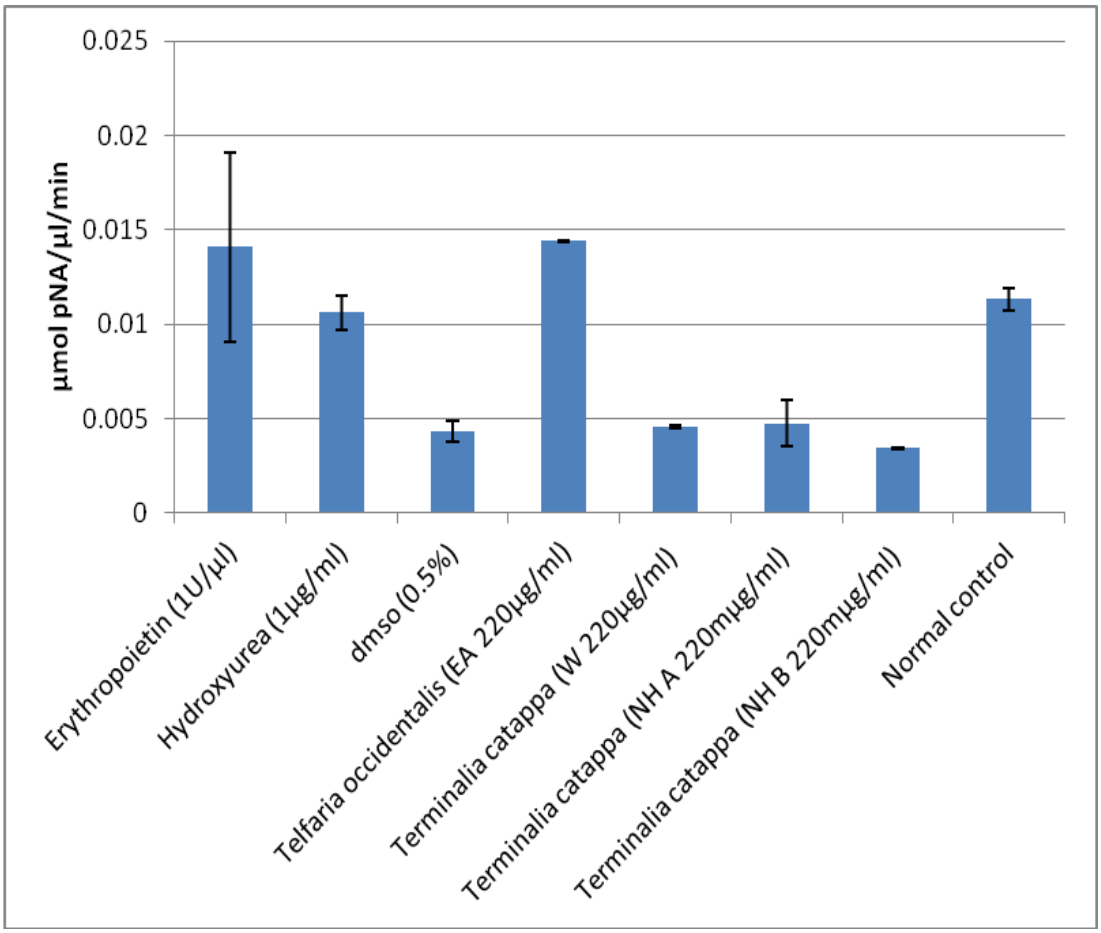


Figure 4.15: Effect of Foetal Haemoglobin inducing active solvent fractions on Caspase activity of erythropoietin dependent hEPSCs

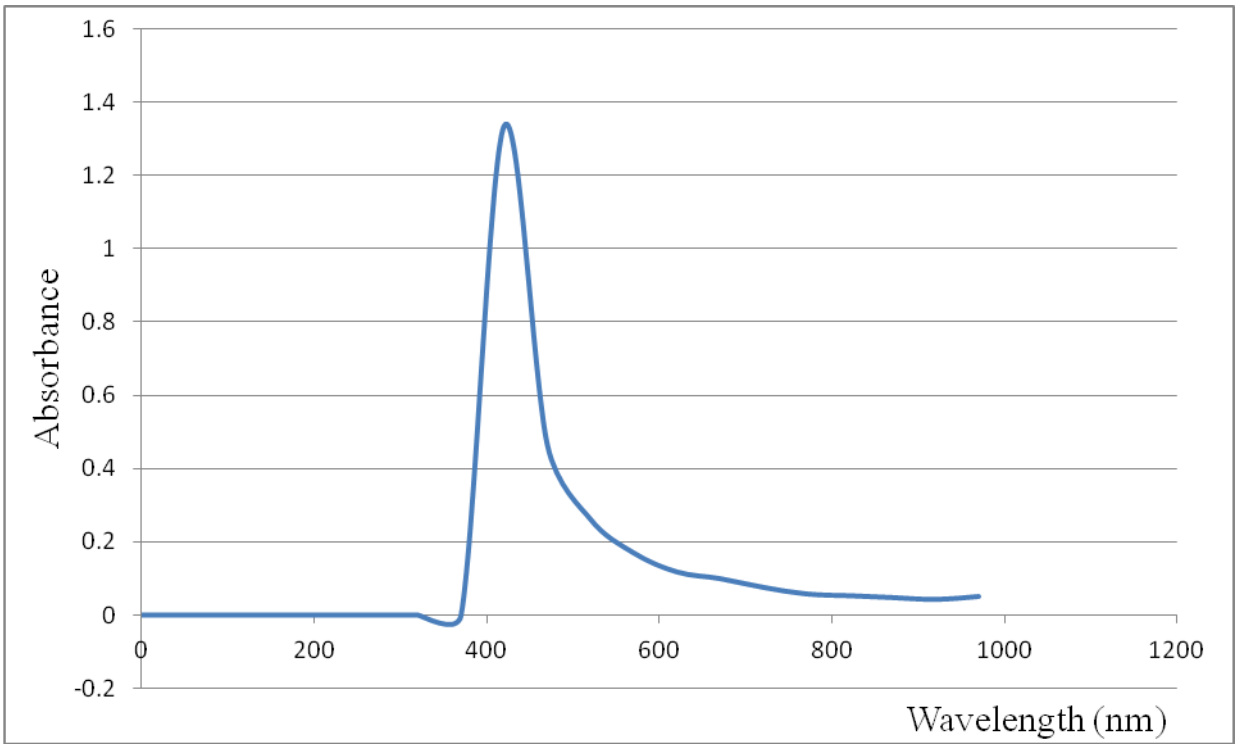


Figure 4.16: UV-Visible spectra of *Terminalia catappa* distilled water fraction.

4.15 GAS CHROMATOGRAPHY AND MASS SPECTRA OF *TERMINALIA CATAPPA* DISTILLED WATER FRACTION

A structural qualitative analysis of TCDWF was done using GCMS, and the data revealed three distinctive peaks of key interest, with retention times of 20min for the first peak and with peaks 2 and 3 occurring at the 22nd minute of the run. Mass spectra of the GC eluates were compared using NIST2011 spectral library; peak 2 corresponded to Pentadecanoic acid and Hexadecanoic acid with a similarity index of 95 (Table 4.3). Peak 3 corresponded to 2-hexadecenoic acid, 9-octadecdenoic acid, cis-vaccenic acid and 10-octadecenoic acid with a similarity index of 91, 92, 92 and 93 respectively peak 4 corresponded to Octadecanoic acid with a similarity index of 94. (Figure 4.17).

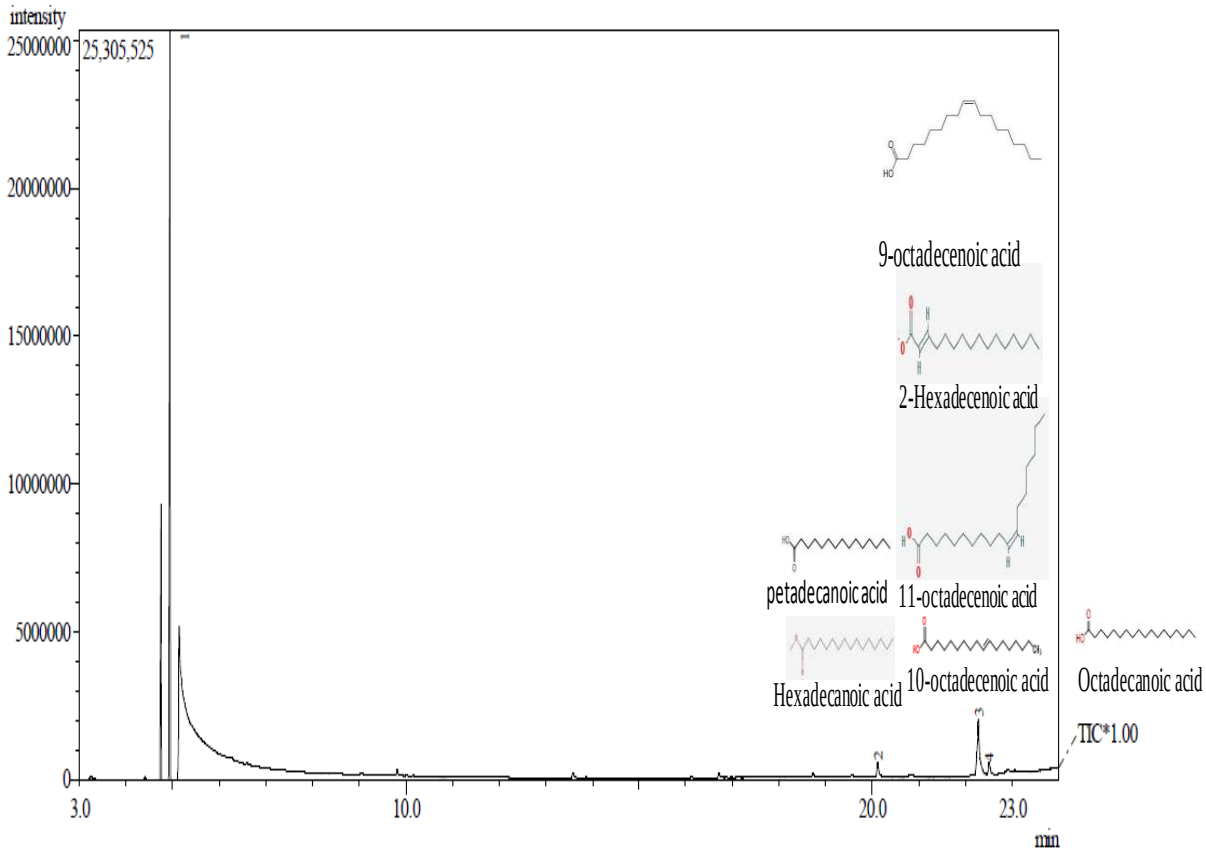


Figure 4.17: Gas chromatogram and Mass spectra identified compounds of non-derivitized *T. catappa* distilled water fraction

Table 4.3: Potential foetal haemoglobin inducing active compounds identified from GCMS of TCDWF.

S/No.	PEAK No	Possible Compounds	Similarity Index
1	2	Pentadecanoic acid	95
		Hexadecanoic acid	95
2	3	Cis-vaccenic acid	92
		9-octadecdenoic acid,	93
		2-hexadecenoic acid	91
		10-octadecenoic acid	92
3	4	Octadecanoic acid	94

4.16 EFFECT OF CIS-VACCENIC ACID (CVA), 2-HEXADECENOIC ACID (2-HDA), OLEIC ACID (OA), APIGENIN (AP), DOCOSAHEXANOIC ACID (DHA), EICOSAPENTANOIC ACID (EPA), STEARIC ACID (SA) AND GENTISIC ACID (GA) ON K562 CELL DIFFERENTIATION

Figure 4.18 shows the effect of the compounds identified from TCDWF on the viability of K562 cells induced for 96 hours using two different concentrations of each compound. Concentrations were selected based on previous studies of the compounds using human cell types (Tricon *et al*., 2004; Renner *et al*., 2013).

Gentisic acid, Stearic acid, Oleic acid, Cis vaccenic acid (CVA) and 2-hexadecenoic acid (2-HDA) showed no significant impact on cell viability at both concentrations employed, Docosahexanoic acid (DHA), Eicosapentanoic acid (EPA) and Apigenin reduced K562 cell viability at 60, 60 and 150μM respectively (Figure 4.18).

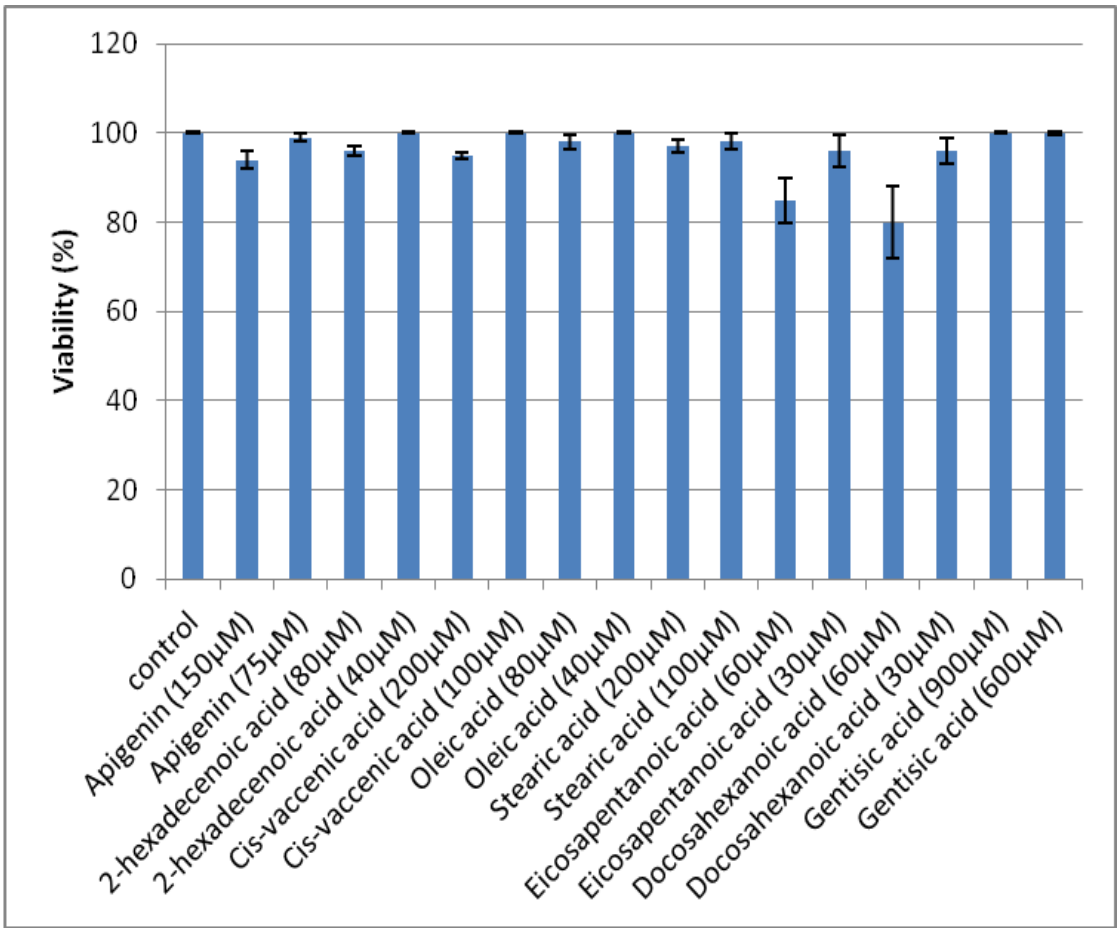


Figure 4.18: Effect of identified compounds on K562 cell viability 96 hours post incubation

4.17 EFFECT OF CIS-VACCENIC ACID (CVA), 2-HEXADECENOIC ACID (2-HDA), OLEIC ACID (OA), APIGENIN (AP), DOCOSAHEXANOIC ACID (DHA), EICOSAPENTANOIC ACID (EPA), STEARIC ACID (SA) AND GENTISIC ACID (GA) ON K562 CELL DIFFERENTIATION

CVA induced differentiation and increased the percentage (15%) of benzidine positive cells in K562 cells relative to un-induced and Hemin (40µM) (Benz *et al*., 1980) induced cells. CVA K562 induced differentiation was concentration dependent (Plate 4.6). 2-HDA also induced differentiation and increased the percentage of benzidine positive cells in K562 cells comparable to Hemin (40µM). 2-HDA K562 induced differentiation was concentration dependent (Plate 4.7). it was also observed that OA induced differentiation and increased the percentage of benzidine positive cells in K562 cells comparable to Hemin (40µM). Oleic acid K562 induced differentiation was concentration dependent (Plate 4.8). Apigenin induced differentiation and increased the percentage of benzidine positive cells in K562 cells comparable to Hemin (40µM). Apigenin K562 induced differentiation was concentration dependent and Apigenin appeared to alter the morphology of K562 cells as seen on Plate 4.9. DHA showed slight induction of K562 cell differentiation although using MGG staining DHA appeared to be inhibiting K562 cell growth (Plate 4.10). EPA, SA and GA however failed to induce K562 differentiation at all the concentrations assayed (Plates 4.11, 4.12 and 4.13 respectively).

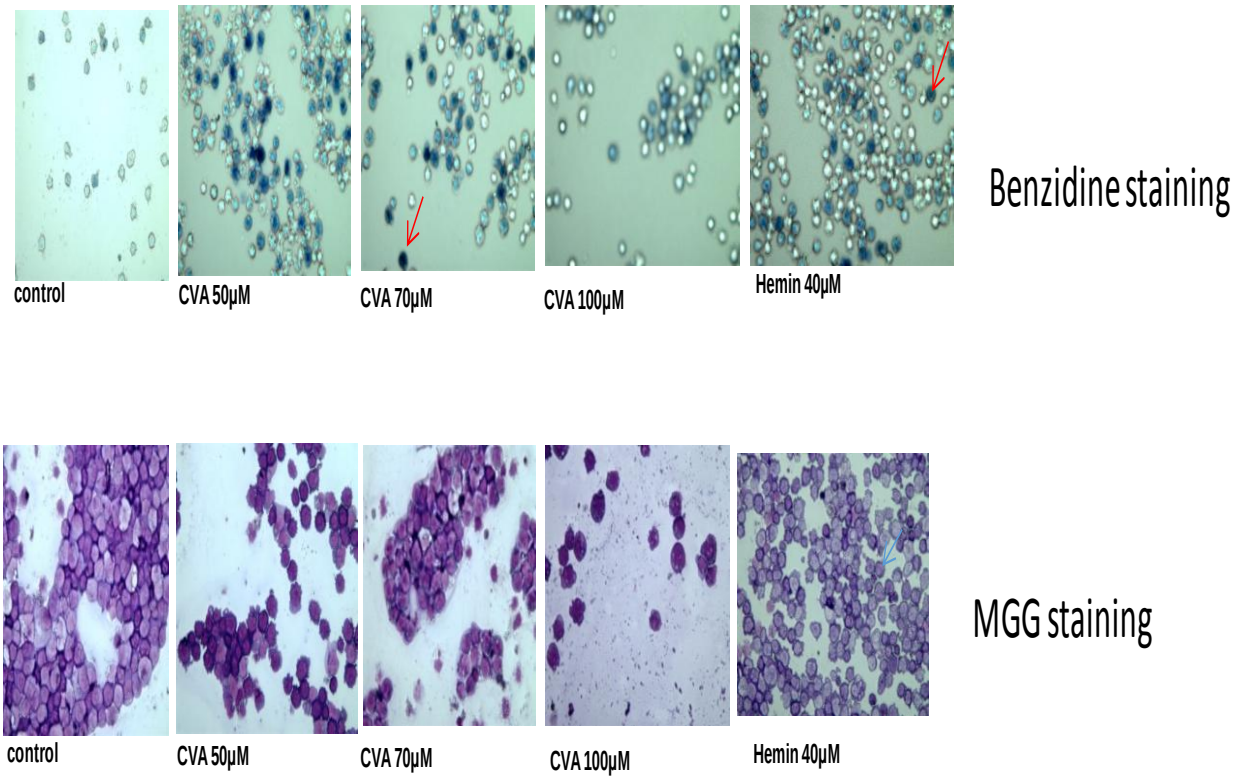
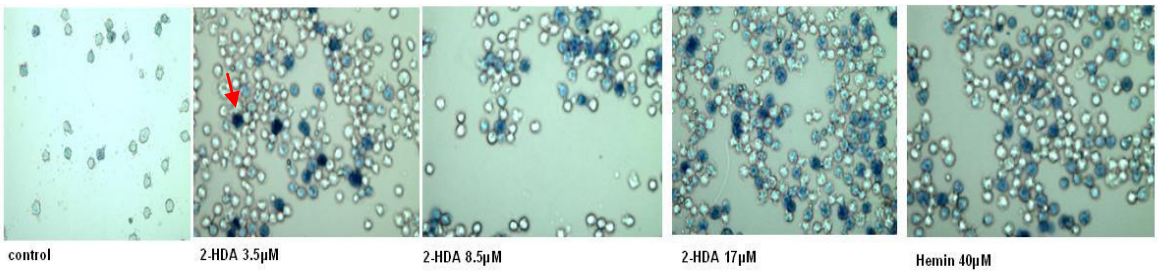
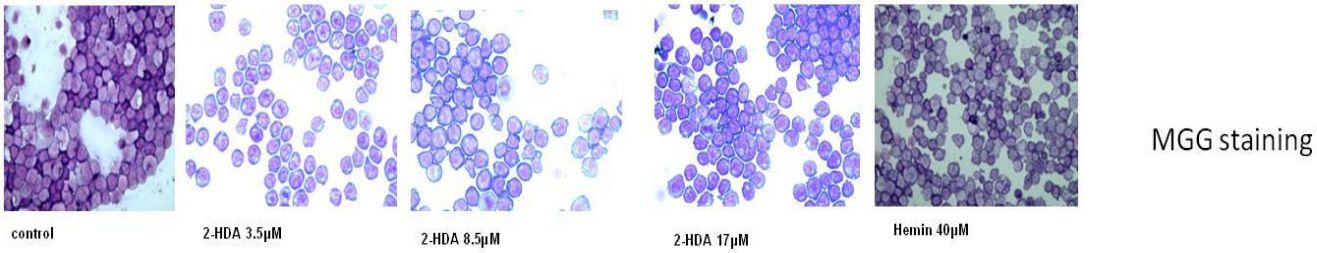


Plate 4.6: Effects of Cis vaccenic (CVA) on K562 cell differentiation. Magnification X 400. Benzidine staining was used to assay the effect of CVA on K562 cell differentiation and commitment to the erythroid lineage **(arrow indicate benzidine positive erythroid committed cells).** MGG staining was used to determine the effect of CVA on the morphology of K562 cells





Magnification X 400 Benzidine staining was used to assay the effect of 2-HDA on K562 cell differentiation and commitment to the erythroid lineage **(arrow indicate benzidine positive erythroid committed cells).** MGG staining was used to determine the effect of 2-HDA on the morphology of K562 cells

