

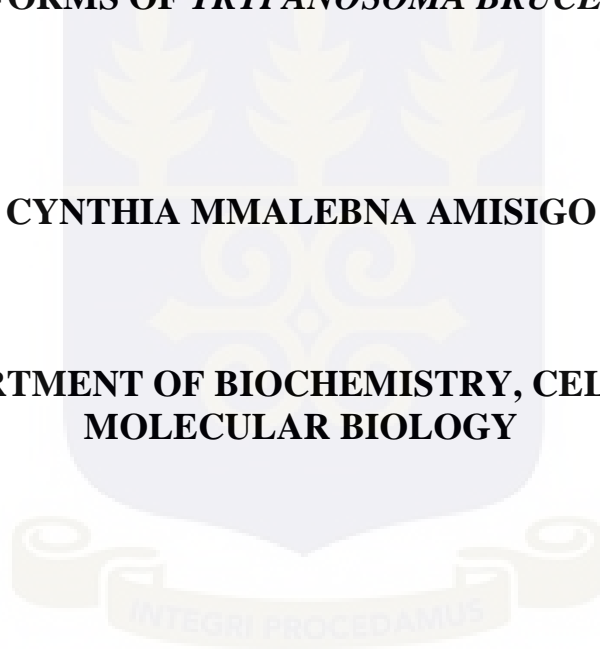
UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

**EFFECTS OF IRON CHELATORS ON BLOODSTREAM
FORMS OF *TRYPANOSOMA BRUCEI***

CYNTHIA MMALEBNA AMISIGO

**DEPARTMENT OF BIOCHEMISTRY, CELL AND
MOLECULAR BIOLOGY**



JULY 2018

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*TRYPANOSOMA BRUCEI***

BY

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(10374698)**

**A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN
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BIOLOGY OF INFECTIOUS DISEASES**

**DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR
BIOLOGY**

JULY 2018

DECLARATION

I, Cynthia Mmalebna Amisigo, do certify that this project aside other cited works was conducted by me under the supervision of Dr. Theresa Manful Gwira and Dr. Jonathan P. Adjimani in the Department of Biochemistry, Cell and Molecular Biology, University of Ghana.

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ABSTRACT

African trypanosomiasis still remains a lethal disease to both human and livestock. The disease persists due to limited drug availability, toxicity and emergence of drug resistance, hence the need to provide alternative forms of therapy. Studies have shown that the iron chelator deferoxamine exhibited anti-trypanosomal effects by inhibiting cell growth and interfering with the activity of some iron dependent enzymes. In this study, the *in vitro* effects of different phenolic acids, which are known to complex iron, were assessed for their trypanocidal activities against *Trypanosoma brucei brucei* using the alarmaBlue assay. The cell cycle effects of the chelators were determined by flow cytometry and parasite morphological analysis was done by microscopy. Gallic acid was the most potent phenolic acid with an IC₅₀ value of 14.2µM. The compounds showed a dose dependent effect on the cell viability and the mitochondrion membrane potential. There was also a significant loss in kinetoplast and an increase in the S phase of the cell cycle. mRNA sequencing and RT-qPCR data showed an upregulation of almost all the transcripts and a corresponding increase in the iron related genes such as the ribonucleotide reductase and cyclin 2 genes. The chelators also showed moderate toxicity against macrophages. The results throw more light on the possible mechanism of action of the chelators and provide alternative therapeutic approaches in the treatment of African trypanosomiasis which might include interfering with the iron metabolism of the parasite.

DEDICATION

I dedicate this work to the Almighty God. His steadfast love for me has never ceased and I dedicate this to His glory.

I also dedicate this work to my parents for their unconditional love and support. I am very grateful.

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LIST OF ABBREVIATIONS

AAT	Animal African Trypanosomiasis
APOL1	Apolipoprotein L1
CA	Caffeic Acid
CC ₅₀	Cytotoxic Concentration (50%)
CDK	Cyclin Dependent Kinase
CGA	Chlorogenic Acid
CYC	Cyclin
DA	Diminazene Aceturate
DFO	Deferoxamine
EDDA	Ethylenediamine di-o-hydroxyphenylacetic acid
EDTA	Ethylenediaminetetraacetic acid
ESAG	Expression-site-associated- genes
FA	Ferulic Acid
Fe-SOD	Iron Superoxide Dismutase
GA	Gallic Acid
HAT	Human African Trypanosomiasis
IC ₅₀	Inhibitory Concentration (50%)
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRP	Iron regulatory proteins
kDNA	Kinetoplast DNA

MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]
PA	Protocatechuic Acid
QA	Quinic Acid
RA	Rosmarinic Acid
RNR	Ribonucleotide Reductase
RT-qPCR	Reverse Transcriptase Quantitative PCR
SI	Selectivity Index
SOD	Superoxide Dismutase
TAO	Trypanosome Alternative Oxidase
TR	Transferrin Receptor
vPBS	Voorhei's modified PBS
VSG	Variant Surface Glycoprotein

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Trypanosomes are protozoan parasites that belong to the class Kinetoplastida. Disease caused by these organisms include African and South American trypanosomiasis (Stijlemans *et al.*, 2015). Other members of this class include *Leishmania spp.* which cause visceral and cutaneous leishmaniasis in humans (Jain and Jain, 2015). *Trypanosoma brucei (T. brucei) spp.* are the only species that are able to cause infection in both human and livestock. There are at least three sub species of *T. brucei*, which includes *T. brucei rhodesiense (T. b. rhodesiense)*, *T. brucei gambiense, (T. b. gambiense)* and *Trypanosoma brucei brucei (T. b. brucei)*. The human trypanosomiasis is caused by *T. b. rhodesiense* and *T. b. gambiense* (Kaiser *et al.*, 2011) which poses major health problems in sub-Saharan Africa (Chamond *et al.*, 2010). *Trypanosoma brucei brucei* on the other hand causes Animal African Trypanosomiasis (AAT) or nagana in cattle (Giordani *et al.*, 2016). The effect on animals hinders livestock productivity leading to serious economic losses (Chamond *et al.*, 2010).

These pathogenic eukaryotic organisms are able to thrive successfully in the host by evading the host immune system. They achieve this by changing their surface coats known as the variant surface glycoproteins (VSG) and evade immune responses mounted against them by the host. These features make vaccine production difficult (Black and Mansfield, 2016). Chemotherapy plays an important role in the control of African Trypanosomiasis (AT). Diminazene aceturate and isometamidium chloride are the most commonly used anti-trypanosomal drugs against different species of animal trypanosomes (Holmes *et al.*, 2004). In human treatment, drugs such as suramin

and pentamidine are used against the early stages of the infection while melarsoprol and eflornithine are used at the late stages (Kaiser *et al.*, 2011). There are however reports of drug resistance and toxicity (Tsegaye *et al.*, 2015; Fairlamb and Horn, 2018) and hence the urgent need for the development of new and effective anti-trypanosomals.

Studies on the biology of *T. brucei*, host-pathogen interactions, as well as the completion of the genome wide project have provided different avenues to explore in the search for effective therapeutic agents. *Trypanosoma brucei brucei* is the model organism used in studying the biology of trypanosomes and some eukaryotes since it is not harmful to humans, can be easily cultured in the laboratory and can be genetically manipulated (Basu *et al.*, 2016).

Trypanosomes require sufficient amount of intracellular iron for cellular activities. The activity of iron dependent enzymes relies on the availability of iron which is essential in the metabolic processes of the parasite. The iron dependent enzymes are very important in the parasite's proliferation and energy metabolism (Breidbach *et al.*, 2002; Merschjohann and Steverding, 2006; Manta *et al.*, 2012; Basu *et al.*, 2016). Depriving the parasites of iron affects their survival (Breidbach *et al.*, 2002). The iron-deprived conditions are created *in vitro* by changing the type of serum in the media or by the use of iron chelators (Mussmann *et al.*, 2004; Carneiro, 2007).