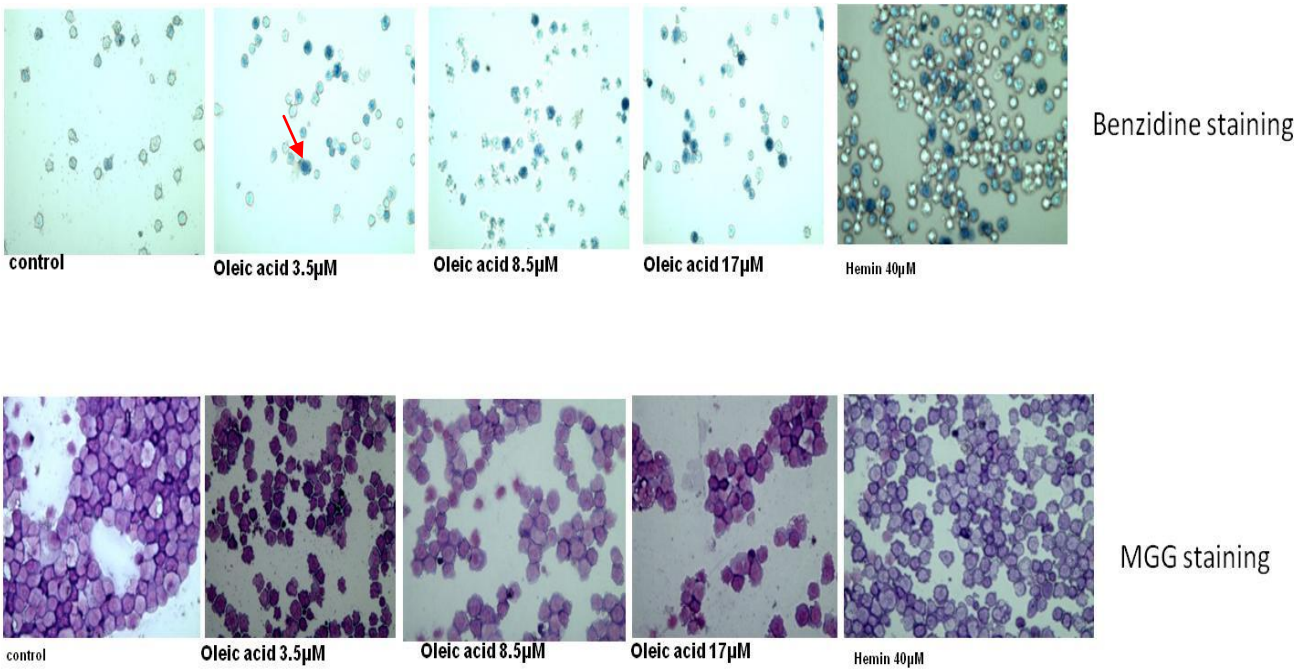


Plate 4.8: Effect of Oleic acid on K562 cells differentiation. Magnification X 400 Benzidine staining was used to assay the effect of Oleic acid on K562 cell differentiation and commitment to the erythroid lineage **(arrow indicate benzidine positive erythroid committed cells).** MGG staining was used to determine the effect of Oleic acid on the morphology of K562 cells

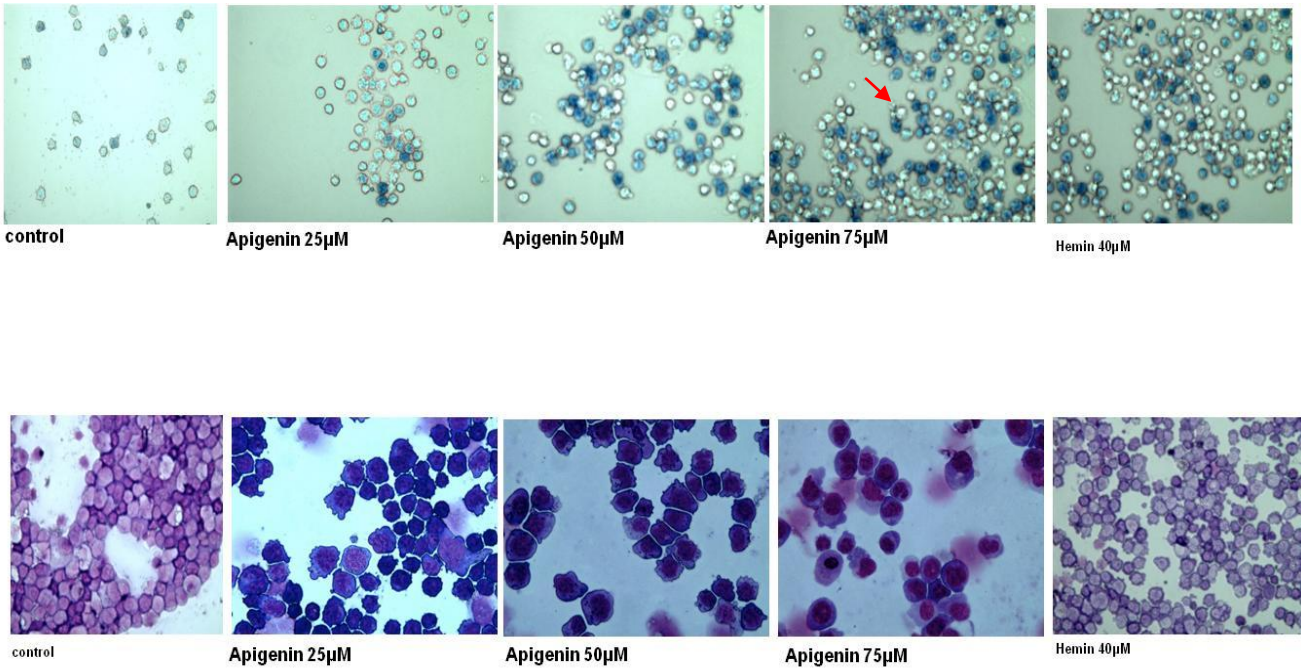


Plate 4.9: Effect of Apigenin on K562 cells differentiation. Magnification X 400. Benzidine staining was used to assay the effect of Apigenin on K562 cell differentiation and commitment to the erythroid lineage **(arrow indicate benzidine positive erythroid committed cells).** MGG staining was used to determine the effect of Apigenin on the morphology of K562 cells

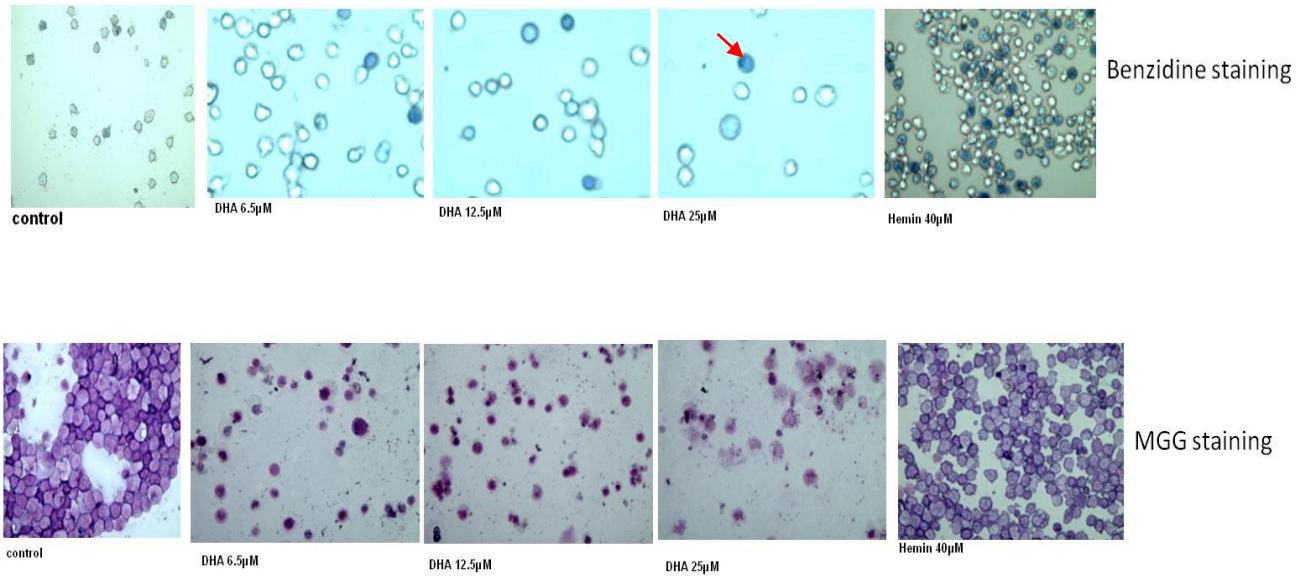


Plate 4.10: **Effect of Docosahexanoic acid (DHA) on K562 cells differentiation. Magnification X 400** Benzidine staining was used to assay the effect of DHA on K562 cell differentiation and commitment to the erythroid lineage **(arrow indicate benzidine positive erythroid committed cells).** MGG staining was used to determine the effect of DHA on the morphology of K562 cells

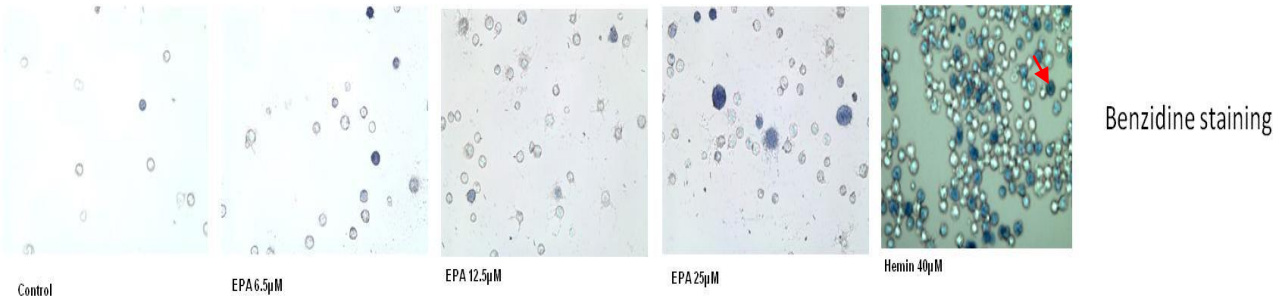


Plate 4.11: Effect of Eicosapentanoic acid (EPA) on K562 cells differentiation. Magnification X 400 Benzidine staining was used to assay the effect of EPA on K562 cell differentiation and commitment to the erythroid lineage **(arrow indicate benzidine positive erythroid committed cells).**

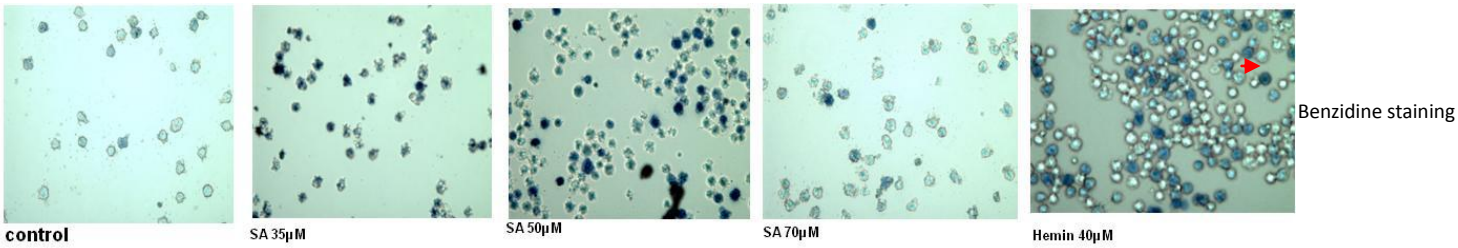


Plate 4.12: Effect of Stearic acid (SA) on K562 cells differentiation. Magnification X 400
Benzidine staining was used to assay the effect of Stearic acid on K562 cell differentiation and commitment to the erythroid lineage **(arrow indicate benzidine positive erythroid committed cells).**

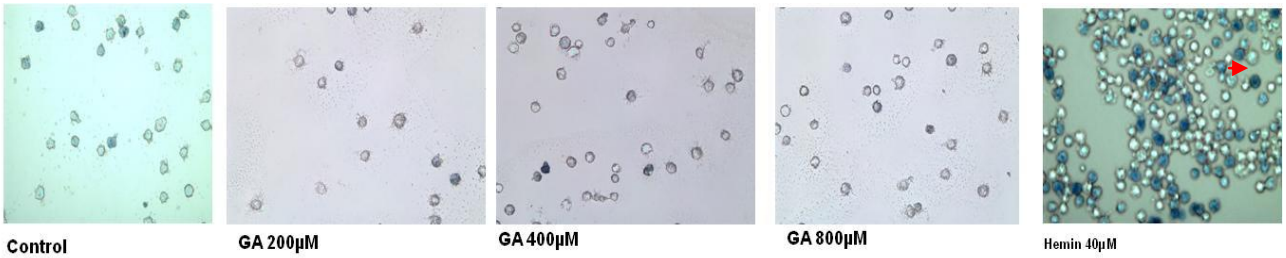


Plate 4.13: Effect of Gentisic acid (GA) on K562 cells differentiation. Magnification X 400
Benzidine staining was used to assay the effect of Gentisic acid on K562 cell differentiation

and commitment to the erythroid lineage **(arrow indicate benzidine positive erythroid committed cells).**

4.18 EFFECT OF CVA, 2-HDA, OLEIC ACID, APIGENIN, DHA, EPA, STEARIC ACID AND GENTISIC ACID K562 ERYTHROLEUKEMIC CELLS DEVELOPMENTAL DIFFERENTIATION

K562 cells were induced for 5 days using varying concentrations of identified compounds. The results (Figure 4.18) revealed that all the compounds assayed induced k562 cell differentiation was time dependent with higher levels of benzidine positive k562 cells observed after 120 hours of induction with the compounds (Figure 4.19). in addition Apigenin induced K562 differentiation revealed a concentration dependent pattern, with Apigenin at 75µM being most effective at inducing differentiation. K562 differentiation was found to be significantly ($P<0.05$) high relative to un-induced and Hemin (40µM) induced controls in all the treatments with the exception of Gentisic acid which appeared to stall K562 differentiation (Figure 4.19).

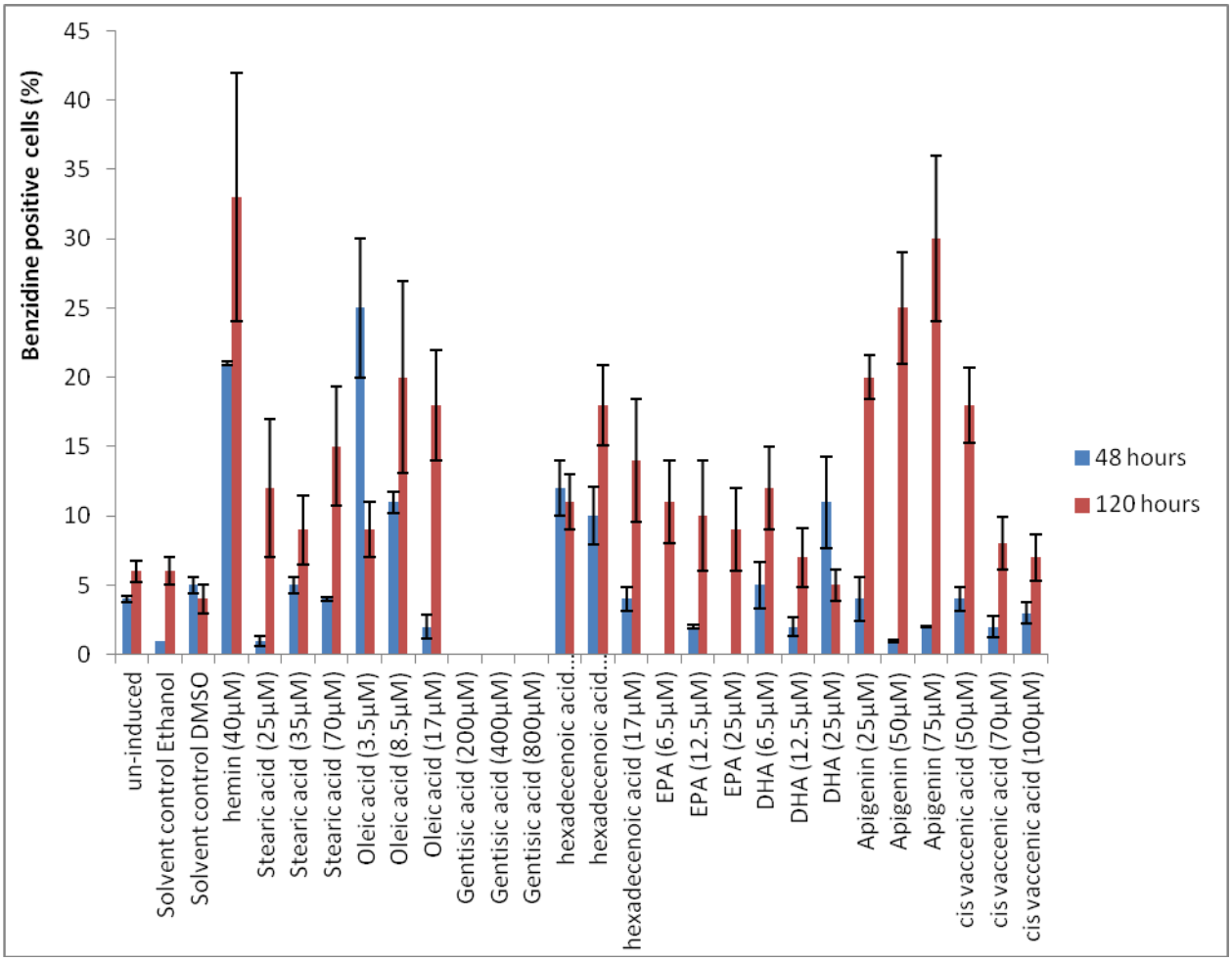


Figure 4.19: Effect of Cis-Vaccenic Acid, 2-Hexadecenoic Acid, Oleic Acid, Apigenin, Docosahexanoic Acid, Eicosapentanoic Acid, Stearic Acid And Gentisic Acid on K562 cell developmental differentiation

4.19 EFFECT OF APIGENIN, 2-HDA, CVA OLEIC ACID JK-1 CELL DIFFERENTIATION

Compounds active in inducing K562 differentiation and commitment to the erythroid lineage were further screened for their ability to induce JK-1 Cell differentiation using Hemin (40μM) as a positive control. CVA induced significant ($P<0.05$) JK-1 cell differentiation after 72 hours (Figure 4.19). Over 19% of JK-1 cells were differentiated to the erythroid linaege, 72 hours post induction with CVA (50μM) see Figure 4.20.

4.20 EFFECT OF ERYTHROPOIETIN ON CVA INDUCED JK-1 CELLS DIFFERENTIATION.

EPO-pre-differentiation of JK-1 cells did not significantly alter the level of CVA induced differentiation of JK-1 cells. The amount of differentiated JK-1 cells in both EPO grown and non- EPO grown cells was not significantly different as assessed by benzidine- giemsa staining (Plate 4.14)

4.21 EFFECT OF APIGENIN, DHA, 2-HDA, OLEIC ACID AND CVA ON K562 CELL FOETAL HAEMOGLOBIN (HBF) SYNTHESIS

CVA (50μM), 2-HDA (17μM) and Oleic acid (8.5μM) induced foetal haemoglobin synthesis in K562 cells 48 hours after induction compared to the un-induced control. This difference was not apparent 72 hours post induction compared to the un-induced control. Apigenin (75μM) and Oleic acid (3.5μM) did not induce K562 foetal haemoglobin synthesis at 24 hours post induction. Whereas Apigenin and Oleic acid induced foetal hemoglobin synthesis in K562 cells at 72 hours post induction at 75μM and 3.5μM respectively similar to the un-induced control (Figure 4.21).

4.22 EFFECT OF APIGENIN, 2-HDA, OLEIC ACID AND CVA ON JK-1 CELL FOETAL HAEMOGLOBIN SYNTHESIS

CVA (50µM) significantly ($P<0.05$) increased HbF levels in JK-1 cells to higher levels relative to un-induced and hemin (40µM) induced JK-1 cells (Figure 4.22). Apigenin failed to induce foetal Hb synthesis in JK-1 cells, likewise.

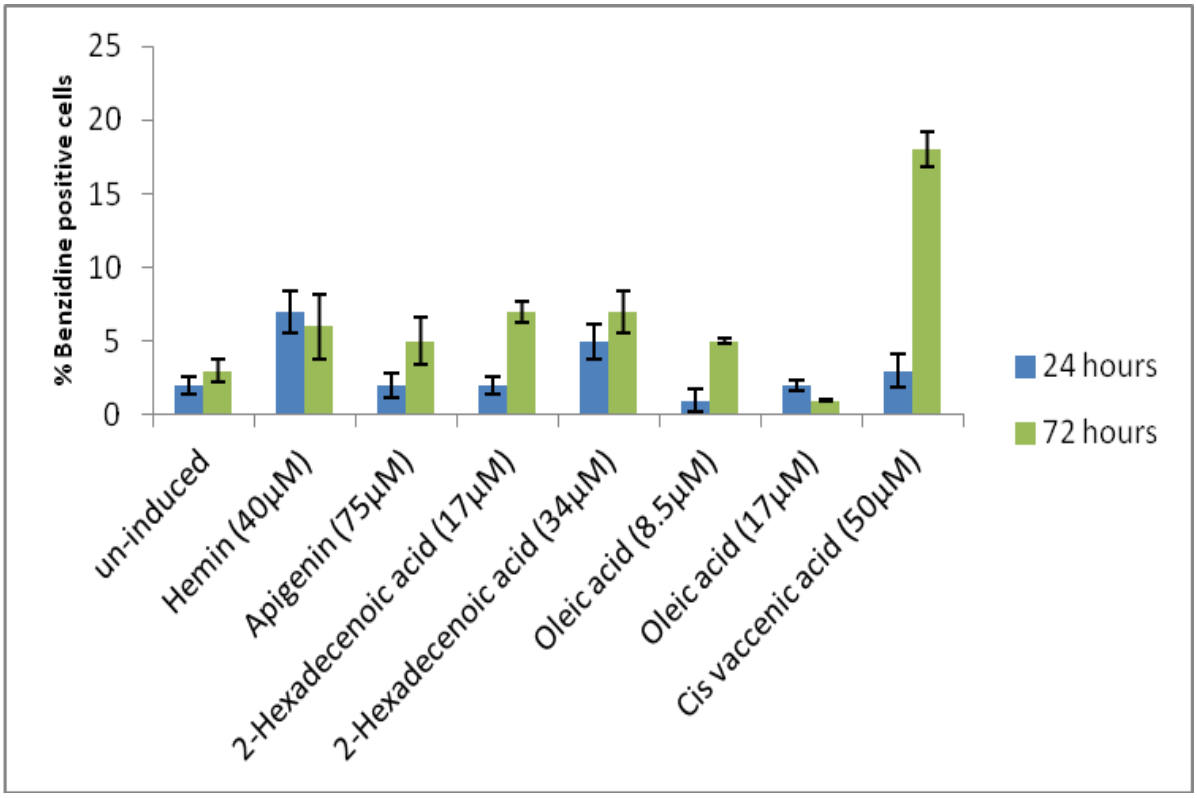


Figure 4.20: Effect of Hemin, Apigenin, 2-hexadecenoic acid, oleic acid and Cis vaccenic acid on JK-1 cell differentiation after 72 hours treatment

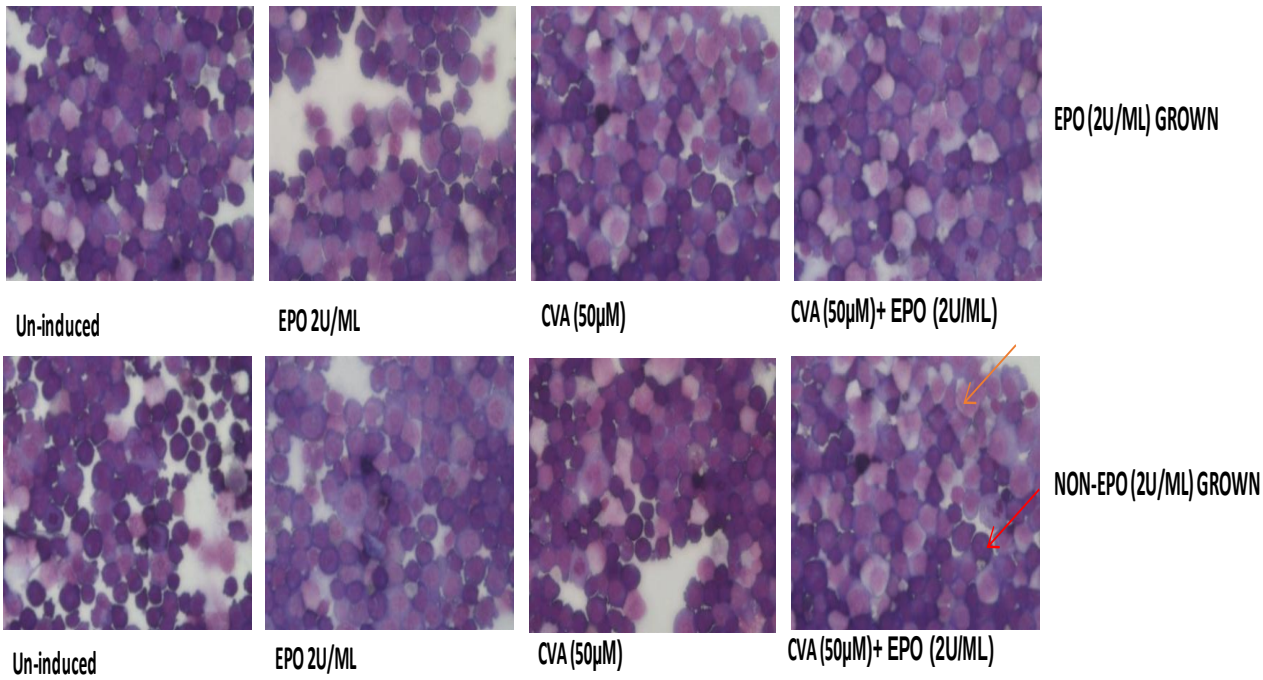


Plate 4.14: Effect of CVA on JK-1 cell differentiation 72 hours treatment (JK-1 cells were either grown in EPO 2U/ml or not before incubation with CVA 50µM). Magnification X 400. *Red arrow indicates un-differentiated JK-1 cells while the orange arrow shows differentiated JK-1 cells.*

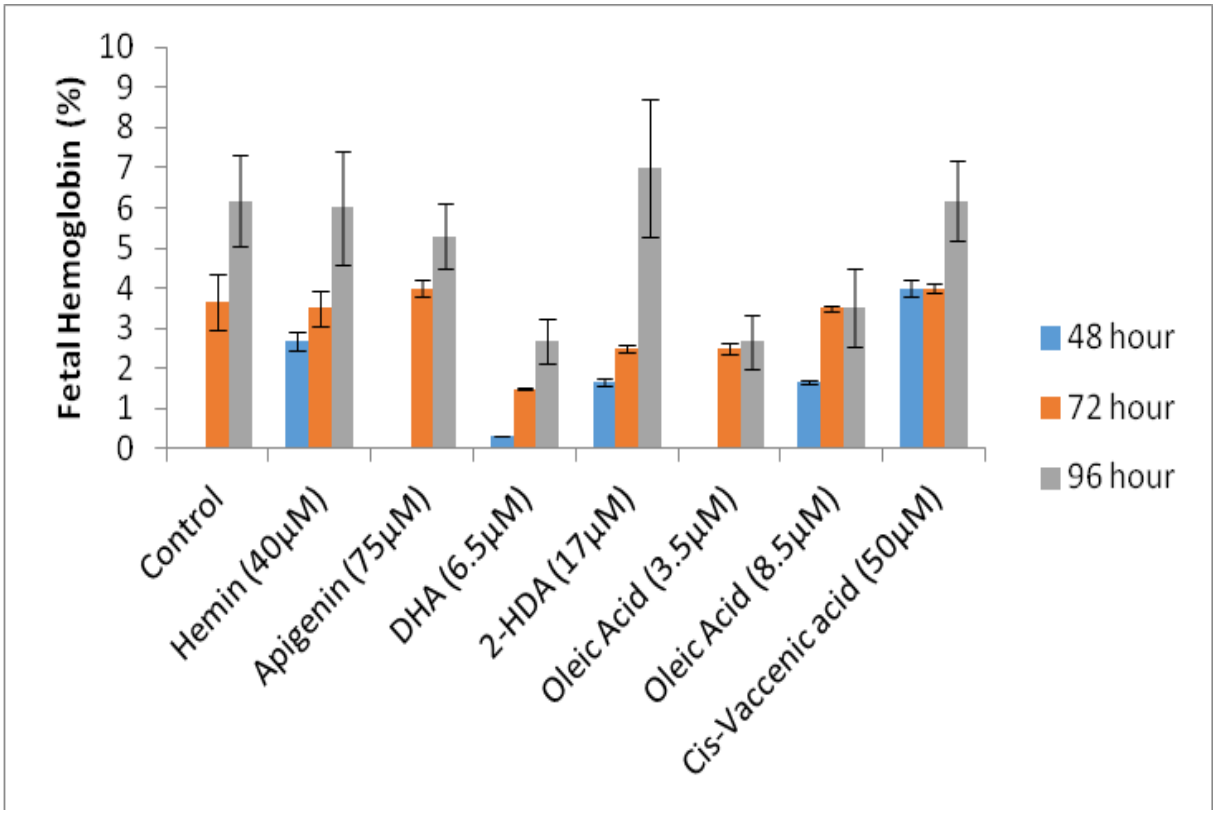


Figure 4.21: Effect of Hemin, Apigenin, 2-hexadecenoic acid, oleic acid and Cis vaccenic acid on K562 foetal haemoglobin synthesis after 96 hours induction

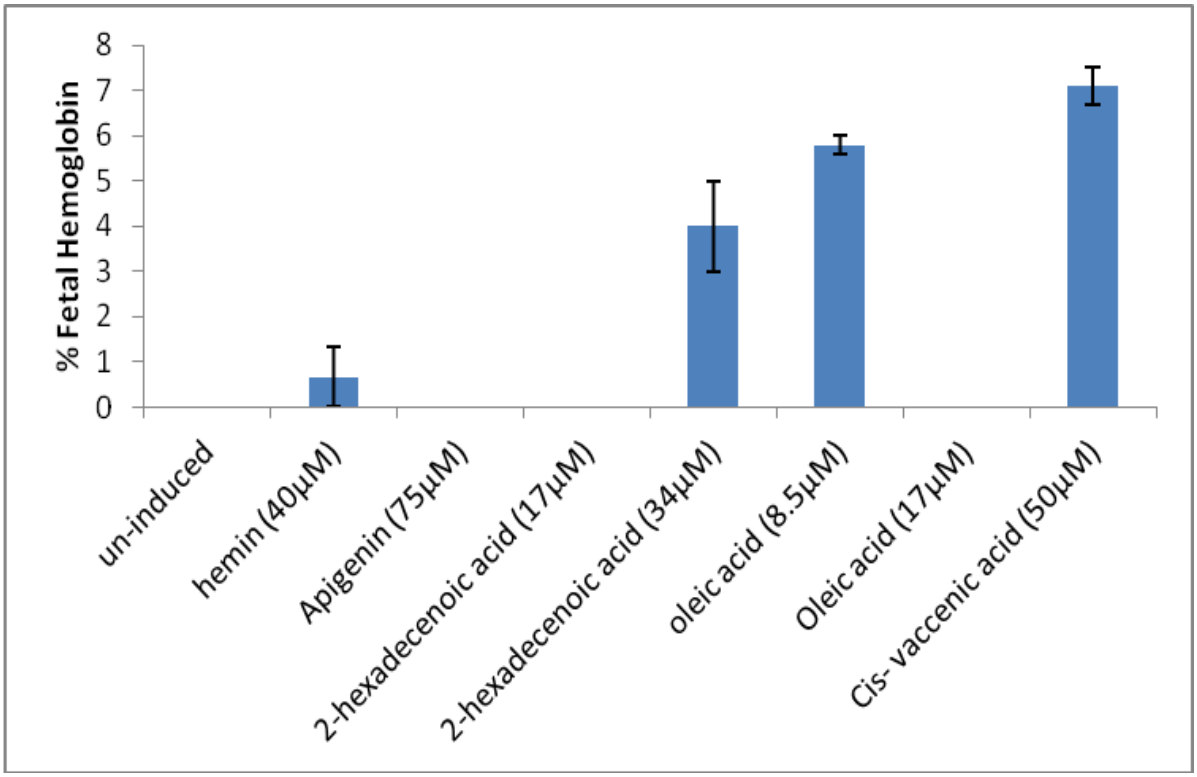


Figure 4.22: Effect of Hemin, Apigenin, 2-hexadecenoic acid, oleic acid and Cis-vaccenic acid on JK-1 foetal haemoglobin synthesis after 96 hours induction

4.23 EFFECT OF CVA, 2-HDA, OLEIC ACID, APIGENIN, DHA, EPA, STEARIC ACID AND GENTISIC ACID ON K562 CELLS γ-GLOBIN GENE EXPRESSION

Compounds were screened for their γ-globin inducing activity on K562 cells. Apigenin induced γ-globin gene expression at 75μM, while 2-HDA induced K562 γ-globin expression at 3.5, 8.5 and 17μM. DHA also induced K562 γ-globin gene expression at 6.5, 12.5 and 25μM. Oleic acid and CVA weakly induced K562 γ-globin gene expression at 3.5μM and 50μM respectively (Plate 4.15).

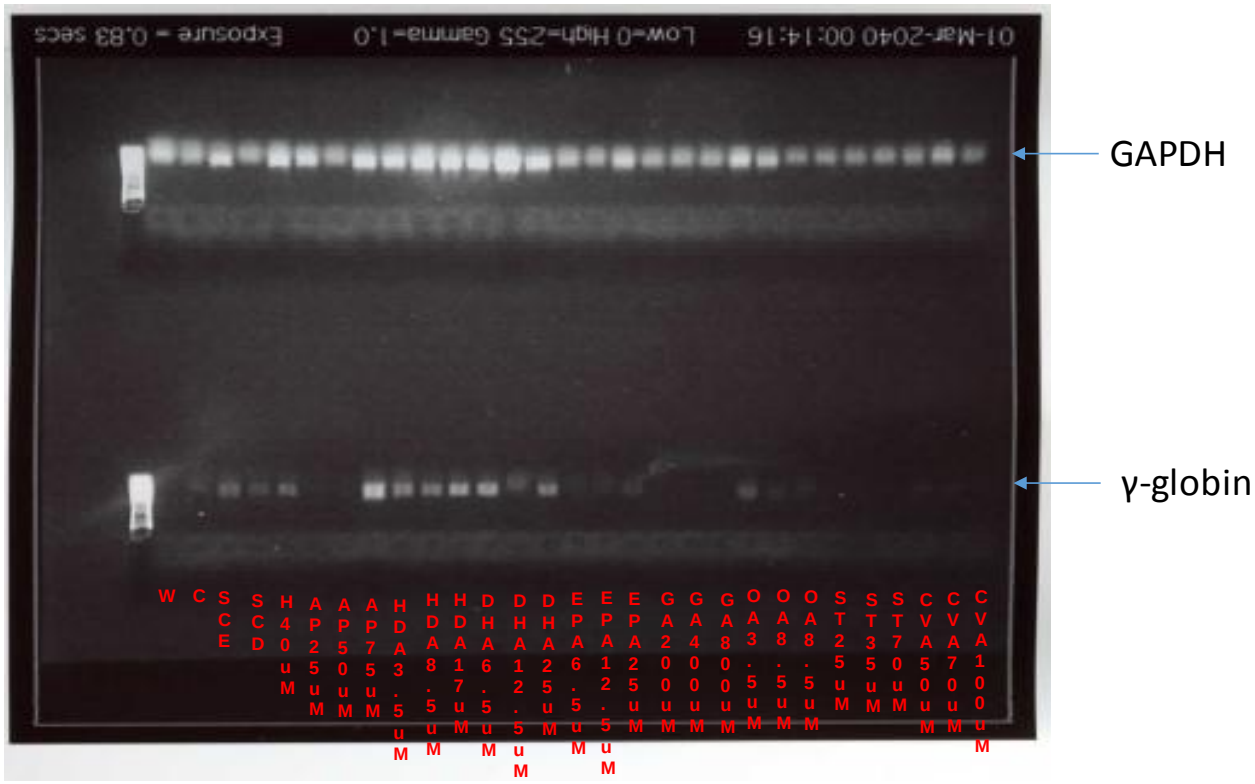


Plate 4.15: Effect of Cis-Vaccenic Acid, 2-Hexadecenoic Acid, Oleic Acid, Apigenin, Docosahexanoic Acid, Eicosapentanoic Acid, Stearic Acid And Gentisic Acid on K562 cell γ-globin gene expression. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene served as an internal control

4.24 EFFECT OF APIGENIN, DHA, 2-HDA, OLEIC ACID AND CVA ON K562 CELL DEVELOMPENTAL γ-GLOBIN GENE EXPRESSION

K562 induced γ-globin gene expression was studied using the compounds active in inducing K562 γ-globin gene expression; Apigenin (75μM), DHA (6.5μM), 2-HDA (17μM), oleic acid (8.5 and 17 μM) and CVA (50μM) which were positive for γ-globin induction in K562 cells. Apigenin induced K562 γ-globin gene expression was strongest at 48 hours and the expression decreased at 72 and 96 hours respectively (Plate 4.16). 2-HDA induced K562 expression was strongest at 48 hours but weaker compared to Apigenin and Hemin induced K562 Expression at 48 hours. However, Oleic acid and Cis-vaccenic acid induced K562 γ-globin gene expression was weak and comparable with the un-induced control (Plate 4.16). Having established the developmental activity of K562 γ-globin inducing active compounds, the capacity of Apigenin and 2-HDA which had a developmental effect on K562 γ-globin gene expression was further assessed for their capacity to alter K562 γ-globin gene expression within 24hours of induction. The results (Plate 4.17) show that Apigenin and 2-HDA up-regulates K562 γ-globin gene expression within 24 hours at 75μM and 17μM respectively (Plate 4.17).

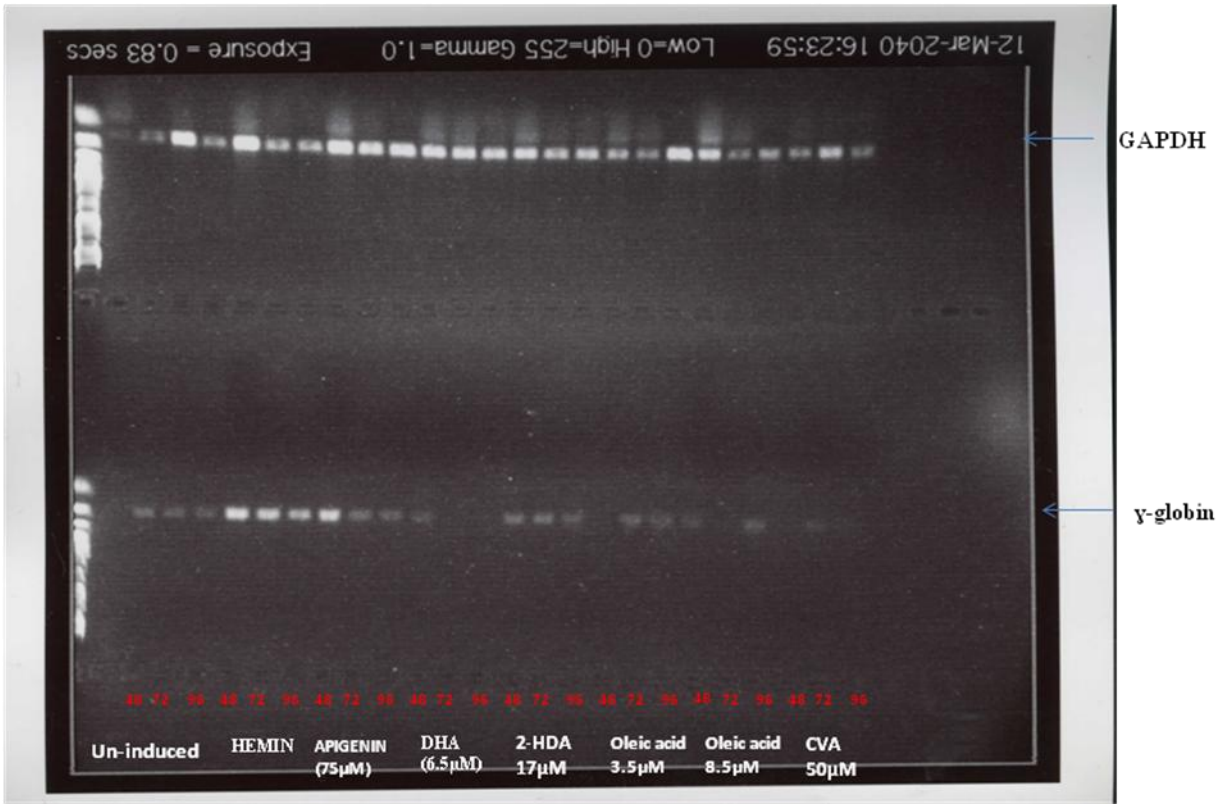


Plate 4.16: Effect of Apigenin, Docosahexanoic acid (DHA), 2-hexadecenoic acid (2-HDA), Oleic acid and Cis-vaccenic acid (50μM) on K562 developmental γ-globin gene expression after 96 hours induction. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene served as an internal control

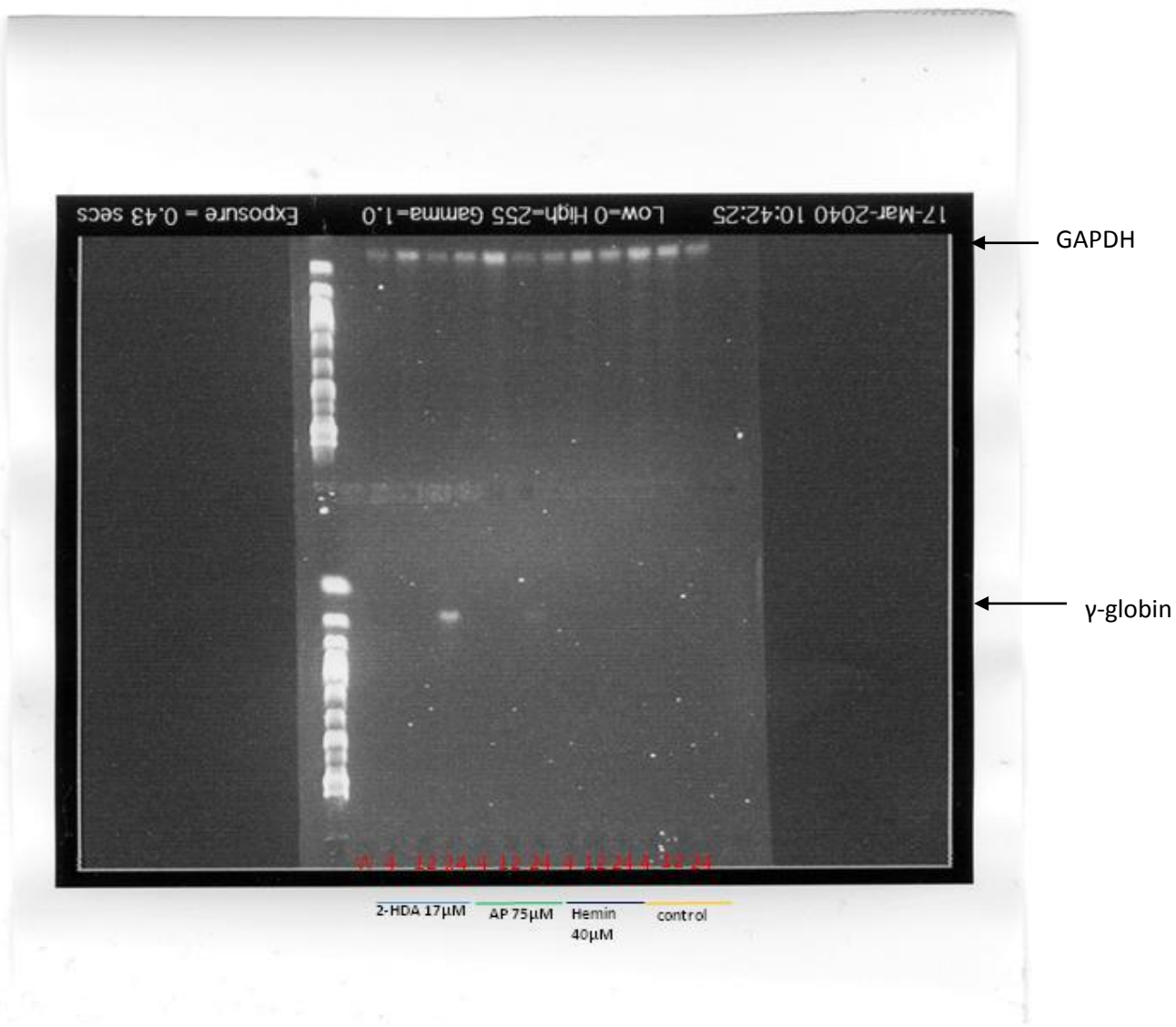


Plate 4.17: Effect of Apigenin and 2-hexadecenoic acid (2-HDA) on K562 developmental γ-globin gene expression after 24 hours induction. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene served as an internal control

4.25 EFFECT OF CIS-VACCENIC (CVA), 2-HEXADECENOIC ACID (2-HDA), APIGENIN (AP) AND OLEIC ACID (OA) ON JK-1 CELL γ-GLOBIN GENE EXPRESSION

CVA (50μM) induced increased JK-1 γ-globin gene expression. Very low γ-globin gene expression was also detectable in oleic acid (8.5μM) and 2-HDA (34μM) induced JK-1 cells (Plate 4.18).

4.26 EFFECT OF DIFFERENT CONCENTRATIONS OF CIS-VACCENIC (CVA) ON JK-1 γ-GLOBIN GENE EXPRESSION

CVA induced JK-1 γ-globin and β-globin gene expression was more pronounced when the cells were induced with 50μM CVA, with higher concentrations being less effective in inducing JK-1 globin gene expression (Plate 4.19).

4.27 EFFECT OF CIS-VACCENIC ACID (CVA) ON JK-1 DEVELOPMENTAL γ -GLOBIN GENE EXPRESSION

CVA (50μM) altered γ-globin gene expression was evident 48 hours post induction of JK-1 cells with 50μM CVA (Plate 4.20). CVA (50μM) also induced high level of β-globin gene expression 72 hours post induction.