The iron chelators commonly used are derived from microorganisms (siderophores). The process of acquiring large quantities of siderophore for treatment can be tedious and costly (Merschjohann and Steverding, 2006) hence other sources of iron chelators, especially the plant derived sources are being explored. Phenolic acids are one of the most abundant plant secondary metabolites which have been studied for their iron chelating properties as well as anti-microbial and anti-tumor effects (Andjelković *et al.*, 2006; Kakkar and Bais, 2014; Semaming *et al.*, 2015; He *et al.*, 2016). Iron chelators have been shown to inhibit the growth of trypanosomes *in vitro* (Breidbach *et al.*, 2002;

Merschjohann and Steverding, 2006). There is however limited information on the effect of chelators on the parasite's morphology, cell cycle process and gene expression.

In this study, the effects of phenolic acids with iron binding potential were investigated for their trypanocidal and cytotoxic activities. The effect of the chelators on the parasite's morphology, mitochondrion membrane integrity, presence or absence of nuclear material and the shape of the parasite was examined. The cell cycle changes and the effect of the compounds on trypanosomes' gene expression were also analyzed. The data generated will help to understand the biology of the parasite with respect to iron deprivation and also provide more insight for alternative therapeutic approaches.

1.2 Problem statement

In the absence of an effective vaccine, chemotherapy based treatment for African trypanosomiasis seems to be the way out. However, drugs used in the treatment trypanosomiasis are limited. Only a few drugs are able to treat the chronic and acute forms of human trypanosomiasis while the current treatments for animal trypanosomiasis are not effective against all the trypanosome species (Mergia *et al.*, 2016). These drugs are toxic, have numerous side effects that are harmful to the host and there have also been reports of treatment failures (Giordani *et al.*, 2016). Hence the need to develop new anti-trypanosomal drugs which are safe, efficacious and inexpensive.

1.3 Justification

Iron is essential for most living organisms of which trypanosome is no exception. Compared to its host, only little amount of iron is required for the trypanosome survival hence depriving the parasite of iron through the use of chelators would inhibit its survival. Iron chelators from plant sources may also add to the group of potent anti-trypanosomals and further studies on the effect of iron chelators on the parasite's biology will provide information on therapeutic interventions. Iron deprivation may represent a new strategy for treatment of African trypanosomiasis.

1.4 Hypothesis

Depriving the parasite of iron through the use of iron chelators could affect the viability, morphology and cell cycle of the parasite as well as the gene expression of some iron dependent enzymes.

1.5 Aim

To determine the effects of iron chelators on bloodstream forms of *Trypanosoma brucei brucei* (*T. brucei brucei*) *in vitro*

1.6 Objectives

1. To determine the effect of iron chelators on the viability of the cell, the cell cycle and morphology of the bloodstream forms of *T. brucei brucei*.
2. To determine the effect of chelators on the expression levels of iron dependent enzymes: ribonucleotide reductase (RNR), superoxide dismutase and alternative oxidase using mRNA sequencing and Reverse Transcription quantitative Polymerase Chain Reaction PCR (RT-qPCR).
3. To determine the cytotoxic effect of iron chelators on murine macrophages cell lines

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 African Trypanosomiasis

African Trypanosomiasis (AT) is an infectious disease that affects humans, domestic and wild animals in sub-Saharan Africa and its transmitted by the tsetse fly (Mulatu *et al.*, 2016). Several trypanosome species including *Trypanosoma brucei* (*T. brucei*) is responsible for causing AT in both cattle and humans (Basu *et al.*, 2016). Human African Trypanosomiasis (HAT) can be chronic or acute depending on the type of infecting trypanosoma species. *T. brucei gambiense* causes the chronic form of sleeping sickness in West and Central Africa while *T. brucei rhodesiense* causes the acute form of the disease in East and Southern Africa, (WHO, 2017). About 60 million people are at risk with the disease (Brun *et al.*, 2010). There are two clinical stages of HAT. The first clinical stage is known as the hemo-lymphatic stage where the trypanosome resides in both the bloodstream and lymphatic system. This is accompanied by symptoms such as headaches, anemia general weakness (Brun *et al.*, 2010). The second or late stage is the meningo-encephalitic stage where the parasites move to the blood-brain barrier and affect the central nervous system leading to symptoms such as sleep disorders (Brun *et al.*, 2010; Franco *et al.*, 2014).

Animal African Trypanosomiasis (AAT) is one of the most important livestock related diseases in sub-Saharan Africa. *Trypanosoma brucei brucei* is the causative organism of AAT or nagana in cattle. About 55 million cattle are at risk with the disease leading to a loss of three million animals annually (Tihon *et al.*, 2017). Other species that cause nagana include *Trypanosoma congolense* (*T. congolense*) and *Trypanosoma vivax* (*T. vivax*). *T. brucei evansi* and *T. brucei equiperdum* are also subspecies of *T. brucei brucei* that cause animal trypanosomiasis in horses, camels, cattle,

buffalo and donkeys. (Auty *et al.*, 2015). AAT disease (if not treated) can lead to fever, anemia, infertility and eventually death hence having a great economic impact on animal production. Animals are also known to be good reservoirs of the human infectious species especially *T. b. rhodesiense* (Auty *et al.*, 2015; WHO, 2017). The *T. brucei brucei* are only pathogenic to animals and not humans. This is because the parasite is lysed by a component of the trypanosome lytic factors (TLF) in the human serum known as the Apolipoprotein L1 (APOL1) (Capewell *et al.*, 2014). *T. brucei rhodesiense* contains a serum resistance antigen (SRA) which is encoded by SRA gene. This protein binds to the APOL1 neutralizing its effect. *T. brucei gambiense* on the other hand does not have the SRA gene hence cannot encode for the SRA. One mechanism of resistance by *T. b. gambiense* is that, it reduces the expression of the haptoglobin-hemoglobin receptor, which required for TLF uptake hence conferring resistance.

2.2 Life cycle of *Trypanosoma brucei*

Transmission of the parasite starts when the mammalian host gets infected with the metacyclic trypomastigotes. The metacyclic trypomastigotes, located in the salivary glands of the vector are transferred to the bloodstream of the mammal through the bite of an infected tsetse fly during a blood meal. (CDC, 2015). In the bloodstream, the parasites undergo asexual replication by binary fission forming the long slender and short stumpy forms (Figure 1). This is also accompanied with the loss of their surface coats and changes in their metabolism. When the tsetse fly feeds on an infected mammal, the fly becomes infected with the bloodstream trypomastigotes which transform into the procyclic trypomastigotes through binary fission in the midgut of the fly (CDC, 2015). Upon leaving the midgut, the procyclic trypomastigotes are transformed into the epimastigotes

then into the metacyclic trypomastigotes. Then they enter the salivary gland of the vector (CDC, 2015) ready for another infection (Figure 1).

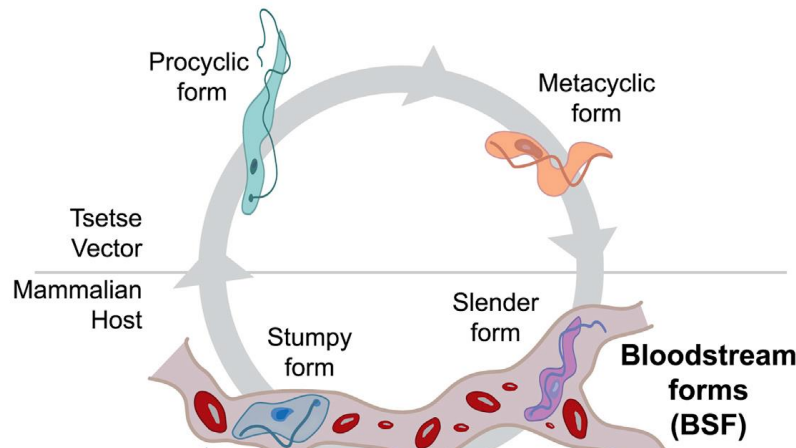


Figure 1: Life cycle of *Trypanosoma brucei* (Trindade *et al.*, 2016).

2.3 Control of African trypanosomiasis

Control of African trypanosomiasis is either by drug treatment, vector control or the use of trypanotolerant breeds (Bett *et al.*, 2010). Due to the antigenic variation exhibited by the parasites, there is currently no vaccine against trypanosomes hence the mode of treatment is mainly chemotherapeutic (Black and Mansfield, 2016; Ezeh *et al.*, 2016). The entire cell surface of trypanosome is surrounded by a surface coat of which majority is made up of the variant surface glycoprotein (VSG). These VSGs enable the parasite to escape immune responses mounted by the host. During an infection, the host produces antibodies such as the immunoglobulin M (IgM) and immunoglobulin G (IgG) which recognizes and binds to these surface coats causing a decrease in parasitemia (Brun *et al.*, 2010). Few trypanosomes however are able to encode for new set of VSGs, which are not immediately recognized by the host immune system, hence are able to multiply and continue the infection. This continuous cycle makes it difficult for the host immune

system to eliminate the parasites (Brun *et al.*, 2010). Trypanosomes contain about 2000 VSGs and only one VSG is expressed at a time (Horn and McCulloch, 2010). These VSGs are expressed from the gene expression sites together with other expression site associated genes (ESAGs) such as the transferrin receptor. The switch in VSG expression involves three mechanisms: gene conversion, telomeric conversion, segmental conversion or transcriptional switching (Aitcheson *et al.*, 2005) all of which lead to antigenic variation making vaccine development difficult (Black and Mansfield, 2016). Chemotherapy is currently the main treatment method used in controlling trypanosomiasis (Ezeh *et al.*, 2016).

Pentamidine is the first line treatment for the early-stage of *T. b. gambiense* infection and it is given intramuscularly. Treatment of the early stage of *T. b. rhodesiense* infection is by the use of suramin and this first-line treatment is administered intravenously (Adeyemi and Sulaiman, 2012; Kennedy, 2013). Both drugs, suramin and pentamidine, are also used as the second line treatment of *T. b. gambiense* and *T. b. rhodesiense* HAT respectively. Treatment for the late stage *T. b. rhodesiense* HAT is melarsoprol which is administered intravenously while melarsoprol, nifurtimox and eflornithine are used in the treatment of the late stage *T. b. gambiense* HAT (Denise and Barrett, 2001; Kennedy, 2013). The mechanism of action of suramin remains unclear, however, uptake of this drug by the trypanosome is through receptor-mediated endocytosis of bound low density lipoproteins (Denise and Barrett, 2001). Changes in this endocytic pathway have been associated with suramin resistance (Barrett *et al.*, 2011). Pentamidine is taken via the P2 amino- purine transporter or the high affinity pentamidine transporter (HAPT1) and loss of both transporters is necessary for pentamidine resistance (Barrett *et al.*, 2011). A recent study by Song *et al.* (2016) reported that *T. brucei* aquaglyceroporin 2 (TbAQP2) which also mediates the drug uptake through receptor-mediated endocytosis, is also implicated in the pentamidine and

melarsoprol cross resistance hence trypanosome without the P2 and HAPT1 transporters are still sensitive to pentamidine (Munday *et al.*, 2013; Song *et al.*, 2016). Melarsoprol is also taken via the P2 amino purine transporter and acts on the parasite's trypanothione, which is essential for prevention of oxidative stress (De Koning *et al.*, 2012; Kennedy, 2013; Beig *et al.*, 2015; Giordani *et al.*, 2016). Eflornithine diffuses through the plasma membrane or via the *T. brucei* amino acid transporter 6 (TbAAT6) hence a loss in the transporter is associated with its resistance (Baker *et al.*, 2011). The drug inhibits ornithine decarboxylase enzyme (ODC). The activity of nifurtimox is linked to the presence of the nitro group which produces reactive oxygen species upon reduction (Barrett *et al.*, 2011). It affects the activity of the trypanothione reductase, an enzyme needed in the trypanothione synthesis. Nifurtimox also used in the treatment of Chagas' disease (Kansiime *et al.*, 2018)

The most common drugs used in the treatment of AAT include diminazene aceturate and isometamidium chloride (Holmes *et al.* 2004; Adeyemi and Sulaiman, 2012). Diminazene aceturate also known as diminazen is an aromatic dimaidine joined by a tribenzene bridge (Figure 5C) and is one of the commonly used synthetic anti-trypanosomal drugs in livestock (da Silva Oliveira *et al.*, 2015; Giordani *et al.*, 2016). Apart from *T. brucei brucei*, it is also known to be effective against *T. congolense*, *T. vivax* and *T. brucei evansi* (Reid, 2002). In *T. brucei* the uptake of the diminazene aceturate is predominantly by an aminopurine transporter (De Koning *et al.*, 2012). Loss of this transporter confers resistance to the parasite (Denise and Barrett, 2001). Once the drug is inside the parasite, diminazene aceturate binds to the minor groove of the DNA located in the kinetoplast leading to an irreversible loss in the kinetoplast (Zuma *et al.*, 2015). Isometamidium chloride also has a similar mechanism of action to that of diminazene aceturate, where it also affects the mitochondrial DNA (Tihon *et al.*, 2017). The drug inhibits the activity of

the type II topoisomerase of the kinetoplast DNA (kDNA) leading to the loss of the kDNA and the subsequent death of the parasite (Chowdhury *et al.*, 2010). There have been numerous reports of drug resistance in most African countries making the treatment of this disease challenging (Tihon *et al.*, 2017).

Vector control is also another strategy used in controlling trypanosomes. One of the most efficient method is the use of insecticides which is used to reduce the tsetse fly population in an effected area. Other vector control strategies involve the removal of breeding sites or the disruption of their natural habitat (Esterhuizen, 2015). The use of trypanotolerant breeds such as N'Dama West African Longhorn and Dwarf West African Shorthorn also helps in controlling the disease (Berthier *et al.*, 2015).

2.4 Morphology of *Trypanosoma brucei*

In the mammalian bloodstream, trypanosomes exist as the proliferative long slender and the non-diving short stumpy forms (Matthews, 2005). The long slender bloodstream forms have a free flagellum attached to their long undulating membrane and also contain one mitochondrion. The size and activity the mitochondrion varies depending on the developmental stage of the parasite (Jakob *et al.*, 2016). The bloodstream forms of the parasite depend on glycolysis for its energy metabolism hence the mitochondrion activity is reduced and its size is also decreased. The activity of the mitochondrion in the insect form of the parasite (procyclics) is high since its relies solely on the mitochondrion for its energy requirements (Jakob *et al.*, 2016). Other essential organelles include the nucleus, endosomes, lysosomes, Golgi bodies and the endoplasmic reticulum which are functionally related to most eukaryotic organelles.

Trypanosomes also contain other unique features which makes their biology interesting. The kinetoplast is the DNA of the mitochondrion and this is located close to the posterior part of the cell (Matthews, 2005). The genome is made up of an unusual network of circular DNA known as the mini and maxi circles which encodes various mitochondrion proteins (Simpson *et al.*, 2000).

The mini circles encode for the guide RNA which is used during RNA editing while the maxi circles encode for the genes responsible for the respiratory chains as well as ribosomal RNA (Jakob *et al.*, 2016). The transcripts from the maxi circles undergo post transcriptional processing via the RNA editing process (Read *et al.*, 2016). This process leads to insertion or deletion of uridine on the transcripts generating a functional mRNA (Read *et al.*, 2016). The procyclic forms of the parasite are not able to survive when the kinetoplast is absent. However, bloodstream forms of other species of the parasite can still survive even in the absence of the kinetoplast, though other studies have also highlighted the importance of the kinetoplast to the survival of the bloodstream forms (Naula *et al.*, 2003). The kinetoplast serves as a potent drug target since it is not present in the mammalian host. The flagellum constitutes 9+2 microtubule axoneme together with a para flagella rod (Broadhead *et al.*, 2006). It is needed by the parasite for its motility, viability, cytokinesis and vesicular transport (Kohl *et al.*, 2003; Broadhead *et al.*, 2006). The flagellum is also necessary for disease transmission and immune evasion (Langousis and Hill, 2014). Another unique feature of the trypanosome is the compartmentalization of most of its glycolytic enzymes in the glycosome. The glycosomes are essential to the parasite's survival and have been studied as possible drug targets (Bauer and Morris, 2017) (Figure 2).