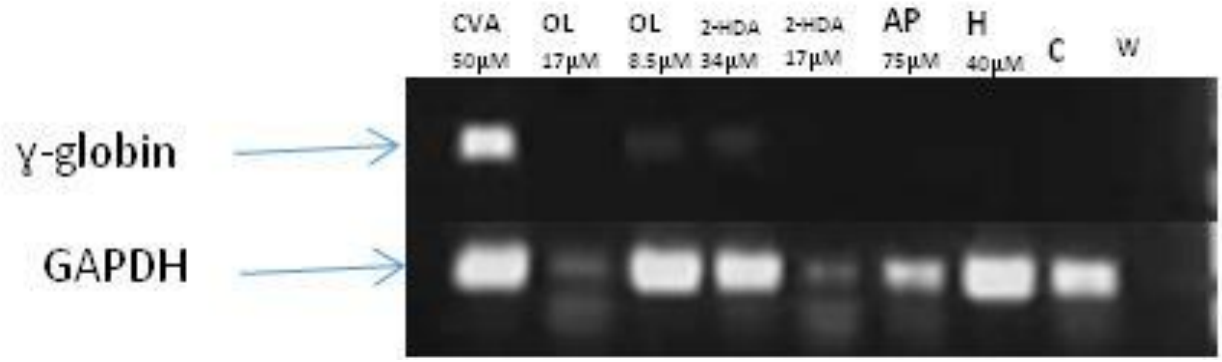


Plate 4.18: Effect of Apigenin, 2-hexadecenoic acid (2-HDA), Oleic acid and Cis-vaccenic acid (50µM) on JK-1 γ-globin gene expression after 96 hours induction. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene served as an internal control

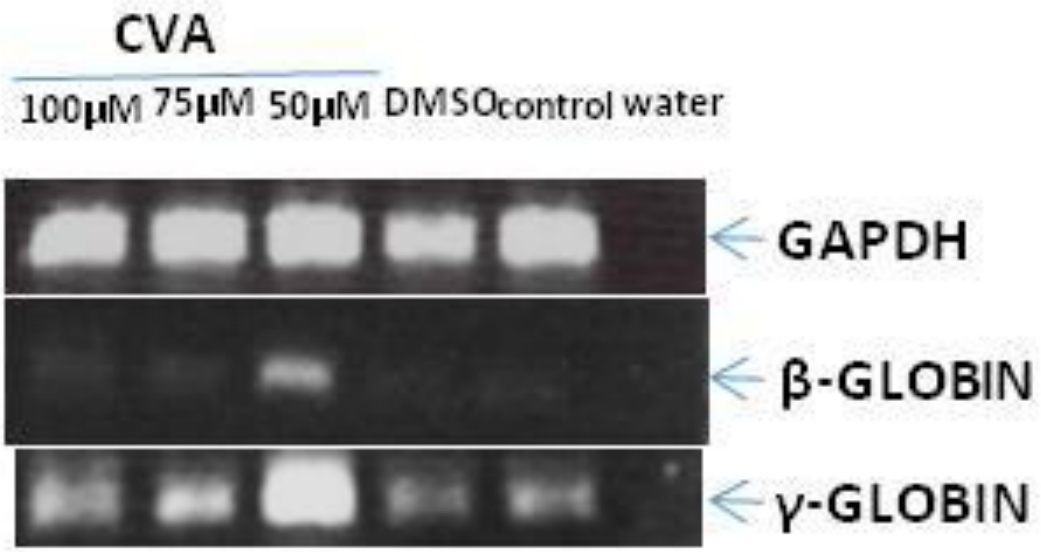


Plate 4.19: Effect of varying concentrations of Cis-vaccenic acid on JK-1 globin gene expression. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene served as an internal control

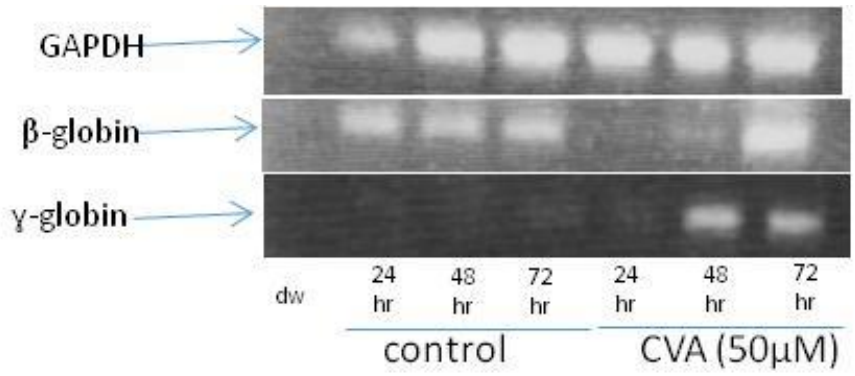


Plate 4.20: Effect of Cis-vaccenic acid (50µM) on JK-1 developmental globin gene expression. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene served as an internal control

4.28 EFFECT OF CVA ON TIME DEPENDENT RELATIVE EXPRESSION OF ɣ -GLOBIN mRNA IN JK-1 CELLS

CVA (50µM) significantly ($P<0.05$) increased γ-globin gene expression in JK-1 cells, with the highest level of γ-globin gene expression evident at 48 hours post induction (Figure 4.23). γ-globin gene levels slowly tapered off with time of induction.

4.29 EFFECT OF ERYTHROPOIETIN ON CIS-VACCENIC ACID INDUCED ɣ -GLOBIN GENE EXPRESSION in JK-1 CELLS

Significantly higher γ-globin fold expression was observed when JK-1 cells were firstly grown in EPO (2U/ml) compared to JK-1 cells which were directly induced using CVA (50µM) (Figure 4.24)

4.30 EFFECT OF CIS VACCENIC ACID (CVA) ON TRANSGENIC MOUSE BONE MARROW ERYTHROID PROGENITOR CELLS DIFFERENTIATION AND ɣ-GLOBIN GENE EXPRESSION

CVA (50µM) induced transgenic mouse bone marrow erythroid progenitor stem cells differentiation (Figure 4.25). Differentiation (accessed by BFUe colony formation) was highest 24 hours after induction with over 180 BFUe colonies observed. 2-HDA likewise induced transgenic mouse bone marrow stem cell BFUe colony formation (Figure 4.25) at 24 hours post induction (110%). However the percentage of BFUe formation induced by CVA and 2-HDA on day 2 and day 3 was significantly ($P<0.05$) lower compared to the levels induced by this compounds on day 1. Transgenic mouse bone marrow progenitor stem cell γ-globin gene expression was also significantly ($P<0.05$) increased when the cells were induced with 50µM CVA (Figure 4.26) compared to un-induced control. The increase was highest at 96 hours post induction.

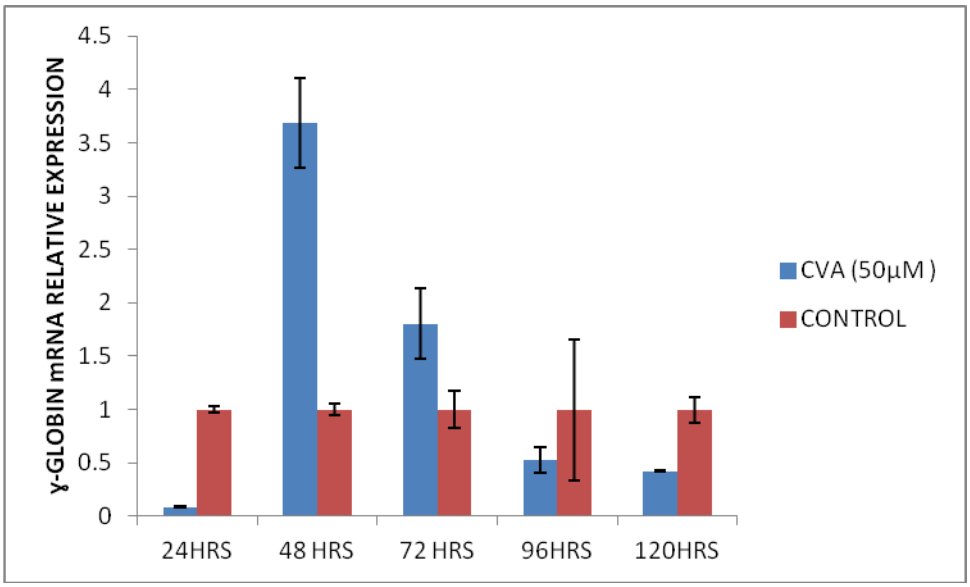


Figure 4.23: Effect of Cis-vaccenic acid (50µM) on the relative expression of γ-globin mRNA in JK-1 cells.

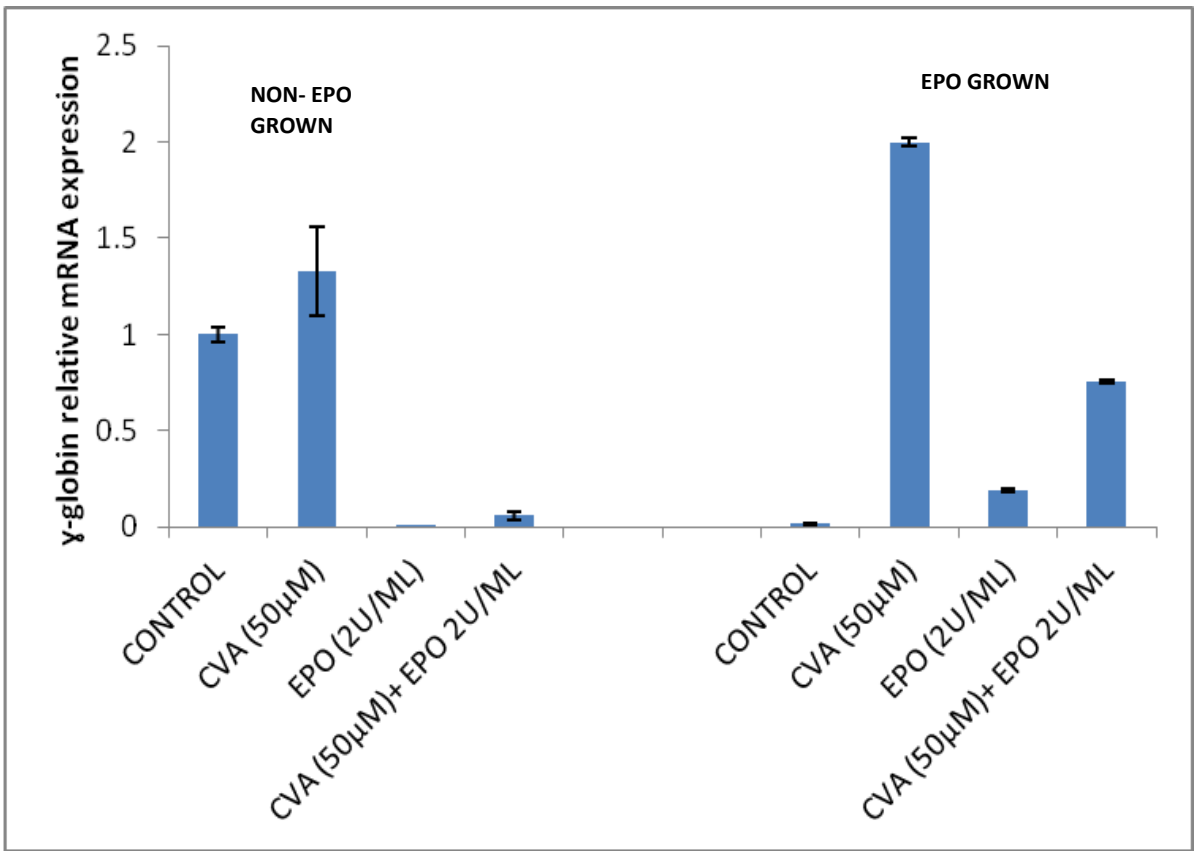


Figure 4.24: Effect of erythropoietin on Cis-vaccenic acid (50μM) induced relative expression of γ-globin mRNA in JK-1 cells.

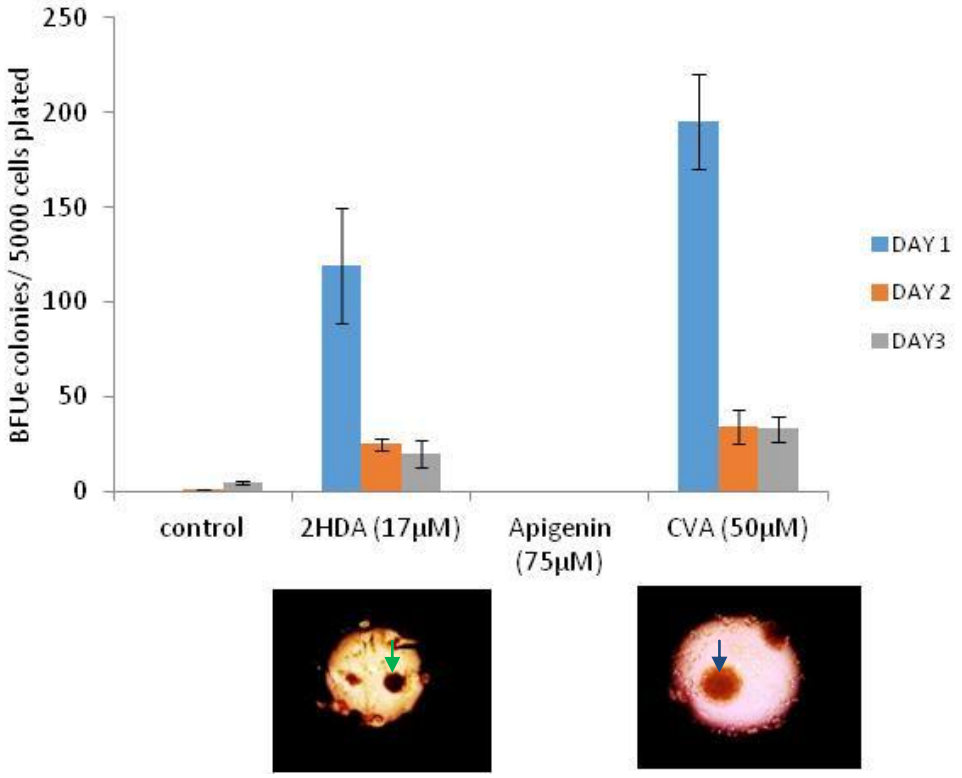


Figure 4.25: Effect of 2-hexadecenoic acid, Apigenin and Cis-vaccenic acid on transgenic mouse bone marrow progenitor stem cell differentiation. (*Blue arrow depicts BFUe colony induced with CVA green arrow depicts BFUe colony induced with 2-HDA*)

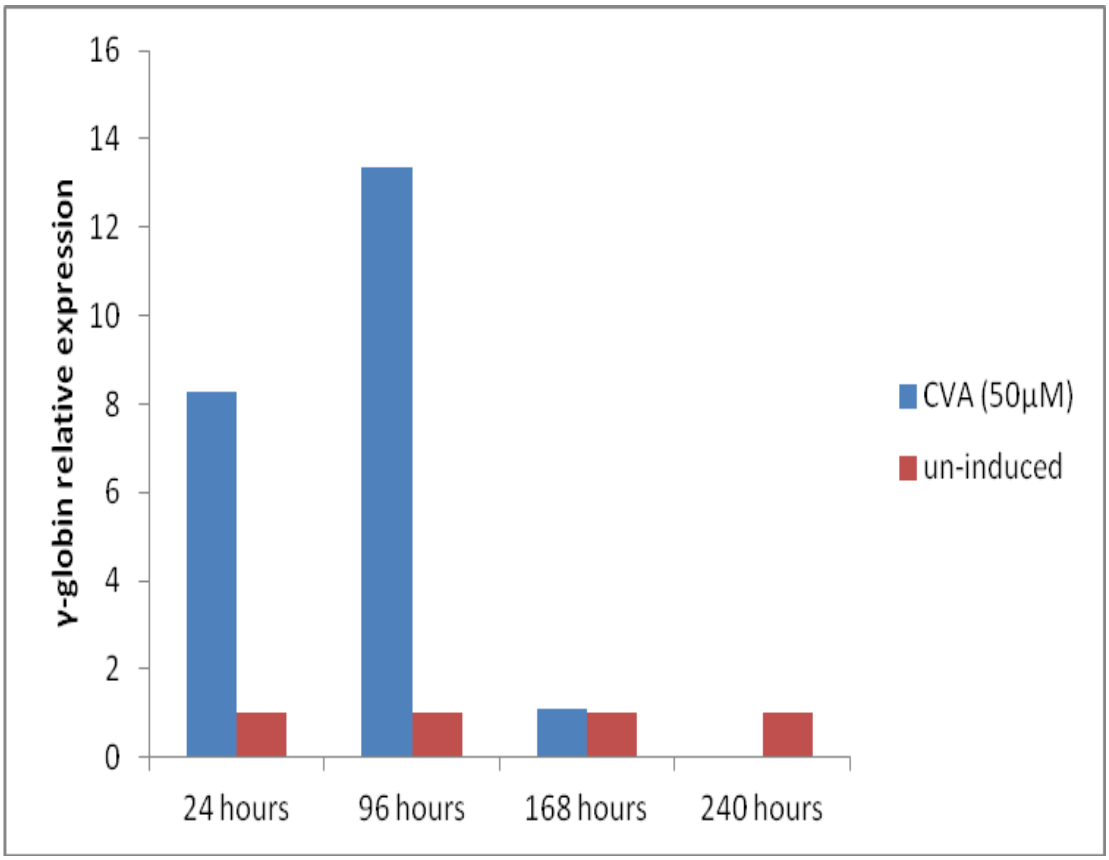


Figure 4.26: Effect of Cis-vaccenic acid on transgenic mouse bone marrow progenitor stem cell developmental γ-globin mRNA expression.

4.31 EFFECT OF CIS-VACCENIC ACID (CVA) ON DIFFERENTIATION OF EARLY AND LATE TRANSGENIC MOUSE PRIMITIVE ERYTHROID PROGENITORS STEM CELLS

CVA (50µM) was used to induce differentiation and γ-globin gene expression of transgenic mouse bone marrow progenitor stem cells. CVA (50µM) was added to progenitor stem cell semi-solid cultures at varying time points of the culture (at start of culture and 48 hours after commencement of culture). Erythroid burst forming units (BFUe) was enumerated 24 hours after induction with CVA (50µM). The results revealed that CVA (50µM) significantly ($P<0.05$) increased BFUe colony formation in both cases. However, BFUe colony formation was significantly higher when transgenic mouse bone marrow progenitor stem cells were induced with CVA (50µM) at 0 hours of the start of culture (Figure 4.27).

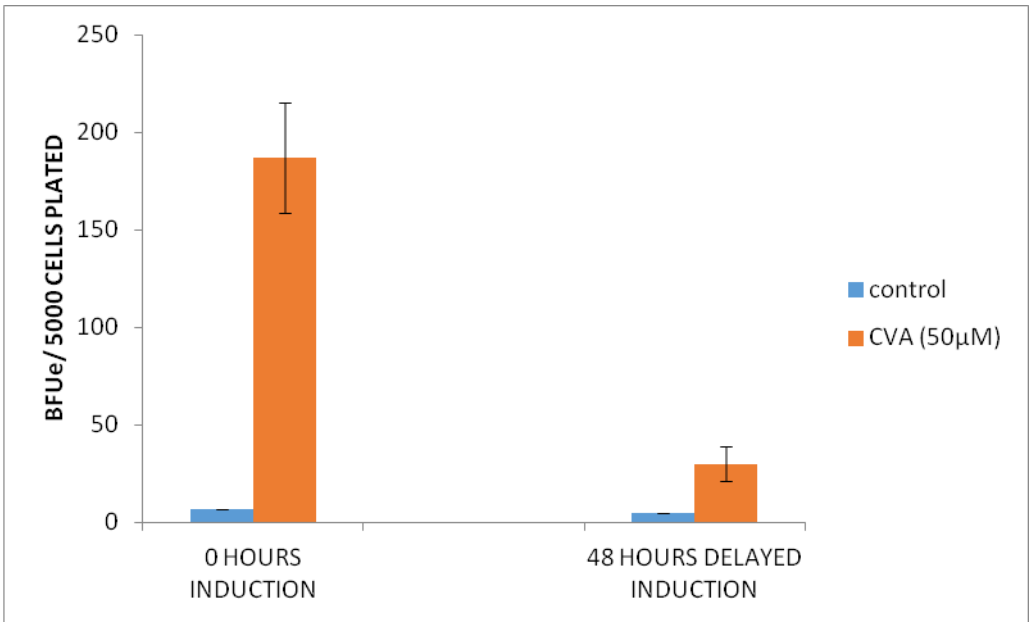


Figure 4.27: Effect of Cis-vaccenic acid on transgenic mouse bone marrow progenitor stem cell differentiation and commitment to the erythroid lineage

4.32 EFFECT OF CVA ON EARLY AND LATE TRANSGENIC MOUSE ERYTHROID PROGENITORS STEM CELLS γ-GLOBIN GENE EXPRESSION

CVA (50μM) was used to induce γ-globin gene expression of transgenic mouse bone marrow progenitor stem cells. CVA (50μM) was added to progenitor stem cell liquid cultures at varying time points of the culture (at start of culture and 48 hours after commencement of culture). γ-globin gene expression was assessed 24 hours after induction with CVA (50μM). The results revealed that CVA (50μM) selectively up-regulates γ-globin gene expression in early transgenic mouse bone marrow progenitors compared to late transgenic mouse progenitor stem cells. CVA induced transgenic mouse bone marrow progenitor bone γ-globin mRNA expression was significantly higher when transgenic mouse bone marrow progenitor stem cells were induced with CVA (50μM) at 0 hours of the culture compared to cells which were induced 48 hours after commencement of culture (Figure 4.28).

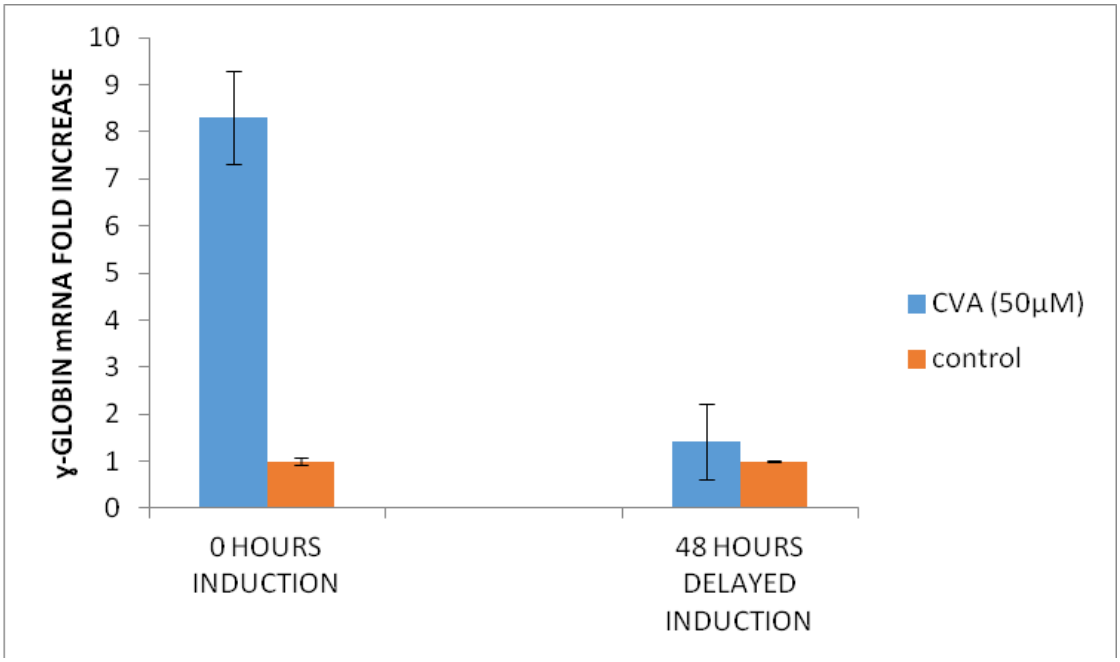


Figure 4.28: Effect of Cis-vaccenic acid on transgenic mouse bone marrow progenitor stem cell γ-globin mRNA expression.

4.33 EFFECT OF FATTY ACID ELONGASE 5 (Elovl5) AND Δ^9 DESATURASE INHIBITION ON CVA INDUCED γ-GLOBIN GENE EXPRESSION IN JK-1 CELLS

Inhibition of Elovl5 and Δ^9 desaturase activity in JK-1 cells using Isoxyl and Cycloate silenced CVA induced γ-globin mRNA expression in JK-1 cells (Figure 4.29). When the cells were first treated with inhibitors, and CVA (50μM) was used to rescue γ-globin mRNA expression in JK-1 cells 24 and 48 hours post inhibition, the results demonstrate that γ-globin mRNA expression was slightly elevated to 0.25 and 0.36 fold γ-globin mRNA expression in Elovl5 and Δ^9 desaturase inhibition respectively 24 hours post inhibition (Figure 4.30). However CVA (50μM) failed to completely restore JK-1 γ-globin mRNA expression in both Elovl5 and Δ^9 desaturase inhibition in JK-1 cells when CVA (50μM) was added 48 hours post inhibition (Figure 4.31).