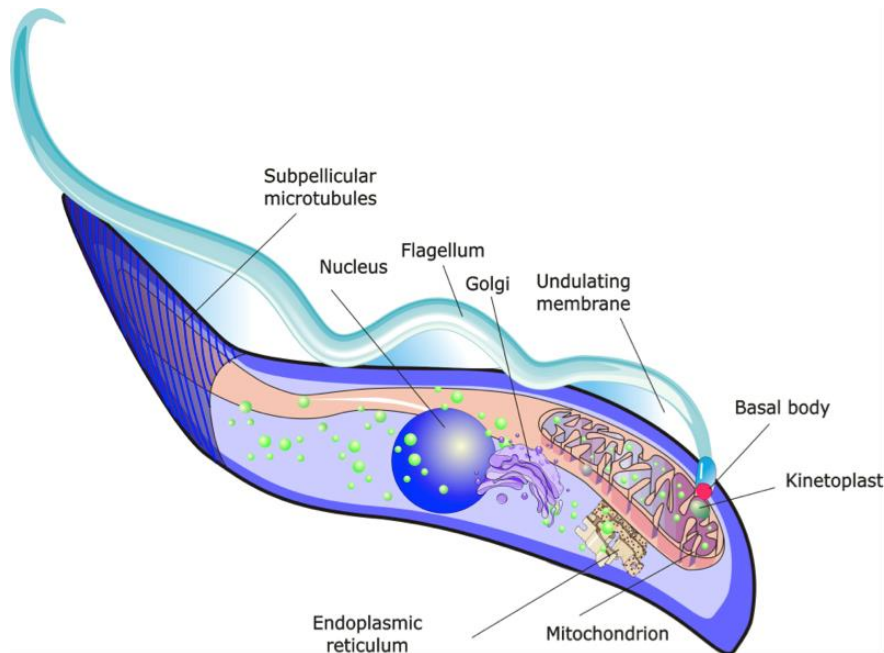


Figure 2: Morphology of *Trypanosoma brucei* (<https://sites.dundee.ac.uk/ian-gilbert/ian-gilbert-research>)

2.5 Cell cycle of *Trypanosoma brucei*

The kinetoplast, nucleus and flagellum are essential organelles that play important part in the cell cycle of the parasite. The cell cycle of trypanosome is highly regulated to ensure that each daughter cell has the equal number of organelles. The cycle is made up of the G1 (Gap 1), S (Synthesis), G2 (Gap 2) and M (Mitosis) phases. The G1 phase is first gap phase in the cell cycle where the cell gets ready for DNA synthesis. At the S phase there is DNA replication and the kinetoplast begins to divide. The G2 phase is the second gap phase where the cell prepares to undergo mitosis and cytokinesis. In the G1 phase, cells contain 1 nucleus and 1 kinetoplast denoted as 1N1K and contains one flagellum (Figure 3). Cells in the G2/M phase have 1 nucleus and 2 kinetoplasts denoted as 1N2K and the mitotic cells contain 2 nuclei 2 kinetoplasts denoted as 2N2K as well as two flagella. (Jones *et al.*, 2014); an abnormal cell will have a different configuration of the cell organelles. All the other organelles equally divide before the final cell division process. The cell

cycle process of trypanosome is a highly regulated process and involves cyclins (CYC) and cyclin dependent kinases (CDK). These proteins promote the progression from one phase to the other. So far 8 cyclin proteins have been identified ranging from CYC2-CYC9 (McKean *et al.*, 2003).

CYC2 aids in the G1/S cell cycle transition while mitosis is initiated by CYC8 (Li and Wang, 2003). The S phase is initiated by CYC4 while CYC6 is involved in G2/M transition (Li, 2012).

CYC3, CYC5 and CYC7 have no effect on the cell cycle progression and function of CYC9 is still not known (Li and Wang, 2003). A knockdown of these genes or an overexpression have been shown to interfere with the cell cycle of the parasite (Li, 2012).

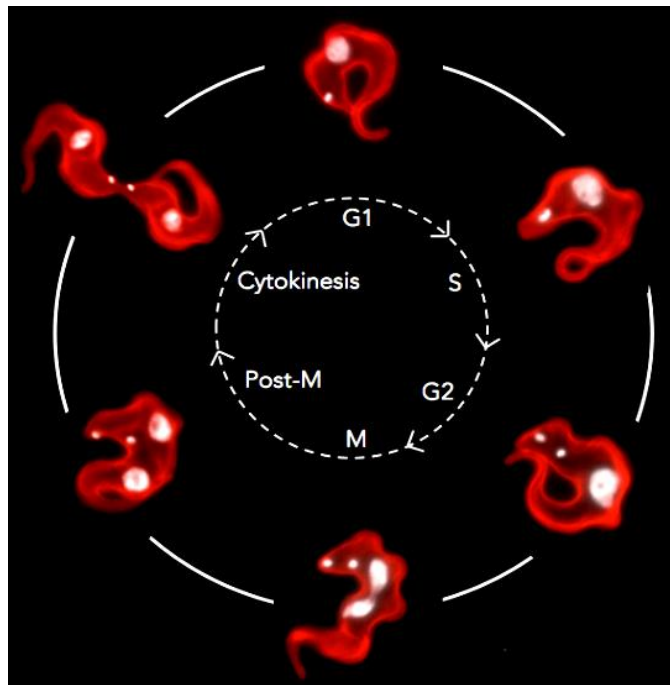


Figure 3: Cell cycle of trypanosome
(<https://research.pasteur.fr/en/team/trypanosomes-molecular-biology/>)

2.6 Gene Expression and regulation in *Trypanosoma brucei*

The process of gene expression in trypanosome is unique due to its polysitronic transcription and trans splicing (Michaeli, 2011; Clayton, 2012). Individual mRNAs generated are processed from mRNA precursors by a process involving 5' trans splicing and 3' polyadenylation. (Clayton, 2014). In trans splicing, a capped splice leader (SL) sequence (39 nucleotides long) is attached to the 5' end of the mRNA (Michaeli, 2011). This protects the transcript from the activity of ribonucleases. During 3' polyadenylation, there is the removal of the 3' end and the subsequent addition of poly adenosine to form the polyA tail. This also protects the 3' end from degradation by ribonucleases. These processes lead to the generation of matured mRNA. The mRNA precursors can also be degraded hence the balance between the precursor degradation and the mRNA processing is necessary to produce the required amount of mRNA needed (Clayton, 2014). The stability degradation and/or translation of the mRNA involves the use of RNA binding proteins which interacts with the 3' untranslated regions of the transcript (Gomez *et al.*, 2010). The mRNA is exported from the nucleus to the cytosol for translation. In the cytosol, the mRNA is translated through the binding of the translation initiation factors at the 5' end and the subsequent binding of the poly A binding proteins (PABP) at the 3' end of the transcript. (Clayton, 2016). The regulation of these genes is controlled at the post transcriptional level and this involves the control of mRNA processing, polyadenylation, mRNA degradation in the nucleus, nuclear export and mRNA degradation in the cytoplasm (Clayton, 2014)

2.7 Iron metabolism in *Trypanosoma brucei*

Iron is essential for the survival of trypanosomes (Manta *et al.*, 2012). Even though it is required in small quantities, the bloodstream forms of the parasite have employed ways to ensure adequate

iron uptake from its host which is used in cellular processes such as DNA synthesis and energy production (Hatcher *et al.*, 2009; Manta *et al.*, 2012). The low iron requirement by the parasite is due to the absence of cytochromes and the presence of few iron dependent enzymes (Steverding, 1997; Stijlemans *et al.*, 2015). The parasite has unique transferrin receptors located at the flagella pocket which are used to internalize holo transferrin (iron loaded transferrin) from the host (Manta *et al.*, 2012). The internalized transferrin is then degraded in the late endosome and through the action of the ferric reductase and a divalent cation transporter, the iron is released into the cytosol while the transferrin receptor is shuttled back to the flagella pocket (Figure 4). The iron then aids in the activity of iron dependent enzymes (Breidbach, 2002; Stijlemans *et al.*, 2015). The parasite also obtains iron through the host's lactoferrin, a glycoprotein similar to transferrin that also binds iron. Iron loaded lactoferrin interacts with the parasite's glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is the receptor for lactoferrin binding and the iron is released for its survival (Basu *et al.*, 2016). Iron can also be obtained through the uptake of heme via the haptoglobin-hemoglobin receptor (HpHbR). This receptor is located on the flagella pocket of bloodstream forms (Stijlemans *et al.*, 2015) and is also responsible for the uptake of APOL1 which makes the *T. b. brucei* unable to infect humans.

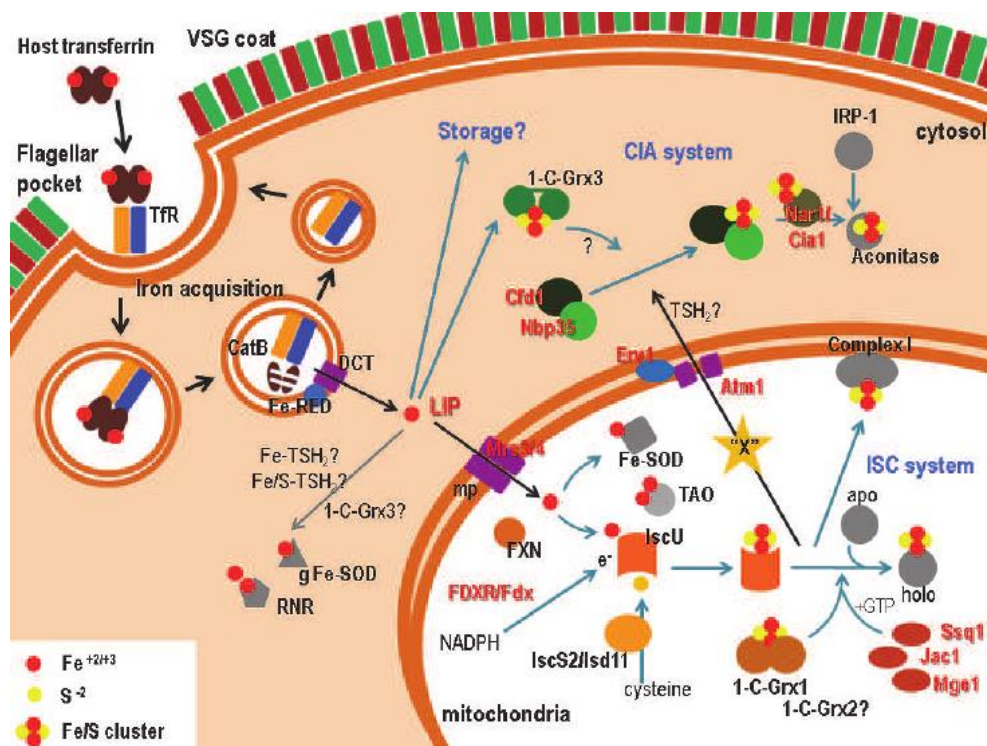


Figure 4: Iron metabolism in *T. brucei* (Manta *et al.*, 2012)

2.8 Transferrin receptor

Bloodstream forms of trypanosome obtain most of their iron requirement from the host transferrin, thereby making transferrin an indispensable growth factor for the parasite (Stijlemans *et al.*, 2015). The heterodimeric transferrin receptor which is encoded by two expression-site-associated genes (ESAGs), ESAG6 and ESAG7 (Beate *et al.*, 1999), is attached to the cell membrane by a glycosylphosphatidylinositol (GPI) lipid. ESAG6 and ESAG7 are homologous expression site associated genes located upstream of the VSG gene (Steverding, 2000; Isobe *et al.*, 2003). The expression and regulation of the receptor is affected by iron availability. There is a 3 to 10-fold increase in the expression levels of the receptor under iron starved conditions such as in the presence of a chelators or absence of serum (Beate *et al.*, 1999; Mussmann *et al.*, 2004; Stijlemans *et al.*, 2015). The increase in expression makes the parasite compete for the limited iron available

making the receptor a useful tool in iron scavenging. The regulation of the transferrin receptor in mammalian cells is via the interaction between the iron regulatory proteins (IRP) and the 3'-UTR of the transferrin receptor transcript. In an iron deprived environment, IRP binds to the 3' of the RNA increasing the stability of the transcript hence a relative increase in the expression of the receptor (Beate *et al.*, 1999; Bergeron *et al.*, 2014). In the case of trypanosomes, the expression of the transferrin receptor is highly dependent on the post transcriptional regulation of the ESAG6 transcript which does not involve the use of IRPs (Beate *et al.*, 1999). The transferrin ligand varies from one mammalian host to another hence trypanosomes have adapted a strategy to obtain transferrin across large range of mammalian host (Beate *et al.*, 1999). The ESAG6 and ESAG7 are part of the 20 VSG expression sites, of which one is expressed at a time (Isobe *et al.*, 2003). Switching between these expression sites leads to differences in the VSG produced as well as the ESAG. With this mechanism, the trypanosome can produce varieties of transferrin receptors to bind different transferrin depending on the mammalian host (Ansorge *et al.*, 1999; Stijlemans *et al.*, 2015).

2.9 Iron dependent enzymes

2.9.1 Trypanosome alternative oxidase

Trypanosome alternative oxidase (TAO) which requires iron as a cofactor. The 33 kDa mitochondrial enzyme, functions by reducing oxygen to water (Chaudhuri, 2006). TAO is similar in size to the alternative oxidase proteins found in other living organisms such as fungi (Chaudhuri, 2006). The expression of the single copy TAO gene (Menzies, 2016) has been shown to be regulated at the transcript level and is more abundant in the bloodstream forms compared to the procyclic forms (Chaudhuri *et al.*, 2002). Due to the lack of cytochromes, trypanosomes depend on

the alternative oxidase for respiration. TAO helps in reducing apoptosis which is a very controlled cellular process. Studies using iron chelators have established the importance of iron in the activity of this enzyme where parasites defective in this enzyme become susceptible to oxidative-induced apoptosis (Ajayi *et al.*, 2001)

2.9.2 Iron superoxide dismutase

There are four different isoforms of the superoxide dismutase (SOD) expressed in trypanosomes (Dufernez *et al.*, 2006). This metalloenzyme protects the cells from reactive oxygen species (ROS) induced cell damage. (Ezzat Abd El-Hack *et al.*, 2016). These ROS such as superoxide are produced during aerobic metabolism or some host immune responses. The SODs converts the excess superoxide anion to hydrogen peroxide and oxygen which is less toxic (Wilkinson *et al.*, 2006). Unlike other microorganisms, all the isoforms of the trypanosome SOD are iron dependent (Bachega *et al.*, 2009) and are found in different organelles. SOD-A and SOD-C are located in the mitochondrion while the glycosomes and cytosol contains the SOD-B1 and SOD-B2 isoforms (Dufernez *et al.*, 2006). Parasites deficient in any of the isoforms have been shown to have increased drug sensitivity as well as exhibiting susceptibility to death stimulated oxidative stress (Prathalingham *et al.*, 2007).