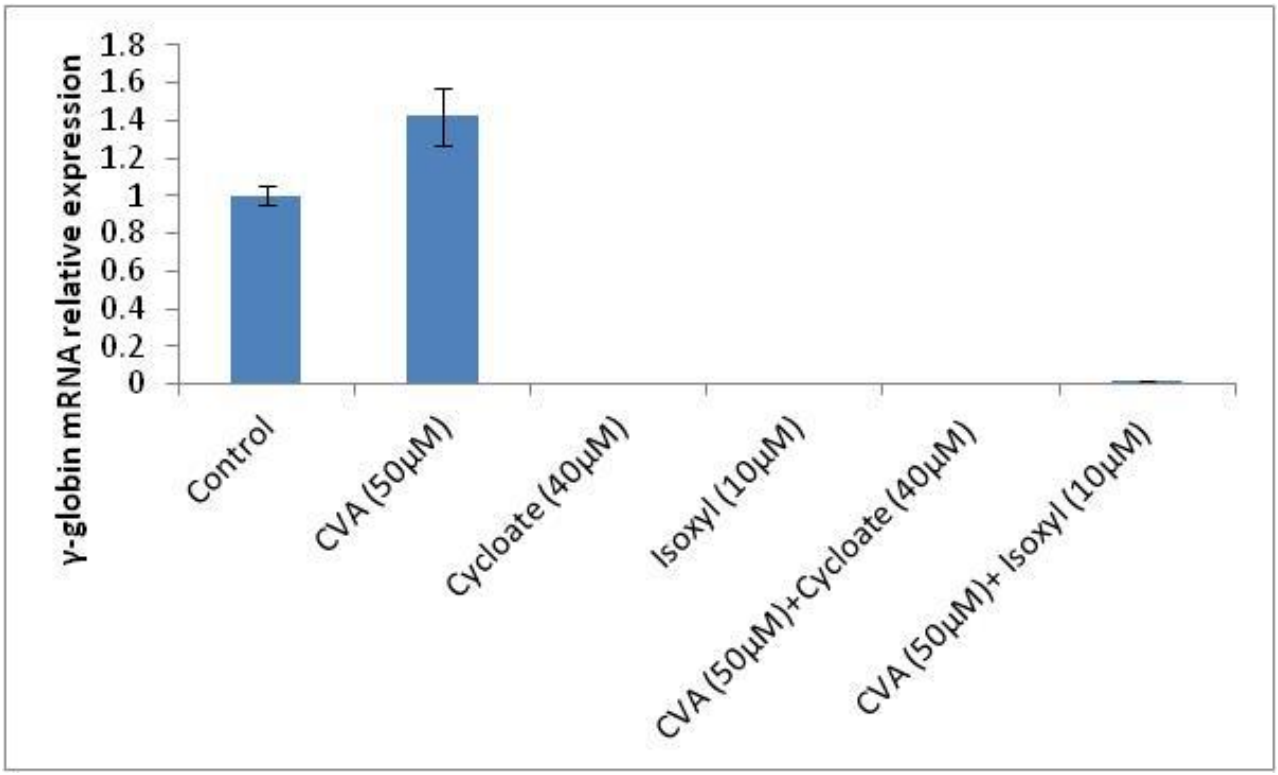


Figure 4.29: Effect of Isoxyl (10μM) and Cycloate (40μM) inhibition of fatty acid elongase 5 and Δ^9 desaturase enzymes respectively on Cis-vaccenic induced γ-globin expression in JK-1 cells.

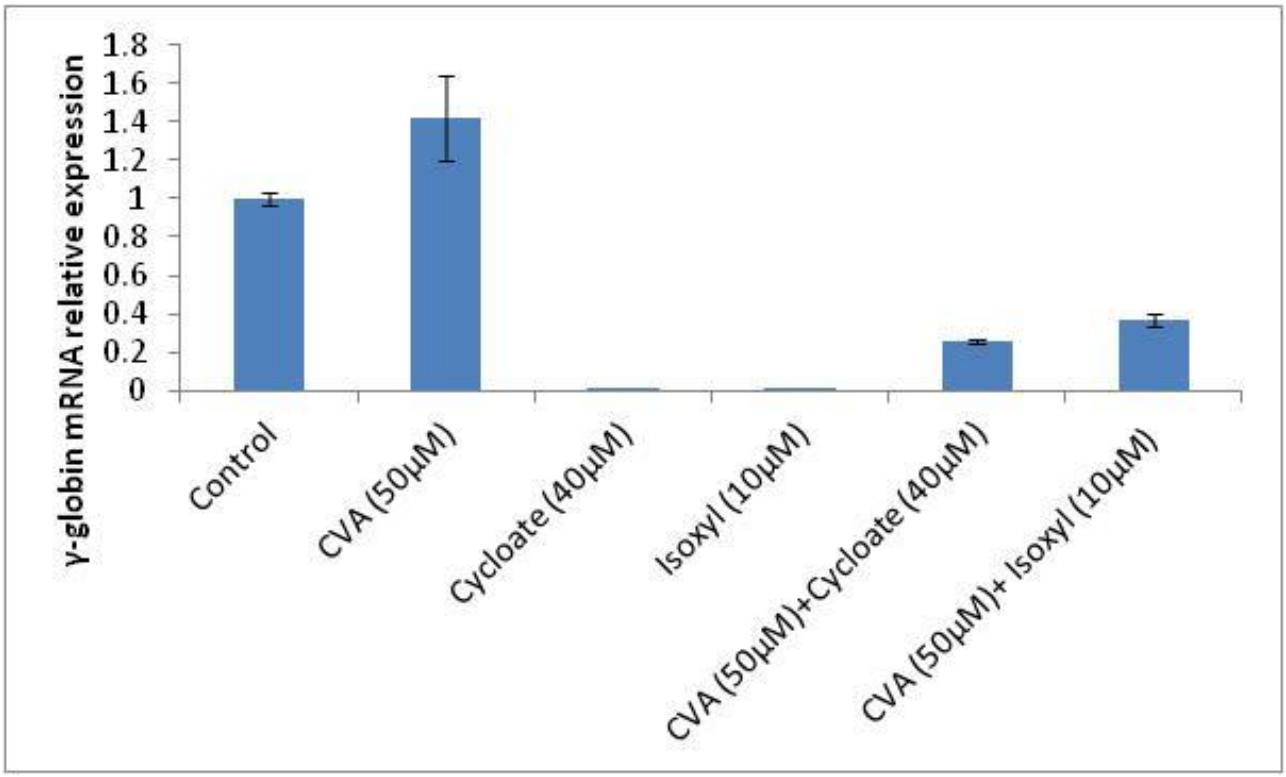


Figure 4.30: Effect of addition of Cis-vaccenic acid (50μM) 24 hours post Isoxyl (10μM) and Cycloate (40μM) inhibition of fatty acid elongase 5 and Δ9 desaturase enzymes respectively on Cis-vaccenic induced γ-globin expression in JK-1 cells.

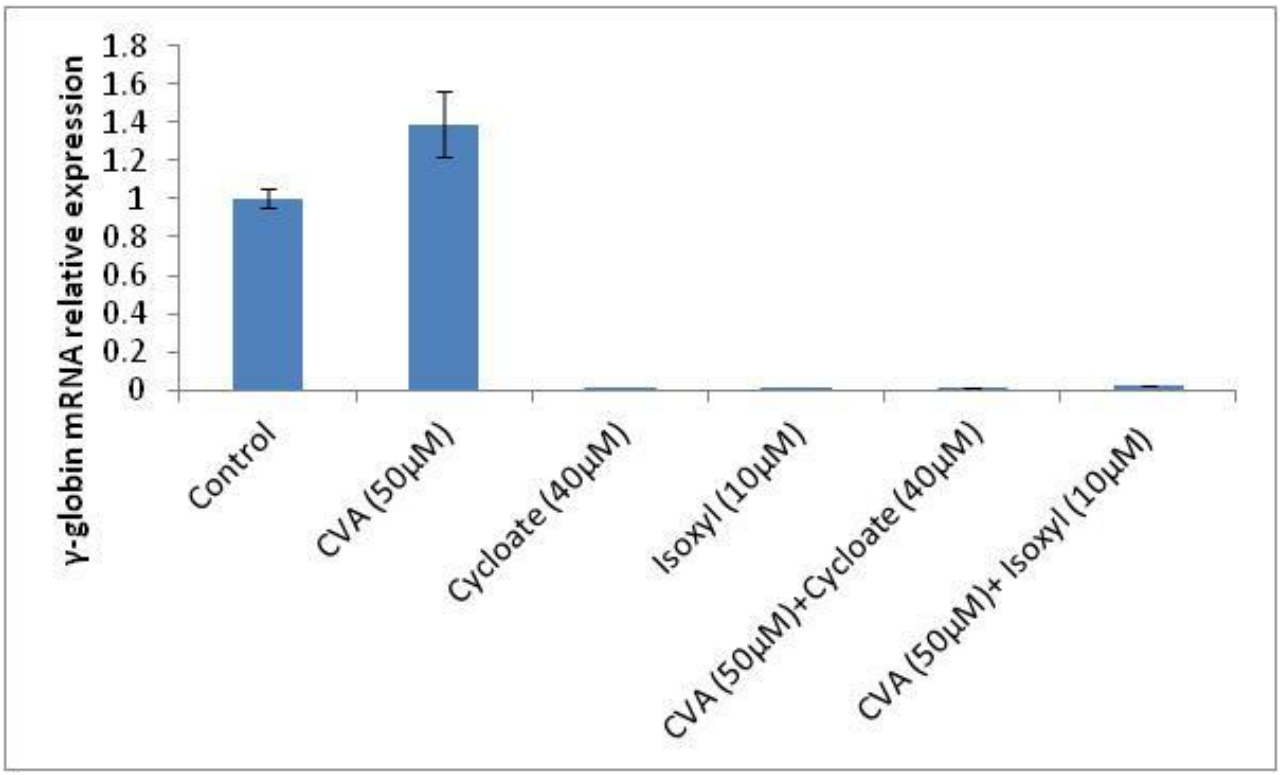


Figure 4.31: Effect of addition of Cis-vaccenic acid (50μM) 48 hours post Isoxyl (10μM) and Cycloate (40μM) inhibition of fatty acid elongase 5 and Δ9 desaturase enzymes respectively on Cis-vaccenic induced γ-globin expression in JK-1 cells.

4.34 EFFECT OF CVA RESCUE ON Elovl5 AND Δ^9 DESATURASE INHIBITION ON DIFFERENTIATION OF TRANSGENIC MOUSE BM ERYTHROID PROGENITOR STEM CELLS (TMbmEPSCs)

Addition of CVA to TMbmEPSCs 48 hours post inhibition of Δ^9 desaturase using 10µM Isoxyl enhanced CVA induced TMbmEPSCs erythroid burst forming unit activity. Fatty Acid Elongase 5 activity inhibition using Cycloate (40µM) inhibited CVA induced transgenic mouse bone marrow progenitor stem cell erythroid burst forming unit activity. Isoxyl and Cycolate on their own induced no significant transgenic mouse bone marrow progenitor stem cell erythroid burst forming unit activity (Figure 4.32).

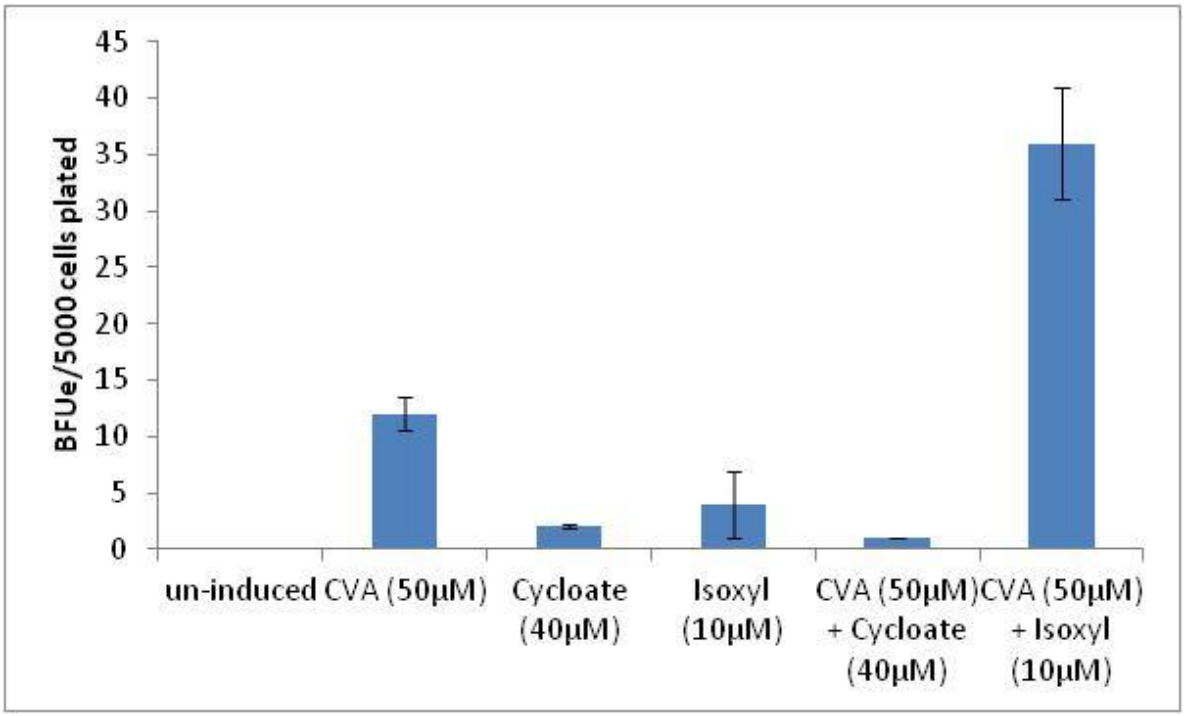


Figure 4.32: Effect of Isoxyl (10µM) and Cycloate (40µM) inhibition of fatty acid elongase 5 and Δ^9 desaturase enzymes respectively on Cis-vaccenic induced BFUe formation of transgenic mouse bone marrow cells.

4.35 EFFECT OF INHIBITION OF Elovl 5 AND Δ^9 DESATURASE ON CVA INDUCED ɤ -GLOBIN EXPRESSION IN TRANSGENIC MICE BONE MARROW ERYTHROID PROGENITOR STEM CELLS

Inhibition of Δ^9 desaturase and ELOVl5 using 10µM Isoxyl and 40µM Cycloate respectively inhibits γ-globin mRNA expression in transgenic mouse bone marrow erythroid progenitor stem cells (Figure 4.33) significantly ($P<0.05$) compared to TMbmEPSCs induced with CVA.

4.36 EFFECT OF CVA ON JK-1 CELLS ERYTHROID KRUPPEL LIKE FACTOR (EKLF) AND B-CELL LYMPHOMA A (BCL11A) GENE EXPRESSION

The results show that CVA (50µM) did not significantly ($P>0.05$) alter EKLF mRNA expression while JK-1 BCL11A gene expression was up-regulated significantly over a 100 fold after 48 hours of JK-1 induction with CVA(Figure 4.34).

4.37 EFFECT OF CVA ON EKLF mRNA RELATIVE EXPRESSION IN TRANSGENIC MICE BONE MARROW PROGENITOR STEM CELLS

CVA (59µM) significantly ($P<0.05$) increased EKLF gene expression in transgenic mouse bone marrow progenitor stem cells to about 9 fold beyond the control (Figure 4.34). Inhibition of Elovl5 enhanced the EKLF inductive activity of CVA while inhibition of Δ^9 desaturase did not differ significantly ($P<0.05$) from EKLF expression in CVA (50µM) induced cells (Figure 4.35).

4.38 EFFECT OF CIS VACCENIC ACID ON JK-1 CELLS VIABILITY

Cis vaccenic acid (CVA) at 50µM did not significantly ($P>0.05$) alter the viability of the un-induced cells (Figure 4.35). A similar scenario was observed when CVA (50µM) was combined with EPO (2U/ml) or EPO alone was used to induce JK-1 cells (Figure 4.36). Cell viability was monitored progressively for 120 hours. No significant drop was observed in JK-1 cell viability within the first 48 hours post incubation with CVA.

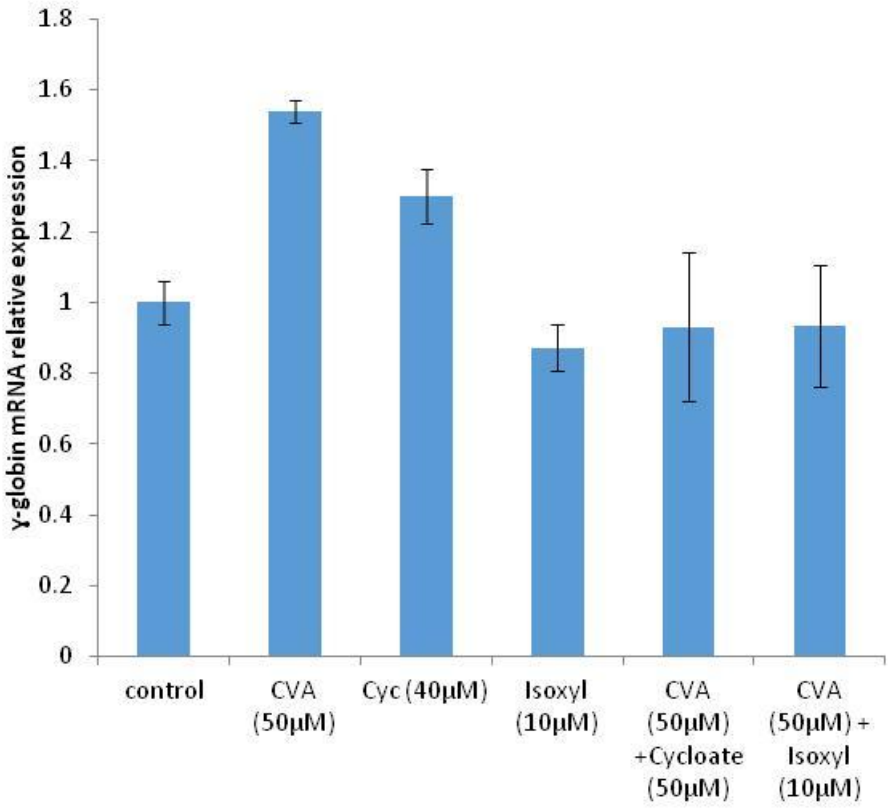


Figure 4.33: Effect of Isoxyl (10μM) and Cycloate (40μM) inhibition of fatty acid elongase 5 and Δ^9 desaturase enzymes respectively on Cis-vaccenic induced γ-globin expression in transgenic mouse bone marrow cells.

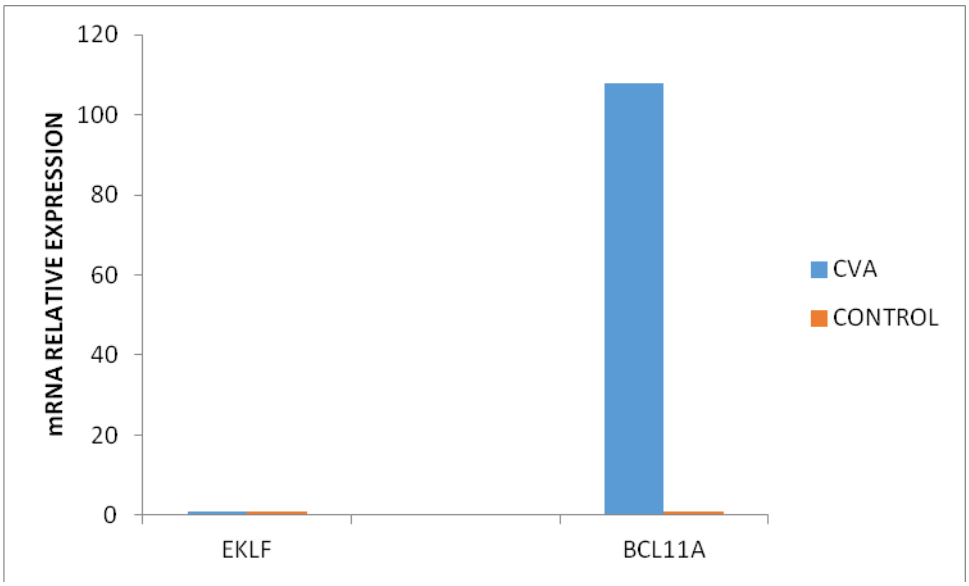


Figure 4.34: Effect of Cis-vaccenic acid on JK-1 EKLF and BCL11A gene expression

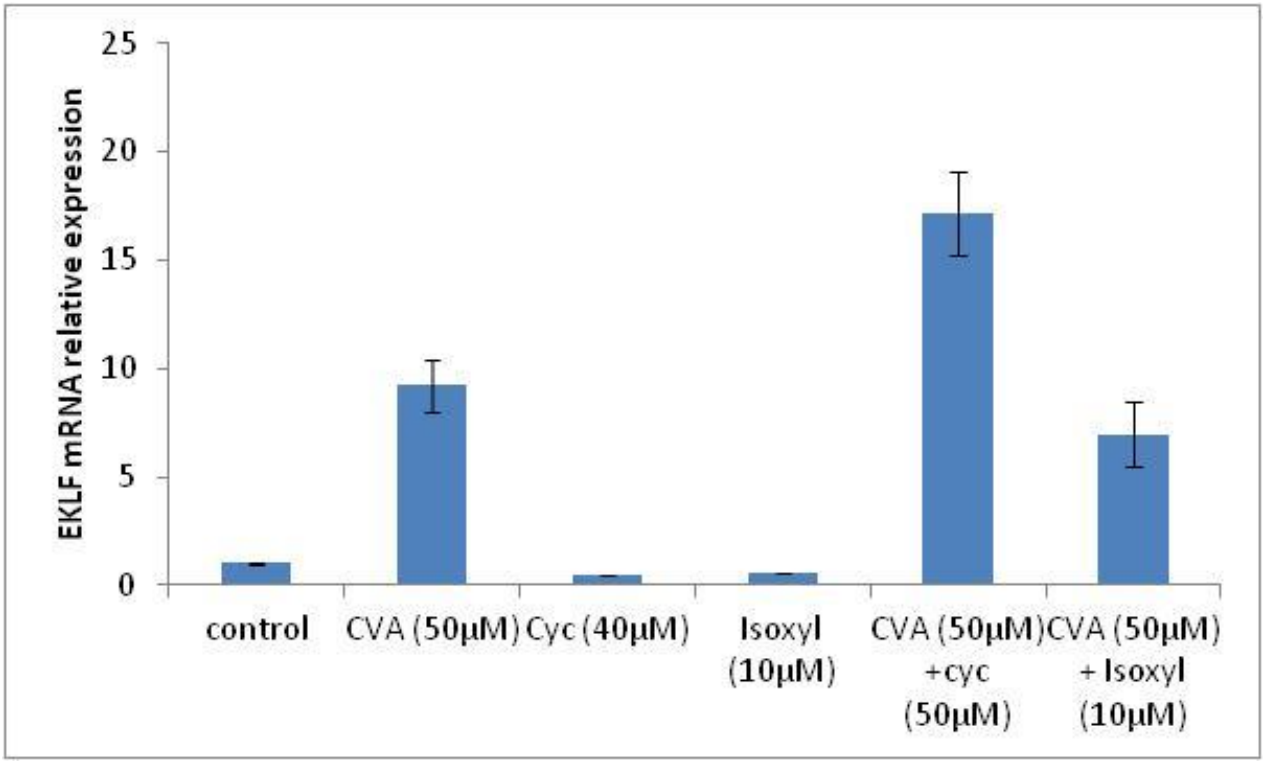


Figure 4.35: Effect of Isoxyl (10µM) and Cycloate (40µM) inhibition of fatty acid elongase 5 and Δ^9 desaturase enzymes respectively on Cis-vaccenic induced EKLF expression in transgenic mice bone marrow cells.