2.9.3 Ribonucleotide reductase

Ribonucleotide reductase (RNR) is an enzyme that reduces ribonucleotides to deoxyribonucleotide in the DNA synthesis process during cell proliferation (Heli *et al.*, 2011; Manta *et al.*, 2012). Based on their oxygen interaction and radical generation, RNR have been grouped into three classes (Class I, II, and III) (Nordlund and Reichard, 2006). Most eukaryotes

contain class I RNR made up of the large subunit R1 and the small R2 subunit. The R1 is for allosteric regulation while the R2 is mainly involved in catalysis (Breidbach *et al.*, 2002; Nordlund and Reichard, 2006). The catalytic subunit is not expressed in the non-dividing short stumpy form of the parasite (Breidbach *et al.*, 2000). The importance of this enzyme was emphasized when parasites treated with iron chelator deferoxamine showed a reduction in DNA synthesis (Breidbach *et al.*, 2002).

2.9.4 Aconitase

Aconitase is an important enzyme in the Krebs cycle which is necessary for energy release. The presence of an iron sulfur cluster is required for the activity aconitase. This cluster binds to the catalytic site of the enzyme and catalyzes the conversion of citrate to isocitrate in the Krebs cycle (Grazina *et al.*, 2013). The enzyme is highly expressed in the short stumpy bloodstream forms as compared to the long slender forms (Saas *et al.*, 2000). Since the aconitase is necessary for differentiation into the procyclic form, the high expression is a pre-adaptation strategy for transmission into the tsetse fly. The link between aconitase and iron, is related to the iron regulation proteins in mammals which acts as an iron sensor (Saas *et al.*, 2000). However, upon a double knockout of aconitase gene in *T. brucei*, there was no change in the regulation of iron uptake (Beate *et al.*, 1999).

2.10 Iron chelation therapy

Irons have two oxidative states, the ferric ion (Fe^{3+}) and ferrous ion (Fe^{2+}). Depending on the pH, the ferric ion can be converted into the ferrous ion (Fazary *et al.*, 2008). The role of iron has been implicated in the development of various diseases including malaria, cancer, neurogenerative

disorders, bacterial and fungal infections (Liu and Hider, 2002; Qiu, *et al.*, 2010; Heli *et al.*, 2011). An example of iron chelation therapy is the current use of deferoxamine mesylate, in the treatment of iron overload diseases (Heli *et al.*, 2011). Trypanosomes are of no exception as studies have shown the anti-trypanosomal activity of different iron chelators against *T. b. brucei* and *T. congolense* (Merschjohann and Steverding, 2006). These iron chelators exhibited toxicity against mammalian cell lines such as human promyelocytic leukemia cells (Merschjohann, and Steverding, 2006; Breidbach *et al.*, 2002), but as compared to the mammalian cells, bloodstream forms of the parasite were more sensitive to the chelators (Breidbach *et al.*, (2002). These studies have implications in iron chelation therapy by providing new therapeutic potentials in the treatment of trypanosomiasis (Stijlemans *et al.*, 2015; Merschjohann, and Steverding, 2006).

2.11 Sources of iron chelators

2.11.1 Siderophores

Iron chelators can be derived from bacteria and fungi (siderophores), plants (phytosiderophores) or can also be synthetically produced. Deferoxamine mesylate (Desferal®, desferrioxamine) is one of the iron chelators derived from bacteria sources (*Streptomyces pilosus*). Even though it is mostly used in the treatment of iron overload diseases, it also acts as an antioxidant and helps to balance inflammation (Arantes *et al.*, 2011). Numerous studies have shown the antimicrobial effects of deferoxamine both *in vitro* and *in vivo* (Heli *et al.*, 2011; Thompson *et al.*, 2012; Jayasinghe *et al.*, 2015). Deferoxamine inhibits the growth of bloodstream forms of *Trypanosoma brucei* (Briedbach *et al.*, 2002), *Trypanosoma congolense* (Merschjohann and Steverding, 2006), *Toxoplasma gondii* (Mahmoud, 1999) and *Plasmodium falciparum* (Jayasinghe *et al.*, 2015). It acts as an intracellular iron chelator by binding ferric ions (Breidbach *et al.*, 2002). Iron has six coordinating sites and

iron chelators are grouped based on their ability to bind to these sites. A hexadentate chelator can bind to all the six sites of the iron forming the most stable iron complex. Deferoxamine is an example of a hydroxamate hexadentate iron chelator (Valdés *et al.*, 2010). Chelators which bind to two or three sites are referred to as bidentate and tridentate respectively (Hatcher *et al.*, 2009). Desferrithiocin is an orally effective tridentate iron chelator obtained from *Streptomyces pilosus* (Hatcher *et al.*, 2009). It has been shown to inhibit proliferation in cancer cell lines making it a good anti-cancer agent (Kicic *et al.*, 2001).

2.11.2 Synthetic chelators

Ethylenediamine di-o-hydroxyphenylacetic acid (EDDA) is a hexadentate iron chelator similar to Ethylenediaminetetraacetic acid (EDTA). It is active against fungal species such as *Cryptococcus neoformans* (Santos *et al.*, 2012) but weakly active against trypanosomes (Merschjohann and Steverding, 2006). Other synthetic chelators include 8-hydroxyquinoline and dimethylglyoxime which have different anti-trypanosomal properties.

2.11.3 Food and plant derived chelators- phenolic compounds

Polyphenols and flavonoids are one of the most abundant secondary metabolites found in plants (Andjelković *et al.*, 2006). They are not just good antioxidants but have potent iron chelating properties (Jayasinghe *et al.*, 2015). Phenolic compounds (a class of polyphenols) are present in most foods. The structures of these phenolic compounds includes a benzene ring with at least one hydroxyl group attached to the carbon atoms. Factors that affect the antioxidant and iron chelating properties of these phenolic compounds include: the number of the hydroxy groups, the position of the hydroxyl groups on the benzene ring, the binding sites and the kind of substituents attached to the ring (Andjelković *et al.*, 2006).

Phenolic acids (a class of phenolic compounds) are divided into hydroxybenzoic acids and the hydroxycinnamic acids and derivatives. The hydroxybenzoic acids are further divided into compounds with the catechol group such as protocatechuic acid and the galloyl group in the case of gallic acid. The catechol groups contain two hydroxyl (OH) moieties while the galloyl groups contain three (Figure 5A). These groups affect the iron binding ability of the compounds (Hynes and O’Coinceanainn, 2004). The hydroxy groups are deprotonated at physiological pH of 7.4 leading to iron binding ability. The hydroxycinnamic acid is a benzene ring attached to propanoic acid. The presence of an additional hydroxy group such as caffeic acid and methoxy group in the case of ferulic acid have an influence on their iron binding abilities (Andjelković *et al.*, 2006).

2.11.3.1 Hydroxybenzoic acids

(A) Gallic acid (GA)

Gallic acid is a 3,4,5-trihydroxybenzoic acid which contains a galloyl group. They are found in plant products which includes grapes, green tea and pineapple (Nowak *et al.*, 2014; He *et al.*, 2016; Sourani *et al.*, 2016). Gallic acid has anti-bacterial and anti-viral properties as well as anti-cancer activity (He *et al.*, 2016). Gallic acid also inhibits the growth of *Trypanosoma brucei in vitro* (Koide *et al.*, 1998). The metal chelation and antioxidant properties of gallic acid is associated with the presence of its galloyl moiety which contains at least two adjacent hydroxy groups. (Fazary *et al.*, 2008; Chuang *et al.*, 2010; Badhani *et al.*, 2015).

(B) Protocatechuic acid (PCA)

Protocatechuic acid (PCA) is a 3,4-dihydroxybenzoic with a catechol group. This natural phenolic acid is found in grapes and brown rice (Tanaka *et al.*, 2011; Kakkar and Bais, 2014). The pharmacological properties of protocatechuic acid cannot be overlooked. With structures similar

to gallic acid, it has also anti-tumor, anti-bacterial and anti-inflammatory properties (Kakkar and Bais, 2014; Semaming *et al.*, 2015). It is also a well-known antioxidant (Psotová *et al.*, 2003). PCA contains two adjacent hydroxyl groups which are responsible for iron binding but unlike gallic acid, it binds iron to a lesser extent (Andjelković *et al.*, 2006) and this could be attributed to the reduced number of hydroxy groups compared to gallic acid (Figure 5A).

(C) *2,3-Dihydroxybenzoic acid (DHBA)*

This is a phenolic acid found in some fruits and aquatic plants. It bears a catechol group which is responsible for iron binding. It has a structure similar to protocatechuic acid with a difference in the position of one hydroxyl group. This phenolic acid is a poor iron chelator and has weak anti-trypanosomal activity (Merschjohann, and Steverding, 2006).

2.11.3.2 Hydroxycinnamic acid and derivatives

(A) *Caffeic acid (CA)*

The 3,4-dihydroxy-cinnamic acid is mostly found in coffee (Shi *et al.*, 2003). It occurs in nature as an ester of chlorogenic and quinic acid (Olthof *et al.*, 2001) (Figure 5B). Its iron binding property is linked to the presence of the two hydroxy groups on the benzene ring and like most phenolic acids, caffeic acid is a good antioxidant through its iron chelation mechanism. (Genaro-Mattos *et al.*, 2015) hence preventing damages caused by reactive oxygen species (Jayasinghe *et al.*, 2015).

(B) *Ferulic acid (FA)*

Ferulic acid is a 4-hydroxy-3-methoxycinnamic acid, a phenolic acid commonly found in grains such as wheat, rice and oats. It is also found in some beverages, fruits and vegetables (Kumar and

Pruthi, 2014). This phenolic acid is effective against both Gram negative and positive bacteria as well as fungal species including yeast (Maurya and Devasagayam, 2010). Ferulic acid has moderate activity against *Leishmania donovani* and weak activity against *T. b. rhodesiense* (Tasdemir *et al.*, 2006). The antioxidant and prooxidant properties makes the chelator a good anti-cancer agent. The only difference between ferulic acid and caffeic acid is the substitution of the 3-hydroxy with a methoxy group leaving it with just one hydroxy group on the benzene ring (Figure 5B). This influences the iron binding ability (Tasdemir *et al.*, 2006)

(C) *Chlorogenic acid (CGA)*

Chlorogenic acid is an ester of caffeic and quinic acid (3-caffeoyl-D-quinic acid) and a precursor of ferulic acid, which is abundant in coffee. Chlorogenic acid acts as an antioxidant by forming complexes with iron by the use of its hydroxy moieties (Olthof *et al.*, 2001; Andjelković *et al.*, 2006).

(D) *Rosmarinic acid (RA)*

The ester of caffeic acid and 3-(3,4-dihydroxyphenyl) lactic acid is one of the important polyphenols found mostly in Lamiaceae herbs like *Perilla frutescens*. It has anti-microbial and anti-tumor activities (Alagawany and Abd El-Hack, 2015). Rosmarinic acid contains four hydroxyl groups two of which is located on both benzene rings (Figure 5B). This makes it a good iron chelator and recommended for the treatment of iron related diseases (Wang *et al.*, 2012).

(E) *Quinic acid (QA):*

This cyclohexane carboxylic acid is made by the hydrolysis of chlorogenic acid. It found in *Moringa oleifera* flowers and is implicated in the treatment of prostate cancer (Inbathamizh and

Padmini, 2013). Quinic acid lacks the benzene ring making it a poor iron chelator (Barton, 2012)

(Figure 5C)

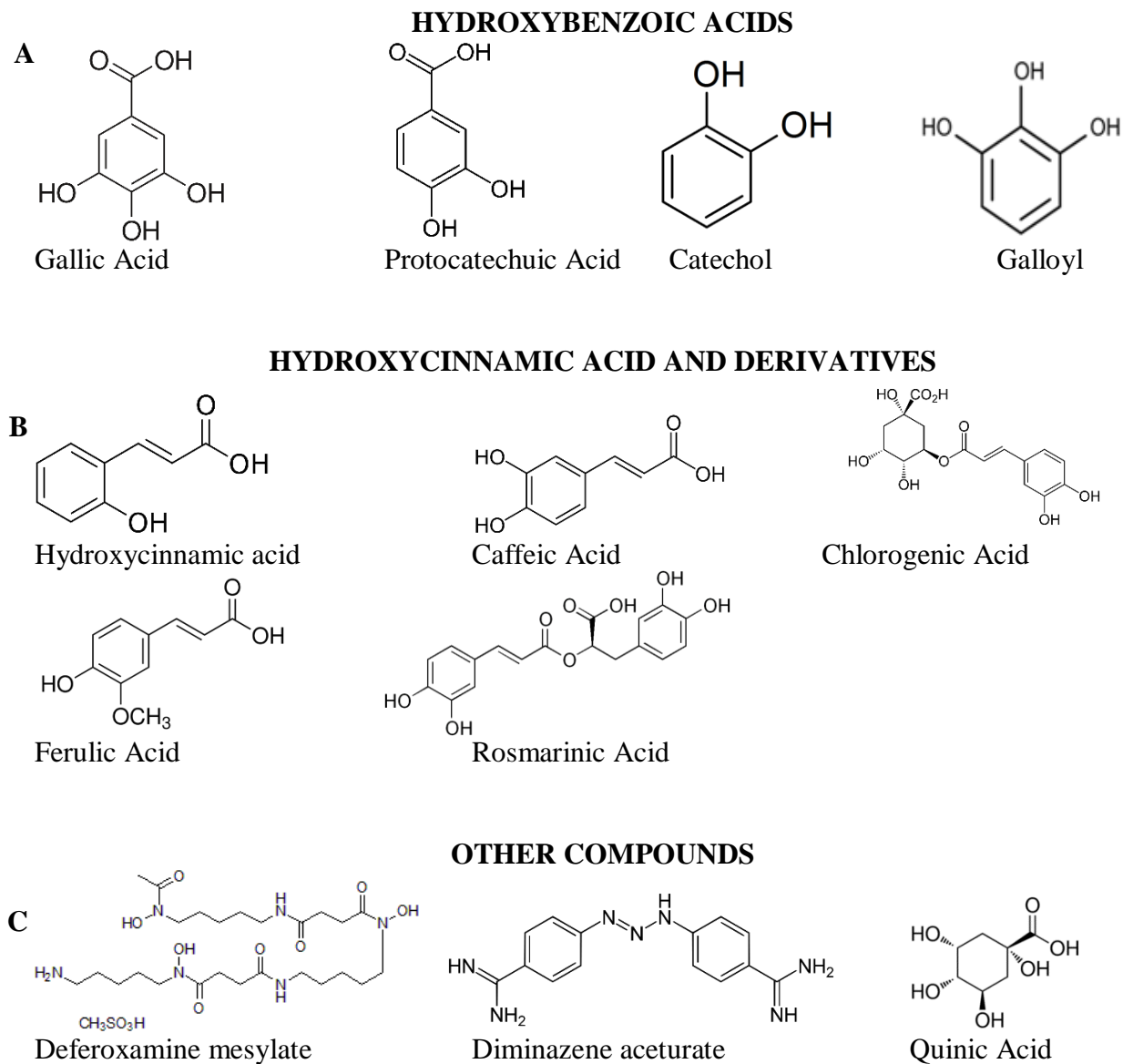


Figure 5: Structures of test compounds used in this study

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Trypanosome strains and culture

Bloodstream forms of *T. brucei brucei* GuTat 3.1 and AnTat 1313 cell lines were used. The cells were cultured in HMI-9 media supplemented with 10% FBS, L-cysteine, β -mercaptoethanol and 100x streptomycin/penicillin. The cell cultures were grown at 37 °C in a humidified atmosphere containing 5% CO₂. AnTat 1313 cell lines were used for the northern blot analysis and RNA sequencing.