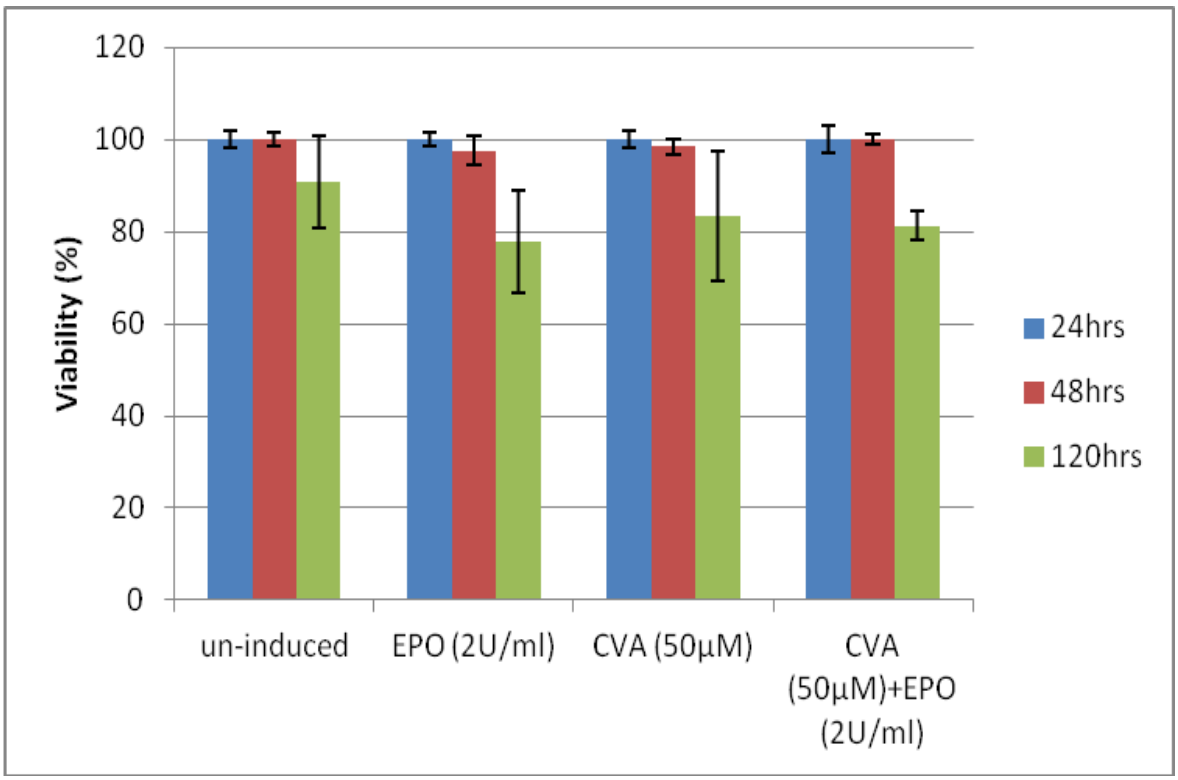


Figure 4.36: Effect of Cis- vaccenic acid (50µM), Erythropoietin (2U/ml) and combined (50µM CVA + 2U/ml Erythropoietin) on JK-1 cell viability.

CHAPTER 5

DISCUSSION

Increase in HbF level of SS and β-thalassemia patients have been shown to be clinically beneficial for such individuals(Musallam, *et al*., 2013; Xu *et al*., 2013) which is the major stimulus for the use of hydroxyurea as the sole FDA-recommended treatment modality for SCA and β-thalassemia. Unfortunately, the widespread use of hydroxyurea is being hampered by concerns over its toxicity profile on long-term use, which is the classical requirement for the management of the haemoglobinopathies. Sankaran (2011) has also reported on the limitations on the success of the use of hydroxyurea for the management of β-thalassemia owing to the requirement of much higher HbF, and the inability to heighten hydroxyurea dosing owing to the risk of cytopenias on long-term use. In the present study, a systematic targeted bioassay guided fractionation was used to identify a novel HbF inducing active compound from an array of medicinal plants used to manage sickle cell anaemia.

The results demonstrate that the TCDWF increased HbF synthesis in adult human erythroid progenitor stem cells (hEPSCs) in a dose-dependent pattern. The increase in HbF levels was consistent with known inducers of HbF (Keefer *et al*., 2006). TCDWF was assayed on both EPO-independent and EPO-dependent hEPSC cultures, and it was discovered that TCDWF increased HbF synthesis in both culture models significantly higher ($P<0.05$) than the un-induced control, although its HbF-inducing activity was higher in EPO-independent cultures, suggesting that this active fraction selects primitive haematopoietic stem cells and adapts them for stimulation by EPO see Figures 4.9, 4.19 and 4.11. This could be desirable in a clinical HbF-inducing agent, as studies have shown that β-thalassemia patients have a reduced capacity for synthesis of

new RBCs. Foetal haemoglobin synthesis was concentration dependent, and TCDWF was most active at 220 μg/ml.

The mechanism by which TCDWF increased HbF syntheis in these cells is unclear, but the data suggest a pathway that is linked to the erythroid lineage specific markers expression including erythropoietin receptors (Van den Akker *et al*., 2010) Higher levels of hEPSC differentiation and foetal haemoglobin synthesis in EPO-independent hEPSC cultures compared with EPO-dependent ones suggest a mechanism that is linked to the expression of early erythroid markers as previously highlighted, (Van den Akker *et al*., 2010) although further studies are required to confirm this. Several known HbF inducers have been reportedly cytotoxic to the progenitor cells.(Sankaran *et al*., 2011). Hence, the cytotoxicity of TCDWF on the progenitor stem cells was evaluated as a measure of Caspase 3 elevation (Pham *et al*., 2011) and LDH release (Chao *et al*., 1988). TCDWF appeared to protect phase I (EPO-independent) cells (compared with the normal control and phase II cell cultures) although a moderate amount of Caspase 3 activity was detected in these cells induced with 220 μg/ml, suggesting a possible role of endogenous Caspase 3 activity in early hEPSC differentiation and HbF synthesis mediated by TCDWF, which is similar to the findings of Fernando and colleagues (Fernando *et al*., 2005). A different scenario was observed in phase II (EPO-dependent) erythroid progenitor cell cultures where TCDWF was added to the cells 5 days after the start of the culture; a significantly higher caspase 3 activity was observed, which was accompanied with significantly high levels of LDH release (Figures 4.12-4.15). Previous studies have shown that it takes 5–6 days for hEPSCs to differentiate into immature erythroblast population with the gain of expression of erythroid specific markers like c-kit and EPO receptors. Taken together, it is hypothesized that the expression of early erythroid specific lineage markers lowers the effect of TCDWF

HbF-inducing activity on hEPSCs. Hence the use of TCDWF as an alternative to Hydroxyurea for the clinical induction of foetal haemoglobin may be more effective if it is not administered in conjunction with EPO.

Partial characterization of the HbF-inducing active fraction of *T. catappa* using GCMS revealed a mixture of structural, closely related long-chain fatty acids including; (hexadecanoic acid, 10-octadecenoic acid and 9-octadecenoic acid), 2-hexadecenoic acid, cis-vaccenic acid, pentadecanoic acid, hexadecenoic acid and octadecanoic acid (Figure 4.17) which have not been previously reported to be active HbF-inducing compounds. Both 10-octadecenoic and octadecenoic acids are mono-unsaturated isomeric forms of each other. The biological activities of these compounds are scantily reported, although eicosanoid products have been shown to activate multiple signaling pathways for cell proliferation and transcription (Kinder *et al*., 2010). Pure versions of these compounds including compounds which have been previously reported in the GCMS studies of derivatized aqueous portions of *T. catappa* (Apigenin and Gentisic acid) were obtained and screened for their ability to induce differentiation and up-regulate γ-globin gene expression in erythroleukemic k562 and JK-1 cells and transgenic mouse bone marrow progenitor stem cells. The data demonstrate that long chain c-18 fatty acids differentially induce differentiation in the different erythroid cell types used. Apigenin induced differentiation of K562 (Plate 4.9 and Figure 4.19) but not JK-1 cells and transgenic mouse primary bone marrow progenitor stem cells. Apigenin also up-regulated K562 γ-globin mRNA synthesis of K562 cells but not JK-1 cells (Plate 4.15 and Plate 4.18). The reason for this is un-clear but given that K562 cells already express a level of γ-globin suggests that it does not function as a globin gene inducer but rather enhances globin transcription. Apigenin was found to be

effective at a concentration of 75µM. This implies Apigenin as a weak HbF inducer in human cell system, thus making it a poor candidate for clinical therapy for SCA.

c-16 Δ^9 unsaturated fatty acid 2-hexadecenoic acid (2-HDA) induced differentiation of K562 and transgenic mice bone marrow progenitor stem cells but not JK-1 cells (Plate 4.7, Figure 4.19 and Figure 4.20). 2-HDA also up-regulated K562 γ-globin gene expression comparable to Hemin, a known K562 γ-globin inducer. K562 cells are a human erythroid cell line derived from a patient with chronic myelogenous leukemia (Huhn *et al*., 1987). These cells have been shown to respond to induction by specific agents by producing foetal and embryonic globins (Cioe *et al*., 1981). This contrasts with JK-1 cells that are also erythroid cell lines established from a patient with chronic myelogenous leukemia but in blast crisis. Unlike K562 cells which are unable to express β-globin genes due to the absence of a key erythroid specific trans-acting factor (EKLF), JK-1 cells still retain the complete ability to express the full complement of the human globin genes. The γ-globin inducing activity of 2-HDA on K562 was evident after a few hours of K562 induction (Plates 4.15- 4.17). However, no significant induction of γ-globin expression was observed when JK-1 cells were treated with 2-HAD (Plate 4.18), suggesting that 2-HDA induces γ-globin expression through a mechanism not reliant on EKLF expression. Suggesting that 2-HDA does not directly alter the globin switch and thus would not be a good candidate for the clinical management of SCA. Oleic acid equally induced K562 cell differentiation and weak expression of the K562 γ-globin gene expression (Plates 4.8, 4.16 and Figure 4.18), however Oleic acid failed to induce differentiation of JK-1 cells and only weakly induced JK-1 γ-globin gene expression (Figure 4.20 and Plates 4.18). DHA also induced K562 cell differentiation and up-regulated K562 γ-globin gene expression however MGG staining (Plates 4.10 and 4.15) of K562 cells induced with DHA

revealed cells with fragmented nucleus revealing that DHA was cytotoxic to the cells at the concentrations used. Consequently may lead to erythropoietic suppression in vivo.

CVA induced differentiation of both K562 and JK1 erythroleukemic cells and significantly increased ɣ -globin mRNA and fetal haemoglobin levels in JK1 cells but weakly in k562 cells (Plate 4.6, Figure 4.19, Plate 4.16, Plate 4.18 and Figure 4.23). The reason for this cell selective induction is unclear. CVA induced differentiation and increased ɣ -globin gene expression was shown to be time dependent with a concentration of 50µM CVA being most effective and higher concentrations being much less effective. Earliest detection in γ-globin mRNA increase in JK1 cells was at 48hrs. JK1 cells consists of largely immature cells capable of differentiating to the erythroid lineage and expressing fetal haemoglobin at very low levels when treated with appropriate inducers (Okuno *et al*., 1990). CVA increased the number of differentiated K562 and JK1 cells in culture as assessed with benzidine-giemsa staining, but did not significantly alter cell proliferation. The data suggests that CVA does not alter hEPSCs survival, but induces commitment of these cells to the erythroid lineage and may thus improve erythropoiesis.

JK-1 cells have been shown to express EPO receptors and respond to EPO stimulation by improved growth kinetics. EPO has been shown to act only on committed erythroid progenitors (CFUe) already expressing the EPO receptors and not early erythroid progenitors (Okuno *et al*., 1990). The data (Plate 4.14 and Figure 4.24) suggest that CVA acts preferentially on relatively early erythroid precursors committing them to the erythroid lineage and inducing higher levels of γ-globin gene expression in these cells which is consistent with our earlier findings that TCDWF HbF inducing activity is higher in EPO-independent hEPSCs.

Similarly CVA (50μM) induced transgenic mice bone marrow cell differentiation as assessed by CVA ability to drive BFUe formation of transgenic mice bone marrow erythroid progenitor stem cells (Figure 4.25). Delayed induction of these progenitors using CVA (50μM) in 48 hours methylcellulose supplemented culture resulted in a significantly reduced capacity for the formation of BFUe colonies compared with transgenic mice bone marrow cultures induced at the start of culture which is in line with the observations with hEPSCs and JK-1 cells. This implies strongly that CVA induces preferably early erythroid stem cells which are still largely amenable to genetic reprogramming without significantly altering or suppressing lineage specific cellular events.

Transcriptional down regulation of the γ-globin gene in adults is associated with decreased synthesis of fetal haemoglobin. This leads to increased expression of the faulty β-globin gene in sickle cell anemia individuals and consequently β^S haemoglobin which leads to the classical pathophysiology of the disease (). CVA increased JK1and cells γ-globin gene expression significantly at 50μM (Plate 4.19). Increase in γ-globin mRNA synthesis was not noticeable until 48hrs after induction (Figure 4.23) which was also consistent with significantly ($P<0.05$) higher foetal haemoglobin synthesis induced in these cells as assayed by alkaline denaturation assay (Jonxis and Huisman, 1956). CVA (50μM) also induced significantly ($P<0.05$) higher γ-globin gene expression in sickle cell transgenic mice bone marrow progenitor stem cells. However CVA failed to induce significant ($P<0.05$) γ-globin mRNA expression in K562 cells which is suggestive that CVA induces γ-globin gene expression via a mechanisms which involves a trans-acting factor absent in K562 cells since its been shown that the β-globin gene structure of K562 cells is un-altered and largely the same as that in erythroid cells (Yien & Bieker, 2012). This could be significant in the possible use of

CVA as an alternative to Hydroxyurea in the management of SCA through foetal haemoglobin induction in these patients.

EKLF is a transcriptional regulator that plays a crucial role in lineage-restricted control of gene expression. It is a red cell enriched DNA-binding protein that interacts with its cognate 5'-CCMCRCCCN-3'element at target promoters and enhancers. EKLF has been shown to be absolutely critical for β-globin expression (Zhang and Bieker, 1998). Its critical role for erythropoiesis and β-globin gene expression was further demonstrated in genetic ablation studies in mice in which the most profound phenotypic effect was profound lethal β-thalassemia at the time of the switch to adult β-globin expression (Yien & Bieker, 2012). EKLF has also been shown to modulate the expression of Bcl11a (another erythroid specific transcription factor and key mediator of γ-globin gene expression) (Sankaran *et al*., 2008). Several studies have linked the transcriptional up-regulation of γ-globin gene to lowered expression of EKLF (Zhang and Bieker, 1998; Yien and Bieker, 2011). The data revealed that CVA induced γ-globin gene expression in JK-1 cells was associated with a slight but significant ($P<0.05$) lowering of EKLF expression in JK-1 cells, however CVA significantly ($P<0.05$) elevated Bcl11a expression in JK-1 cells (Figure 4.34). The results does not exactly correlate with previous findings which identified bcl11a as a γ-globin regulatory target and low expressions known to be associated with increased expression of γ-globin mRNA level (Xu *et al*., 2013). Similarly reduced expression of EKLF has been shown to enhance γ-globin gene expression and is associated with reduced expression of the BCL11A gene expression (Tanimoto, 2000). However the data strongly argues that CVA induced γ-globin gene expression although associated with lowered EKLF expression levels was accompanied with increased Bcl11a gene expression unlike previous reports (Xu *et al*., 2013). Suggesting that CVA increases

clinically beneficial foetal haemoglobin synthesis in humans through un-characterized mechanisms reliant on KLF1 expression but not linked to Bcl11a gene expression.

Fatty acid elongase-5 (Elovl5) is vital in modulating hepatic fatty acid and carbohydrate metabolism. It elongates unsaturated fatty acids like palmitoleic acid (16:1, n-7) to form CVA (18:1, n-7). Elovl5 has also recently been identified as a key mediator of the mammalian target of rapamycin Akt linked FOXO1 (mTORC2-Akt-FOXO1) pathway through the control of CVA synthesis (Tripathy and Jump, 2013). Although the role of CVA in modulating γ-globin synthesis has not yet been demonstrated, our data reveal that CVA directly induces erythroid progenitor stem cell differentiation, however inhibition rescue experimental results strongly suggest a downstream product of CVA as the mediator of γ-globin gene expression induction (Figures 4.29, 4.30 and 4.31).

Δ^9 desaturase converts vaccenic acid to conjugated linoleic acid (CLA) in vivo in humans – CLA (9-cis 11-trans octadecenoic acid) is a group of conjugated C18: $\Delta^{9,11}$ fatty acids which are known to mediate various beneficial health effects in humans (Turpenien *et al*., 2001). Inhibition of Δ^9 desaturase using 10μM Isoxyl significantly suppressed γ-globin expression in JK1 and transgenic mice bone marrow progenitor cells, similarly Cyloate inhibition of in vivo biosynthesis of CVA significantly from palmitoleic acid which has been shown to be the precursor of CVA (Tripathy and Jump, 2013) completely suppressed γ-globin expression in JK1 and transgenic mice bone marrow progenitor cells. Addition of CVA (50μM) to the culture medium 24 and 48hrs after inhibition failed to restore γ-globin synthesis in both cell types, suggestive that CVA induced γ-globin expression is mediated primarily by a downstream biosynthetic product of CVA. Inhibition of Δ^9 desaturase and Elongase vl5 also completely suppressed CVA induced BFUe forming capacity of transgenic mice bone

marrow progenitor cells on semisolid IMDM. However addition of CVA (50µM) to the semisolid culture medium significantly restored CVA induced BFUe activity in cells treated with Isoxyl (50µM) but not cells treated with Cycloate (40µM), this suggests that CVA directly modulates transgenic mice bone marrow progenitor stem cell differentiation and commitment to the erythroid lineage.

The identification and isolation of novel chemotherapeutic agents from plant sources are a growing scientific gesture (Wang & Weller, 2006). In this study, a novel HbF and γ-globin inducing activity from *T. catappa*, a plant commonly used to manage SCA in the tropical countries especially in Africa was successfully identified. The findings of this study could be significant in the development of a targeted therapeutic drug for sickle cell anaemia and beta-thalassemia.

CHAPTER 6

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY

SCA remains a huge global public health issue especially in third world countries where Malaria is endemic. An affordable cure still remains largely uattainable. Hydroxyurea the only FDA approved drug is still largely unacceptable to large percentage of people living with SCA, hence the thrust of this study.

The study was able to:

1. Adapt a method to prioritize medicinal plants used for the management of sickle cell anaemia for further downstream scientific studies.
2. Establish a scientific rationale for the ethnomedicinal use of;
    a. *Telfaria occidentalis* use in the management of SCA
    b. *Terminalia catappa* for the management of SCA.
3. Develop a protocol for the isolation of peripheral blood mononuclear cells using glycerol gradient
4. Identify novel differentiation inducing compounds from *Terminalia catappa*
5. Identify Apigenin as a differentiation and γ-globin gene inducer in K562 cells.
6. Identify Mono-unsaturated fatty acids (Oleic acid, 2-hexadecenoic acid and Cis-vaccenic acid as novel inducers of K562 differentiation and γ-globin gene expression

7. Show Cis-vaccenic acid as a novel JK-1 and TMbmEPSCs differentiation, γ-globin and HbF modulatory compound. CVA was identified from *T. catappa* leaves.

8. Show CVA as an effective inducer of human EPSCs differentiation, γ-globin and foetal haemoglobin inducer

9. Show that CVA directly modulates EPSCs differentiation but indirectly controls γ-globin expression via a downstream biosynthetic metabolite.

10. Demonstrate that CVA may alter globin transcription by directly affecting Bcl11a expression and not EKLF.

6.2 CONCLUSION

The study was able to identify a novel (18:1 *n*7) mono-unsaturated fatty acid from *T. catappa* leaf extract distilled water fraction capable of inducing differentiation of K562, JK1cells and TMbmEPSCs expressing the full complement of the human beta globin gene cluster. CVA was able to up-regulate γ-globin gene expression in JK-1 and TMbmEPSCs, and JK-1 foetal haemoglobin synthesis. Other mono-unsaturated fatty acids identified; Oleic acid and 2-hexadecenoic acid and Apigenin also induced K562 differentiation and γ-globin gene expression. CVA appeared to directly induce differentiation but indirectly modulate γ-globin gene expression. The results demonstrated CVA as an effective γ-globin and foetal haemoglobin inducer in human cells thus making ita good candidate for further assessment as a potential therapeutic agent in the management of SCA and β-thalassemia.

6.3 RECOMMENDATIONS

The results of this study demonstrate CVA as a potent modulator of human γ-gamma globin and foetal haemoglobin synthesis; as such the compound may be useful as a potential SCA anemia therapeutic agent. However toxicity profile of exogenous administration of CVA to humans needs to be first ascertained.

The results of this study also calls for more research on;

1. The effects of CVA on acetylation and methylation of the human globin gene cluster
2. The effects of isomers of CVA on hEPSCs γ-globin gene expression
3. Assay for the foetal haemoglobin inducing effects of isomers of CVA in human erythroid progenitor stem cells
4. Possible molecular connections between the control of Elvol5 prenatal expression and the human globin switching.
5. The role of Apigenin and 2-HDA and DHA as possible cancer cells differentiation inducers and potential cytotoxic agents
6. The physiologic and biochemical role of Stearic acid and Oleic acid in human globin expression

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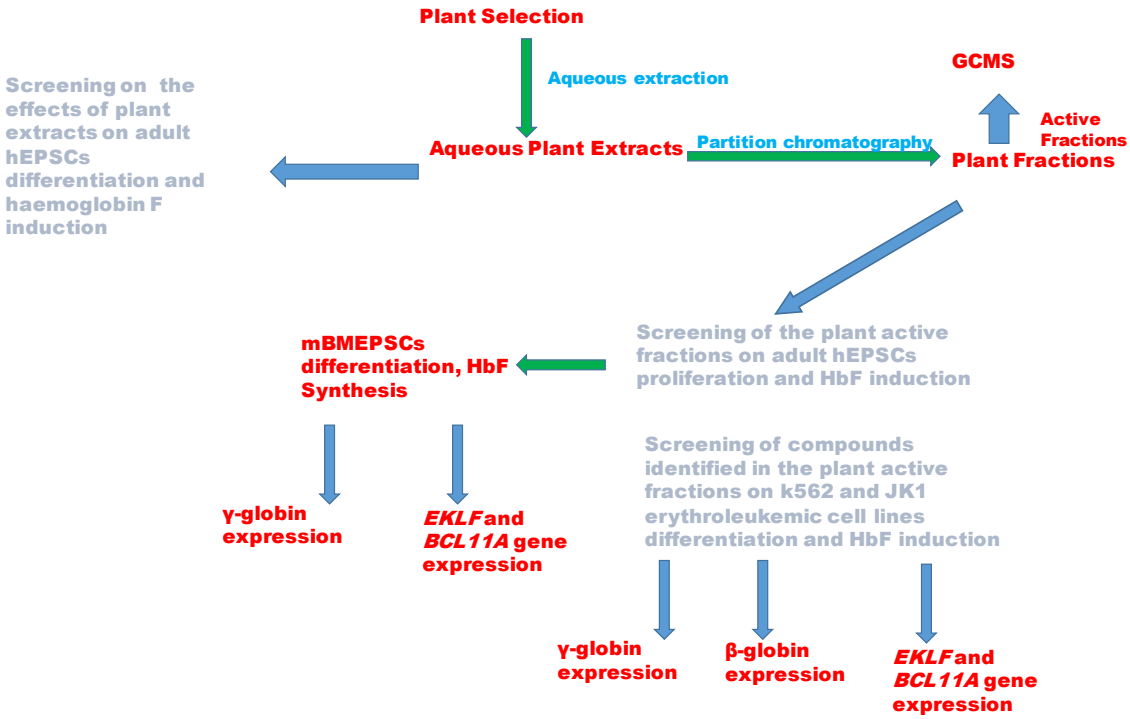
APPENDIX 1

Research Design



APPENDIX 2:

RITAM computation for *Zanthoxylum xanthoxyloides* (Fagara)

Component of Score	Parameter	Score	Reference
Ethnobotanical		4	(Ameh *et al*., 2012; Okpuzor *et al*., 2008)
Efficacy	Erythropoietic and antisickling activity (in vitro)	2	Adesanya and Sofowora, 1983 Sofowora *et al*., 1975
	Erythropoietic activity/ antisickling activity (in vivo)		
Safety	IC_{50}		
	LD_{50}	6	Sofowora *et al*., 1975
Total score		12	

APPENDIX 3

RITAM computation for *Carica papaya* (Pawpaw)

Component of Score	Parameter	Score	Reference
Ethnobotanical		2	Imaga *et al*., 2009
Efficacy	Erythropoietic and antisickling activity (in vitro)	2	Ogunyemi *et al*., 2008 Iweala *et al*., 2010 Mojisola *et al*., 2009
	Erythropoietic activity/ antisickling activity (in vivo)	1	Cyril-olutayo *et al*., 2009
Safety	IC_{50}		
	LD_{50}	6	
Total score		11	

APPENDIX 4

RITAM computation for *Bryophyllum pinatum* (Life plant)

Component of Score	Parameter	Score	Reference
Ethnobotanical		4	Ibile, 2006 http://www.yorubareligion.org/_con/_rubric/detail.php?nr=338&rubric=healing&PHPSESSID=h8ruoe8bab71apf4b2ckd
Efficacy	Erythropoietic and antisickling activity (in vitro)	0.5	Kade, (2003)
	Erythropoietic activity/ antisickling activity (in vivo)		
Safety	IC_{50}		
	LD_{50}	6	Ozolua *et al*., 2010
Total score		10.5	

APPENDIX 5

RITAM computation for *Garcinia kola* (Bitter Kola) seed extracts

Component of Score	Parameter	Score	Reference
Ethnobotanical		5	Okpuzor *et al*., 2008 Adejumo *et al*., 2011
Efficacy	Erythropoietic and antisickling activity (in vitro)	1	Adejumo *et al*., 2011
	Erythropoietic activity/ antisickling activity (in vivo)	7	Ahumobe and Braide, 2009; Kagbo and Ejebe, 2010
Safety	IC_{50}		
	LD_{50}	-5	Kagbo and Ejebe, 2010
Total score		8	

APPENDIX 6

RITAM computation for *Moringa oleifera* (Drumstick plant) leaf extract

Component of Score	Parameter	Score	Reference
Ethnobotanical		2	Imaga *et al*., 2009
Efficacy	Erythropoietic and antisickling activity (in vitro)	0.5	Adejumo *et al*., 2012
	Erythropoietic activity/ antisickling activity (in vivo)		
Safety	IC_{50}		
	LD_{50}	6	Oduola *et al*., 2005
Total score		8.5	

APPENDIX 7

RITAM computation for *Adansonia digitata* (Baobab) Fruit

Component of Score	Parameter	Score	Reference
Ethnobotanical		10	Okpuzor *et al*., 2008 Ibile, 2006 Oloyede *et al*., 2010
Efficacy	Erythropoietic and antisickling activity (in vitro)	0.5	Adesanya *et al* 1988
	Erythropoietic activity/ antisickling activity (in vivo)	0.5	Yusuf *et al*., 2008
Safety	IC_{50}		
	LD_{50}	6	Ramadan *et al*., 1994
Total score		17	

APPENDIX 8

RITAM computation for *Sorghum bicolor* (Sorghum) leaf extract

Component of Score	Parameter	Score	Reference
Ethnobotanical		8	Okpuzor *et al*., 2008 Ibile, 2006 Nwinyi and Kwanashie, 2008
Efficacy	Erythropoietic and antisickling activity (in vitro)	2	Ogwumike, 2002 Akande *et al*., 2010
	Erythropoietic activity/ antisickling activity (in vivo)	7	Ogwumike, 2002 Akande *et al*., 2010
Safety	IC_{50}		
	LD_{50}	-3	Ogwumike, 2002
Total score		14	

APPENDIX 9

RITAM computation for *Azadirachta indica* (Neem) leaf extract

Component of Score	Parameter	Score	Reference
Ethnobotanical		6	Olowokudejo *et al*., 2008
Efficacy	Erythropoietic and antisickling activity (in vitro)	2	Saravannan, 2011 Rahman *et al*., 2001
	Erythropoietic activity/ antisickling activity (in vivo)	3	Macedo *et al*., 2007
Safety	IC_{50}		
	LD_{50}	6	Saravannan, 2011
Total score		17	

APPENDIX 10

RITAM computation of *Telfairia occidentalis* (Fluted Pumpkin) leaf extract

Component of Score	Parameter	Score	Reference
Ethnobotanical		8	Ibile, 2006 Aiyejola and Bello, 2006
Efficacy	Erythropoietic and antisickling activity (in vitro)	0.5	Osuntoki and Sanusi, 2007
	Erythropoietic activity/ antisickling activity (in vivo)	5.5	Hamlin and Dada, 2011 Salman *et al*., 2008 Aiyejola and Bello, 2006
Safety	IC_{50}		
	LD_{50}	6	Ekpeyong *et al*., 2012
Total score		20	

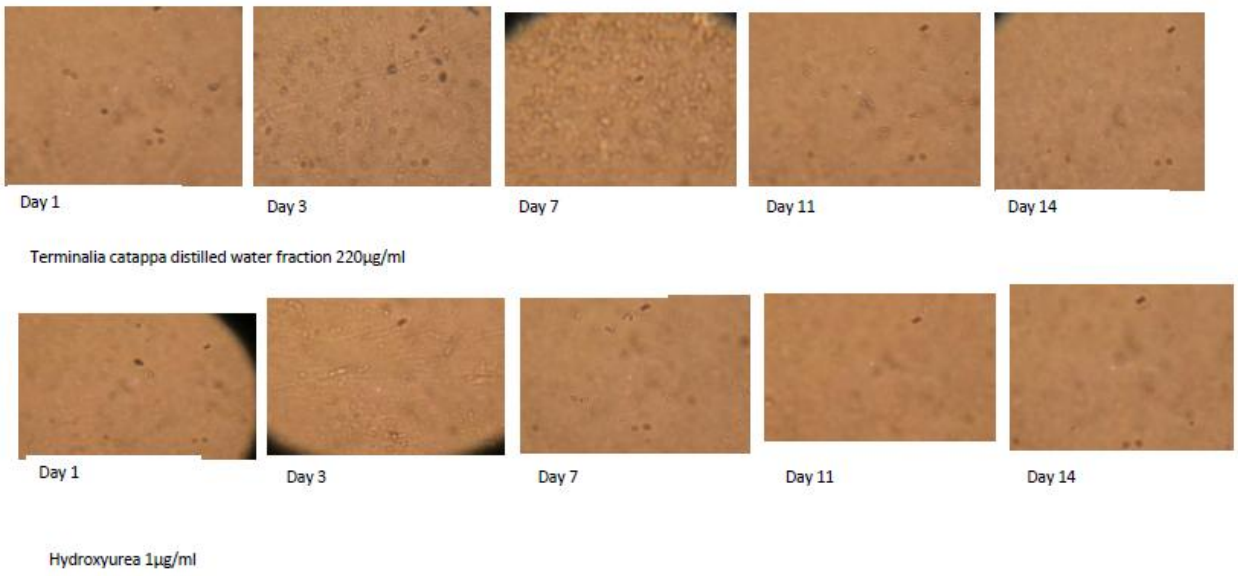
APPENDIX 11

RITAM computation for *Terminalia catappa* (Almond) leaf extract

Component of Score	Parameter	Score	Reference
Ethnobotanical		15	Ibile, 2006 Okpuzor *et al*., 2008 Moody *et al*., 2003 Imaga, 2013
Efficacy	Erythropoietic and antisickling activity (in vitro)	3	Samuel *et al*., 2012 Moody *et al*., 2003 Chikezie *et al*.,2013 Adejumo *et al*., 2010
	Erythropoietic activity/ antisickling activity (in vivo)	2	Ibegbulem 2001
Safety	IC_{50}		
	LD_{50}	-5	Ahmed *et al*., 2005
Total score		15	

APPENDIX 12

Effect of TCDWF on hEPSCs Differentiation

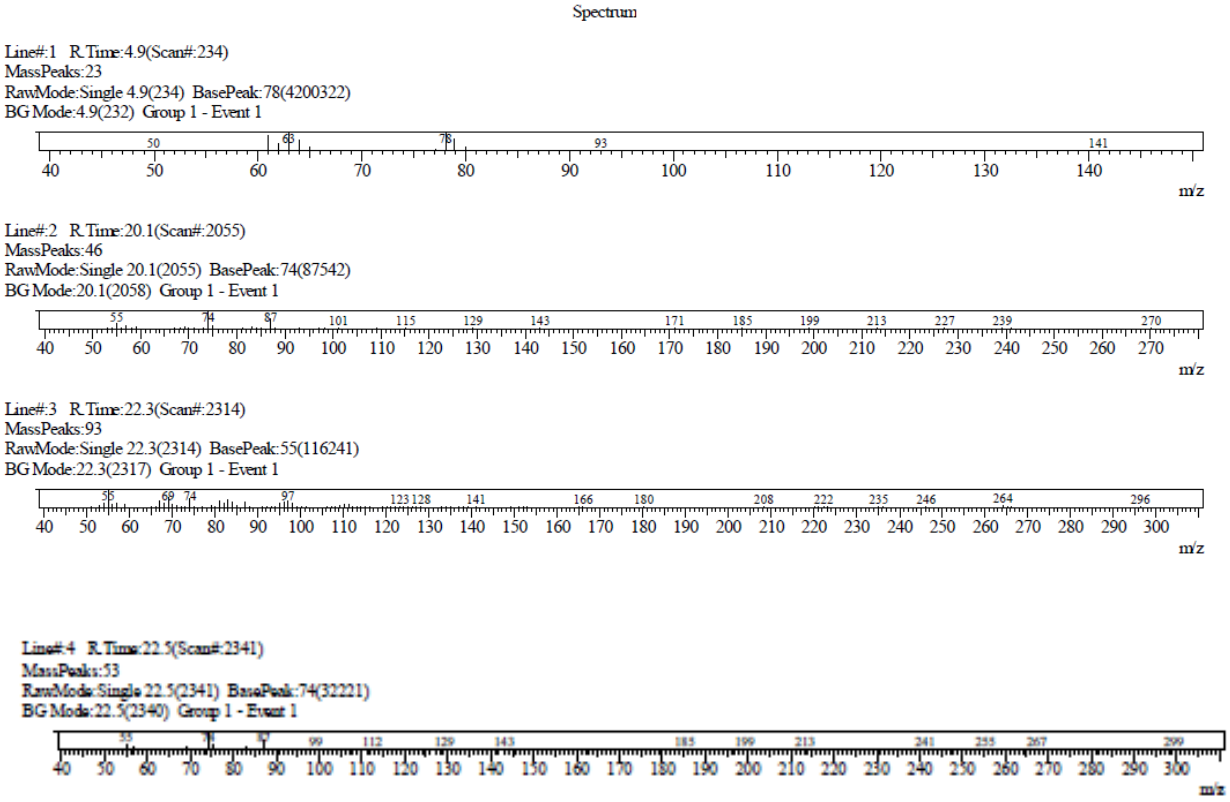


Effect of on *Terminalia catappa* distilled water fraction on HSC differentiation and commitment to the erythroid lineage

APPENDIX 13

Mass Spectrum of *un*-dervatized *Terminalia catappa* distilled water fraction GC

Eluates



Spectrum Comparison

Library

<< Target >>

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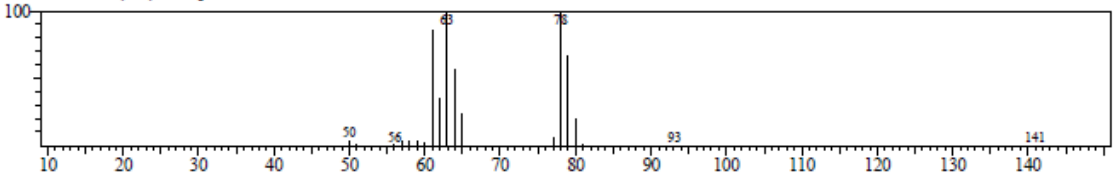

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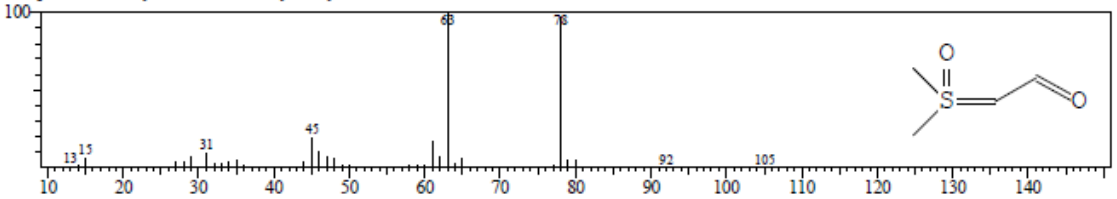

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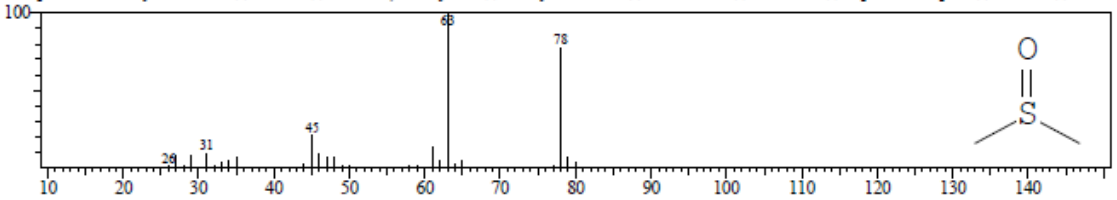

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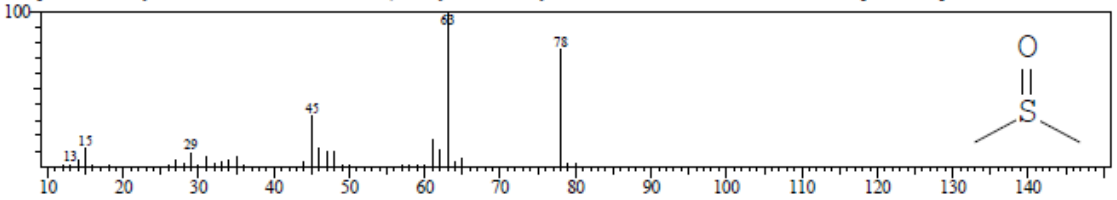

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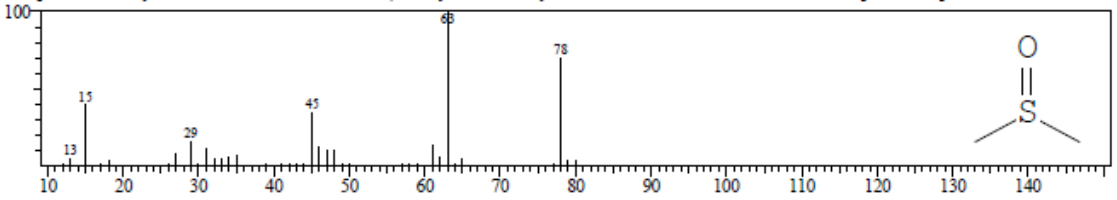
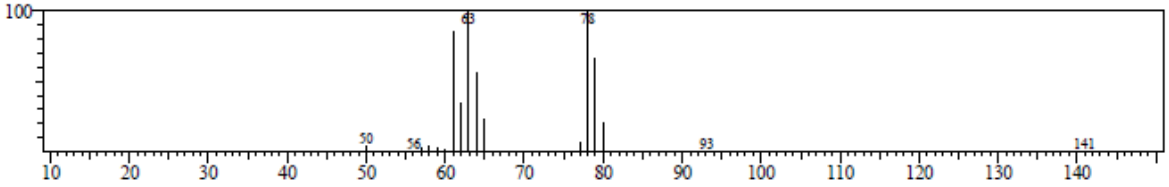
SI:79 Formula:C2H6OS CAS:67-68-5 MolWeight:78 RetIndex:691

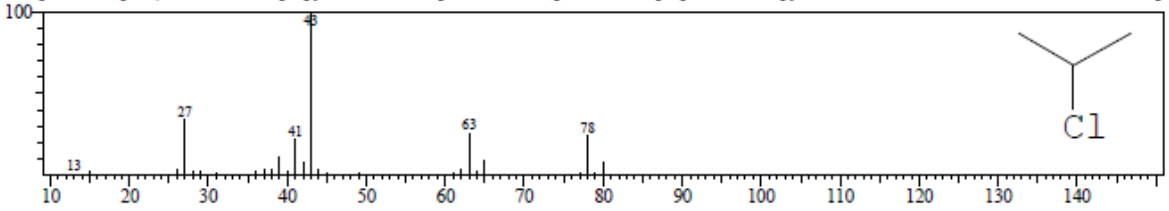
CompName:Dimethyl Sulfoxide $$ DMSO $$ Methane, sulfinylbis- $$ Methyl sulfoxide $$ Demsodrox $$ Dimexide $$ Dipiratril-tropico $$ Dolicur $$ Drom



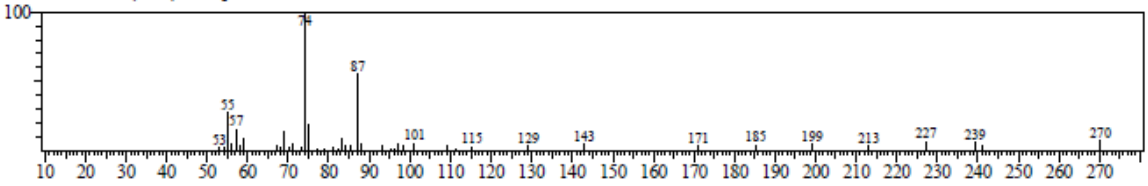
<< Target >>
Line#:1 R.Time:4.942(Scan#:234) MassPeaks:23
RawMode:Single 4.942(234) BasePeak:78.05(4200322)
BG Mode:4.925(232) Group 1 - Event 1



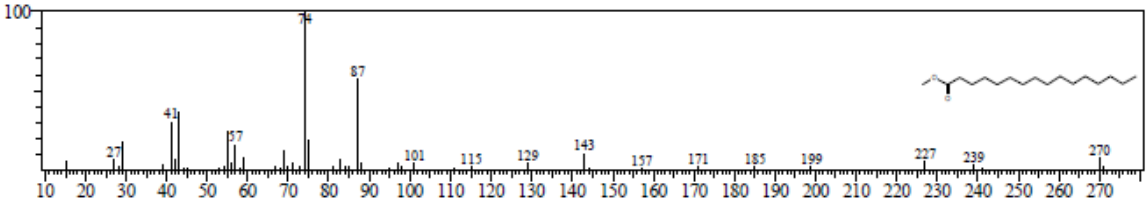
Hit#:5 Entry:525 Library:NIST05s.LIB
SI:79 Formula:C3H7Cl CAS:75-29-6 MolWeight:78 RetIndex:460
CompName:Propane, 2-chloro- $$ Isopropyl chloride $$ Isoprid $$ Narcosop $$ 2-Chloropropane $$ 2-Propyl chloride $$ iso-C3H7Cl $$ UN 2356 $$ sec-Prop



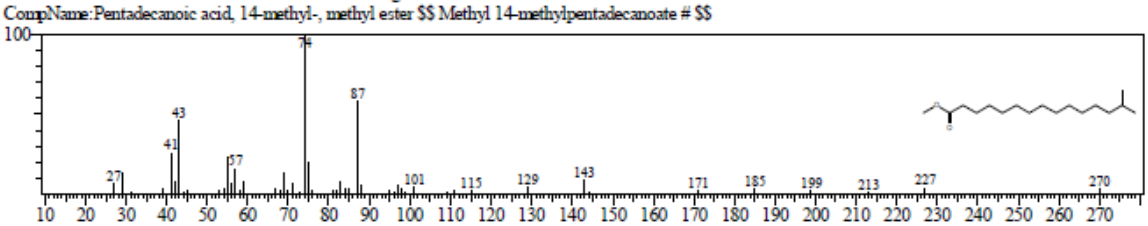
<< Target >>
Line#:2 R.Time:20.117(Scan#:2055) MassPeaks:46
RawMode:Single 20.117(2055) BasePeak:74.05(87542)
BG Mode:20.142(2058) Group 1 - Event 1



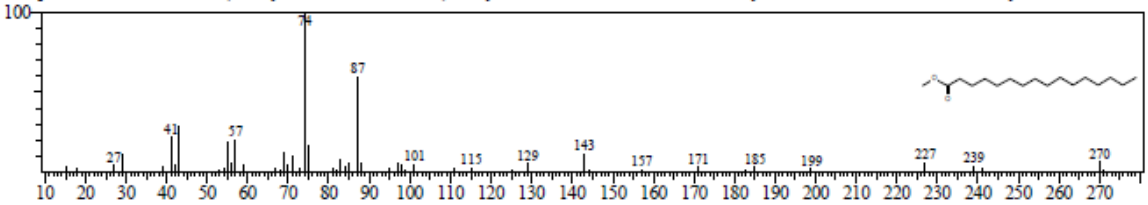
Hit#:1 Entry:22219 Library:NIST05s.LIB
SI:95 Formula:C17H34O2 CAS:112-39-0 MolWeight:270 RetIndex:1878
CompName:Hexadecanoic acid, methyl ester $$ Palmitic acid, methyl ester $$ n-Hexadecanoic acid methyl ester $$ Metholene 2216 $$ Methyl hexadecanoate $



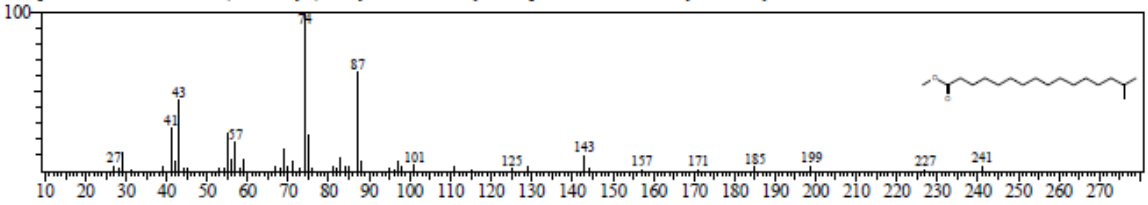
Hit#:2 Entry:22223 Library:NIST05s.LIB
SI:95 Formula:C17H34O2 CAS:5129-60-2 MolWeight:270 RetIndex:1814
CompName:Pentadecanoic acid, 14-methyl-, methyl ester $$ Methyl 14-methylpentadecanoate # $$



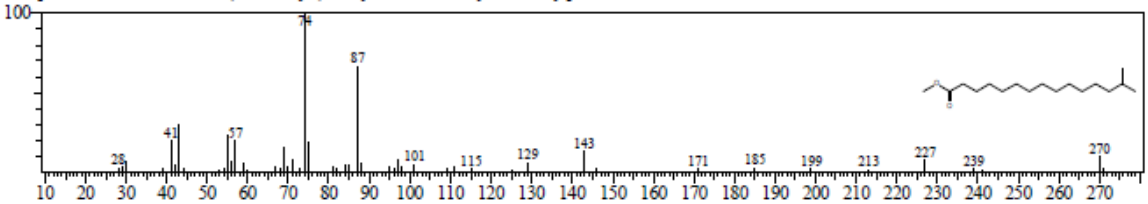
Hit#:3 Entry:22221 Library:NIST05s.LIB
SI:93 Formula:C17H34O2 CAS:112-39-0 MolWeight:270 RetIndex:1878
CompName:Hexadecanoic acid, methyl ester $$ Palmitic acid, methyl ester $$ n-Hexadecanoic acid methyl ester $$ Metholene 2216 $$ Methyl hexadecanoate $



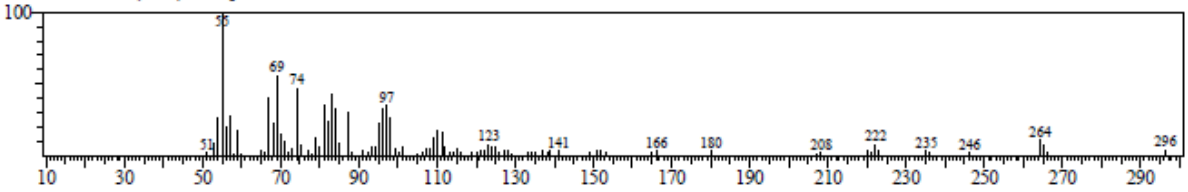
Hit#:4 Entry:22987 Library:NIST05s.LIB
SI:93 Formula:C18H36O2 CAS:6929-04-0 MolWeight:284 RetIndex:1914
CompName:Hexadecanoic acid, 15-methyl-, methyl ester $$ Methyl isoheptadecanoate $$ Methyl 15-methylhexadecanoate $$



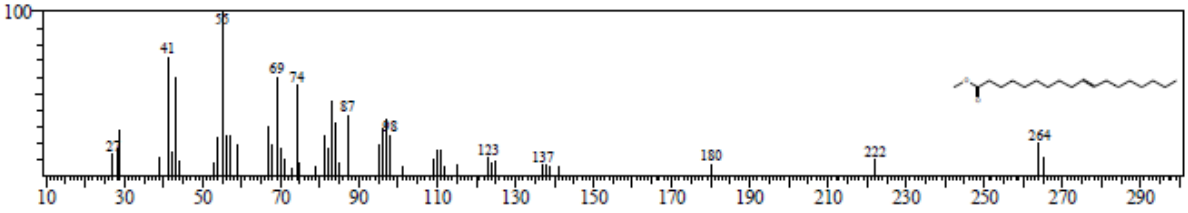
Hit#:5 Entry:22222 Library:NIST05s.LIB
SI:92 Formula:C17H34O2 CAS:5129-60-2 MolWeight:270 RetIndex:1814
CompName:Pentadecanoic acid, 14-methyl-, methyl ester $$ Methyl 14-methylpentadecanoate # $$



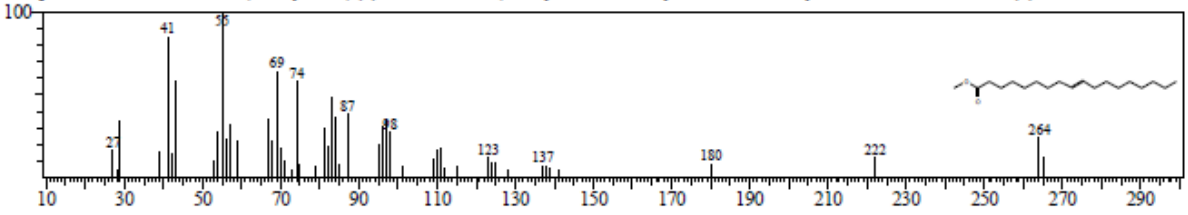
<< Target >>
Line#:3 R.Time:22.275(Scan#:2314) MassPeaks:93
RawMode:Single 22.275(2314) BasePeak:55.00(116241)
BG Mode:22.300(2317) Group 1 - Event 1



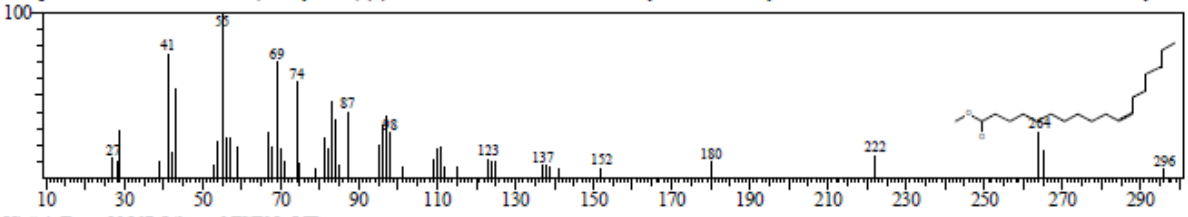
Hit#:1 Entry:98784 Library:NIST05.LIB
SI:93 Formula:C19H36O2 CAS:13481-95-3 MolWeight:296 RetIndex:2085
CompName:10-Octadecenoic acid, methyl ester $$ Methyl (10E)-10-octadecenoate # $$



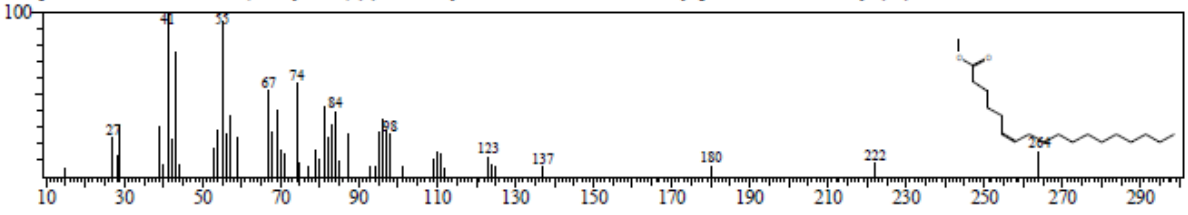
Hit#:2 Entry:23570 Library:NIST05s.LIB
SI:92 Formula:C19H36O2 CAS:1937-62-8 MolWeight:296 RetIndex:2085
CompName:9-Octadecenoic acid, methyl ester, (E)- $$ Elaidic acid, methyl ester $$ Methyl elaidate $$ Methyl trans-9-octadecenoate $$ (E)-9-Octadecenoic aci



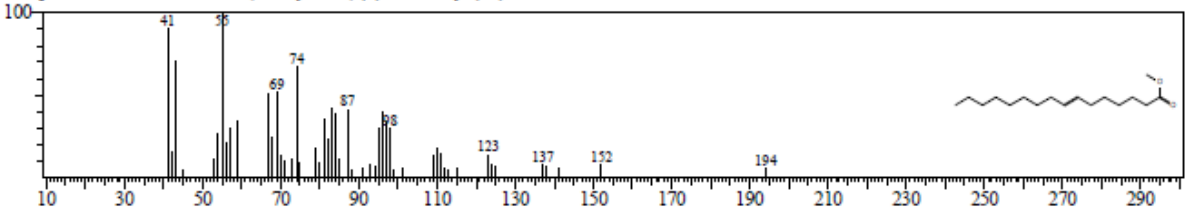
Hit#:3 Entry:98785 Library:NIST05.LIB
SI:92 Formula:C19H36O2 CAS:1937-63-9 MolWeight:296 RetIndex:2085
CompName:11-Octadecenoic acid, methyl ester, (Z)- $$ cis-11-Octadecenoic acid methyl ester $$ Methyl cis-octadec-11-enoate $$ cis-Vaccenic acid methyl est



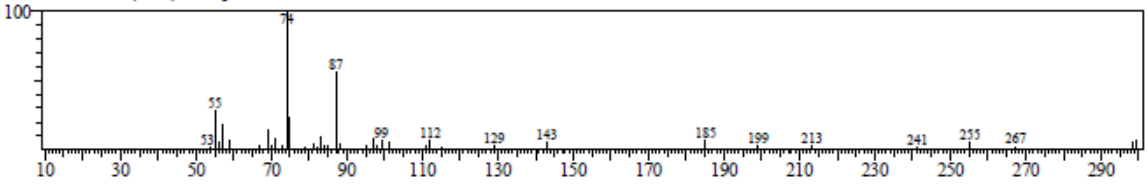
Hit#:4 Entry:23567 Library:NIST05s.LIB
SI:92 Formula:C19H36O2 CAS:2777-58-4 MolWeight:296 RetIndex:2085
CompName:6-Octadecenoic acid, methyl ester, (Z)- $$ Methyl cis-6-octadecenoate $$ Methyl petroselinate $$ Methyl (6Z)-6-octadecenoate # $$



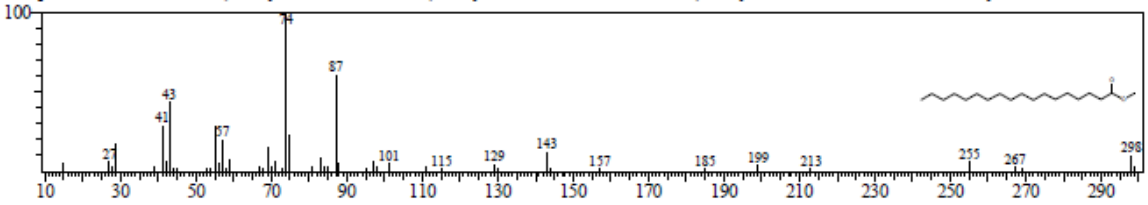
Hit#:5 Entry:82163 Library:NIST05.LIB
SI:91 Formula:C17H32O2 CAS:56875-67-3 MolWeight:268 RetIndex:1886
CompName:7-Hexadecenoic acid, methyl ester, (Z)- $$ Methyl (7E)-7-hexadecenoate # $$



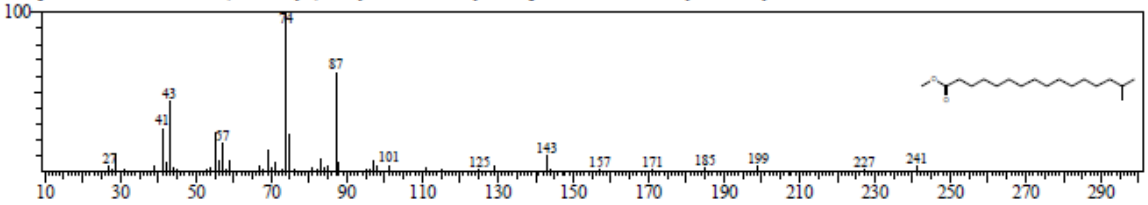
<< Target >>
Line#:4 R.Time:22.500(Scan#:2341) MassPeaks:53
RawMode:Single 22.500(2341) BasePeak:74.05(32221)
BG Mode:22.492(2340) Group 1 - Event 1



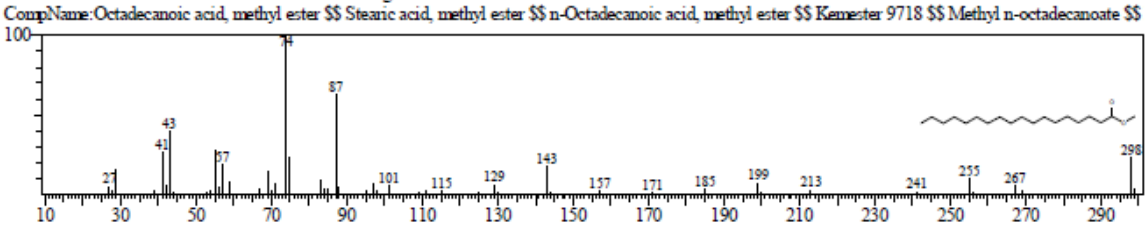
Hit#:1 Entry:100069 Library:NIST05.LIB
SI:94 Formula:C19H38O2 CAS:112-61-8 MolWeight:298 RetIndex:2077
CompName:Octadecanoic acid, methyl ester $$ Stearic acid, methyl ester $$ n-Octadecanoic acid, methyl ester $$ Kemester 9718 $$ Methyl n-octadecanoate $$



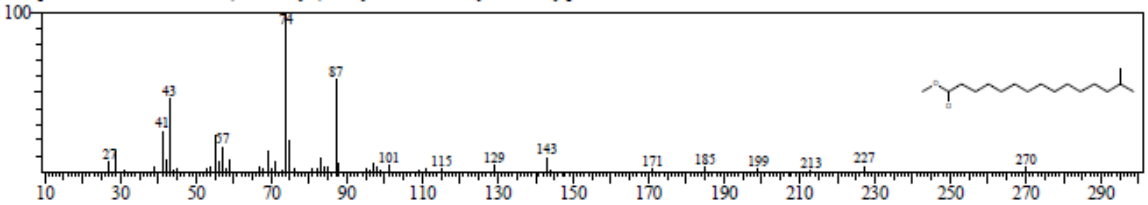
Hit#:2 Entry:22987 Library:NIST05s.LIB
SI:91 Formula:C18H36O2 CAS:6929-04-0 MolWeight:284 RetIndex:1914
CompName:Hexadecanoic acid, 15-methyl-, methyl ester $$ Methyl isoheptadecanoate $$ Methyl 15-methylhexadecanoate $$



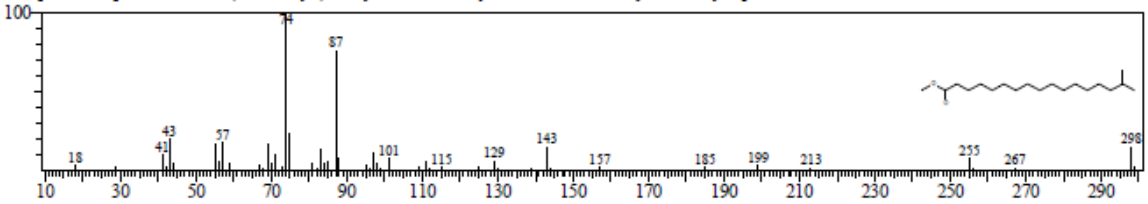
Hit#:3 Entry:23658 Library:NIST05s.LIB
SI:91 Formula:C19H38O2 CAS:112-61-8 MolWeight:298 RetIndex:2077
CompName:Octadecanoic acid, methyl ester $$ Stearic acid, methyl ester $$ n-Octadecanoic acid, methyl ester $$ Kemester 9718 $$ Methyl n-octadecanoate $$



Hit#:4 Entry:22223 Library:NIST05s.LIB
SI:90 Formula:C17H34O2 CAS:5129-60-2 MolWeight:270 RetIndex:1814
CompName:Pentadecanoic acid, 14-methyl-, methyl ester $$ Methyl 14-methylpentadecanoate # $$

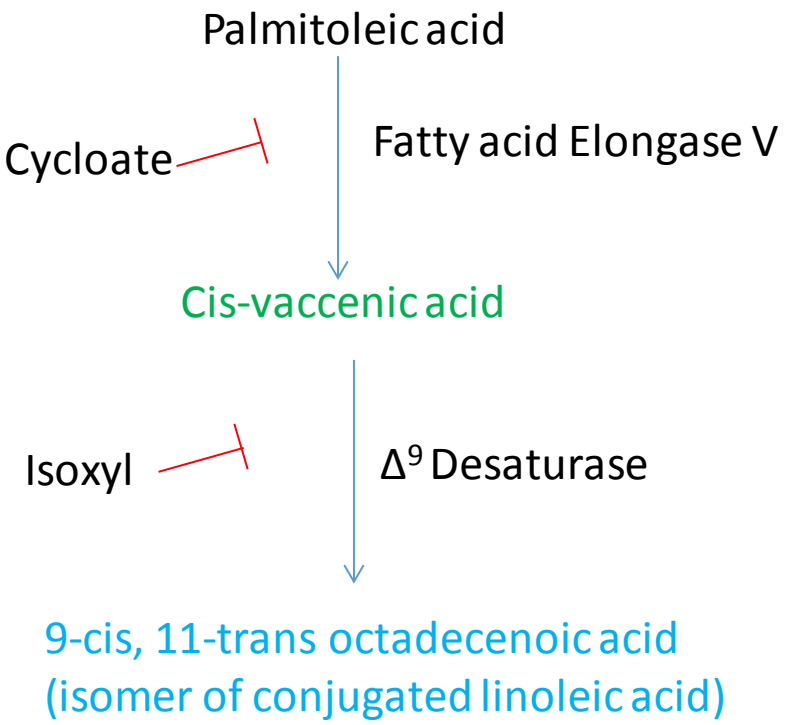


Hit#:5 Entry:100070 Library:NIST05.LIB
SI:90 Formula:C19H38O2 CAS:5129-61-3 MolWeight:298 RetIndex:2013
CompName:Heptadecanoic acid, 16-methyl-, methyl ester $$ Methyl isostearate $$ Methyl 16-methylheptadecanoate $$



APPENDIX 14:

Rescue Experiments Research Concept



APPENDIX 15

Scientific Publications

CELL BIOCHEMISTRY & FUNCTION

CELL BIOCHEMISTRY AND FUNCTION
Cell Biochem Funct (2014)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/cbf.3024

Induction of foetal haemoglobin synthesis in erythroid progenitor stem cells: mediated by water-soluble components of *Terminalia catappa*

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Current novel therapeutic agents for the treatment of sickle cell anaemia (SCA) focus on increasing foetal haemoglobin (HbF) levels in SCA patients. Unfortunately, the only approved HbF-inducing agent, hydroxyurea, has long-term unpredictable side effects. Studies have shown the potential of plant compounds to modulate HbF synthesis in primary erythroid progenitor stem cells. We isolated a novel HbF-inducing *Terminalia catappa* distilled water active fraction (TCDWF) from *Terminalia catappa* leaves that induced the commitment of erythroid progenitor stem cells to the erythroid lineage and relatively higher HbF synthesis of 9.2- and 6.8-fold increases in both erythropoietin (EPO)-independent and EPO-dependent progenitor stem cells respectively. TCDWF was differentially cytotoxic to EPO-dependent and EPO-independent erythroid progenitor stem cell cultures as revealed by lactate dehydrogenase release from the cells. TCDWF demonstrated a protective effect on EPO-dependent and not EPO-independent progenitor cells. TCDWF induced a modest increase in caspase 3 activity in EPO-independent erythroid progenitor stem cell cultures compared with a significantly higher (P 0.05) caspase 3 activity in EPO-dependent ones. The results demonstrate that TCDWF may hold promising HbF-inducing compounds, which work synergistically, and suggest a dual modulatory effect on erythropoiesis inherent in this active fraction. Copyright © 2014 John Wiley & Sons, Ltd.

KEY WORDS—erythroid progenitor stem cells; differentiation; proliferation; foetal haemoglobin; *Terminalia catappa*; sickle cell anaemia; β-thalassemia

INTRODUCTION

Foetal haemoglobin (HbF) has been shown previously to ameliorate the symptoms of sickle cell anaemia (SCA) and β-thalasesemia (haemoglobinopathies),[1–3] and hence, its induction in individuals with either of the haemoglobinopathy is considered a novel therapeutic approach.[3] Foetal haemoglobin (HbF) is the major haemoglobin in human zygotes within the second and third trimesters of gestation, and its expression continues until 4–6 months post-natal before its synthesis is down-regulated to about 1%–2%, and β-haemoglobin becomes the predominant haemoglobin during adult life.[4,5] Several studies have demonstrated the clinical benefits of sustained HbF (hereditary persistence of HbF) synthesis in which the results of genome-wide association studies implicate gene mutations that affect *KLF1* and *Bcl11a* gene expressions or drug-induced reversal of HbF synthesis from adult haemoglobin in β-thalassemia and SCA individuals.[6–10] Hence, current chemotherapeutic approaches to the management of SCA have focused on identifying novel agents that can increase or reactivate the synthesis of HbF in adult β-thalassemia and SCA individuals.[11] To date, hydroxyurea remains the only Food and Drug Administration (FDA)–approved treatment regimen for SCA despite reports of its low specificity and acceptability among SCA patients and its inherent bone marrow suppression activity.[1] Its impacts on organ damage, stroke and carcinogenesis on long-term use in SCA patients are also not well known.[12–15] Hydroxyurea has been shown to induce HbF through the soluble guanylyl cyclase mechanism.[16]

Primary erythroid progenitor stem cells (EPSCs) have been demonstrated as the best *in vitro* model for the screening of HbF-inducing agents.[11,17] Fibach and others developed a two-phase liquid culture system that mimics very closely the *in vivo* erythropoietin (EPO)-dependent and EPO-independent erythroid progenitors proliferation and differentiation *in vitro*.[11,18] This allows for effective *in vitro* screening of potential HbF-inducing agents. Isolation of novel compounds with therapeutic potential from the plants has been emphasized because of the advantages of these compounds in terms of potency and lesser side effects as reported previously.[19–21] Unfortunately, the availability of plant compounds for the induction of HbF synthesis in EPSCs is very scanty.[11] This is despite the abundance of literature on the efficacies of several plant remedies used in the management of SCA.[21–34]

Terminalia catappa (Indian almond), a plant found widely spread in northern Nigeria although it is thought to have its origin in Indonesia and India, has been reportedly used in the management of SCA.[21,22] It has been shown to

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ORIGINAL PAPER

Isolation of Peripheral Blood Mononuclear Cells Using Glycerol Density Gradient

I. A. Almola · H. M. Inuwa · A. J. Nok · A. I. Mamman · N. Habila · A. Muhammad · U. S. Ndidi · B. Ignatius · P. L. Jande · R. Ogbor · L. C. Isaac · N. B. Afolabi-Balogun

Published online: 14 September 2013

Abstract Isolation of peripheral blood mononuclear cells (PBMCs) is fraught with challenges including, but not limited to, the cost of limited gradients available for the isolation of PBMCs. Glycerol gradient (1.077 g/ml) was used to isolate PBMCs from adult peripheral blood. The differentiation potential of the isolated cells was assessed by culturing the cells in MEM at 37 °C in 5 % CO_2. The results demonstrated that the isolated cells could differentiate into committed linages of the erythroid progeny. LDH assay revealed that glycerol was not cytotoxic to the cells. The use of glycerol density as an alternative could be significant in cell culture experiments.

Keywords Peripheral blood mononuclear cells · Isolation · Glycerol density gradient · Stem cells · Hematopoietic progenitor cells

Mononuclear cells are primary sources of high-quality hematopoietic stem cell lines for in vitro cell studies. Several methods exist for the isolation of peripheral blood mononuclear cells (PBMCs) from peripheral blood [1–4], of which most rely on expensive gradients [5] that are difficult to acquire in developing countries [6–9]. Many of these methods rely on the use of density gradients of inert polysaccharides like Ficoll® and Percoll®, which pose little or no toxicity risk to the cells at the desired gradient density and final formed gradient containing PBMCs [10]. Ficoll® has been reported in the separation of PBMCs and granulocytes by the use of a discontinuous gradient [4, 11]. Albumin gradients have been employed for the separation of lymphocytes and for the removal of cell debris and erythrocytes from the cell suspension [12]. Percoll gradient serves as the only medium which can give a gradient that has a low viscosity and can separate cells isopycnic under normal conditions for osmolality and ionic strength [13]. Glycerol is a readily available and cheap reagent, which has several uses including cryopreservation of mice marrow cell [14, 15]. In spite of its numerous uses as reported previously, its use in the isolation of PBMCs has not been reported. Herein, we report for the first time the possibility of using glycerol gradient for the isolation of PBMCs from blood. The separation method described here utilizes peripheral blood obtained from a sickle cell (SS) individual. Human peripheral blood samples were obtained from donors with their informed consent, after the official approval of Institutional Ethics Research Committee. Peripheral blood (2 ml) was collected into a vacutainer tube containing anti-coagulant (EDTA) by venipuncture according to standard procedures (WHO technical report, 1992) and diluted two-fold using PBS pH 7.2. Exactly, 1 ml of this diluted blood was carefully layered on an equal volume glycerol gradient (1.077 g/ml), which was prepared using the following formula as previously described [16]:

$$V_s = V\frac{\rho - 0.1\rho^c - 0.9}{\rho_s - 1},$$

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