3.1 Test compounds

All test compounds used (gallic acid, protocatechuic acid, caffeic acid, ferulic acid, rosmarinic acid, chlorogenic acid, quinic acid, deferoxamine mesylate and diminazene aceturate) were obtained from Sigma-Aldrich (Germany). Diminazene aceturate and deferoxamine were used as a positive controls. Stock solutions of all compounds were made up in dimethyl sulfoxide (DMSO) and working solutions in sterile water.

3.2 Drug sensitivity test using alamarBlue assay

Drug sensitivity test of the compounds against bloodstream forms of *T. brucei brucei* was performed using the alamarBlue assay. Compounds were serially diluted in a flat bottom 96 well plate (Costar®) with HMI-9 medium. Trypanosomes were cultured overnight to a density of 1×10^6 cells/ml and a trypanosome cell suspension (100 μ l) was added to the plates to give a final cell density of 4000 cells/ml. The plates were incubated for 72 hours at 37°C in 5% CO₂. After 5 hours, a final concentration of 44 μ M of resazurin sodium salt (Sigma-Aldrich, Germany) in phosphate

buffered saline was added to each well and the absorbance measured at 570 nm using the Varioskan Lux Elisa plate reader (ThermoFisher Scientific, USA). Data was analyzed using Graphpad prism software and IC₅₀ values (Inhibitory Concentration (50%)) were determined. The IC₅₀ reported for the active compounds are the averages from three independent experiments.

3.3 Analysis of parasite growth

Cells were seeded at an initial density of 1.0×10^5 cells/ml, counted and passed every 24 hours for a period of 5 days in the presence of different concentrations of the deferoxamine, gallic acid and diminazene aceturate. A cumulative growth curve was plotted using Microsoft excel.

3.4 Morphological analysis

3.4.1 DAPI staining

Approximately 5×10^6 cells were harvested by centrifuging at 2700 rpm for 8 minutes. The pelleted cells were washed with Voorhei's modified PBS (vPBS) centrifuged and resuspended in 1 ml vPBS. One milliliter (1 ml) of 8% paraformaldehyde was added and incubated for 10 minutes. An additional 20 μ l volume of 10% Triton X-100 was added to cell suspension and incubated further for 10 minutes. Twelve milliliters (12 ml) of PBS was added and recentrifuged at 2700 rpm for 8 minutes. The pellet was resuspended in 150 μ l of PBS of which 50 μ l was spread on polysine coated slides for 1 hour. The slides were washed twice for 5 minutes and 100 μ l of 1 μ g/ml DAPI (4,6-Diamidino-2-phenylindole dihydrochloride) was added and incubated for 10 minutes. The slides were washed again in PBS for 5 minutes after which 10 μ l of mounting media was added and covered with a cover slip and sealed with nail polish. The nucleus and kinetoplast was examined with the Olympus Fluorescent microscope (100x magnification).

3.4.2 Mitotracker staining

A total of 5×10^6 cells of trypanosome cultures were harvested and re-suspended in 1 ml serum free HMI-9/1% BSA. Ten microliters (10 μ l) of MitoTracker Red CMXRos® (ThermoFisher Scientific, USA) was added to give a final concentration of 100 nM and incubated for 30 minutes. Cells were pelleted and resuspended in 1 ml serum free HMI-9/1% BSA and incubated for another 30 minutes. Pelleted cells were suspended in a mixture of 0.5 ml vPBS and 0.5 ml 6% paraformaldehyde and incubated at 4°C for 1 hour. Cells were washed and resuspended in PBS and 50 μ l was spread on a polysine coated slide, allowed to air dry and DAPI stain (1 μ g/ml) was added. The slides were washed in PBS for 5 minutes, 10 μ l of mounting media was added, covered with a cover slip and sealed with nail polish. The mitochondrion was examined using the Olympus Fluorescent microscope 100x magnification.

3.5 Flow cytometry analysis of the cell cycle

About 5×10^6 cells were harvested after 24 hours of treatment with the test compounds and centrifuged at 2700 rpm, washed twice with PBS and fixed in 70% cold ethanol at -20 °C overnight. The cells were then washed twice in PBS and 200 μ l of guava cell cycle reagent was added and incubated in the dark for 30 minutes. Cell cycle analysis was done using the LSRFortessa™ X-20 flow and FlowJo v10 software. At least 5000 cells were counted for each measurement.

3.6 Northern blot analysis and RNA sequencing

3.6.1 RNA extraction

Total RNA from approximately 5×10^7 cells for treated and untreated samples were extracted using the phenol-chloroform RNA extraction method. Cells were lysed by the addition of 1 ml TriFast reagent (peqlab, GMBH) and incubated on ice for 5 minutes. Two hundred microliters (200 μ l) of chloroform was added, shook vigorously for 5 minutes and incubated for 3 minutes. The cells were centrifuged at 1,000 rpm for 10 minutes at 4 °C. The aqueous phase (clear upper layer) was transferred to an RNase free tube containing 7 μ l of glycogen. The RNA was precipitated by adding 500 μ l of isopropanol, mixed briefly by vortexing and incubated on ice for 10 minutes. The samples were centrifuged at 12,000 rpm for 15 minutes at 4 °C and the supernatant was carefully removed. The pellet obtained was washed by the addition of 1 ml of 75% ethanol, mixed briefly and centrifuged at 10,000 rpm for 5 minutes. After drying, the RNA pellet was dissolved in 50 μ l of sterile water and the concentrations determined using the NanoDrop.

3.6.2 RNase H based rRNA depletion of total RNA

Reaction tubes were set up containing 5X hybridization buffer, anti-rRNA oligo mix, 5 μ g of total RNA and top up to 5 μ l with sterile water. The reactions were hybridized using varying temperatures as follows: Initial temperature at 95 °C for 2 minutes followed by a gradual decrease in temperature to 37 °C for 2 minutes (0.1 °C/sec decrease of temperature from 95 °C). At 37 °C the reaction was paused and 2X prewarmed RNase H mix and RNase H was added and was continued at 37 °C for 20 minutes and stored at 4 °C. DNase treatment was done by adding DNase I mix and incubated at 37 °C for 20 minutes. The RNA was cleaned up and concentrated using the

RNA purification kit (ZYMO Research, USA) following the manufacturer's protocol and the eluted RNA was quantified using the NanoDrop.

3.6.3 Northern Blot analysis

A northern blot analysis was done to confirm the rRNA depletion and to check the integrity of the RNA prior to sequencing. In designing and labeling the probe, beta tubulin gene was PCR amplified with gene specific primers. The PCR product was extracted from the gel and radioactively labelled using the Random Primer Labelling Kit, (Stratagene) following the manufacturer's protocol. The probes were labelled with α -³²P-dCTP DNA radioactive probe. The extracted RNA was loaded onto a 1.6% formaldehyde agarose gel and run at 60 V for 4 hours. The gel was transferred overnight to Nytran membrane (GE Healthcare), stained with methylene and cross linked using the UV-cross linker. The membranes were washed in 2X SSC and prehybridized in 10 ml of northern hybridization solution for 1 hour at 65 °C. The probe was denatured for 5 minutes at 95 °C and placed back on ice for 2 minutes. The probe was then added carefully into the hybridization solution and hybridized overnight at 65°C. The blot was rinsed with 2X SSC/0.1% SDS and washed for 15 minutes with 2X SSC/0.1% SDS and further washed for 10 minutes at 65 °C. The blot was wrapped in a cling film and placed in a cassette. The radioactivity was detected using the PhosphorImager (Fuji, FLA7000)

3.6.4 Illumina sequencing and bioinformatics

Illumina RNA-seq was performed at the CellNetworks Deep Sequencing Core Facility at the University of Heidelberg and the final read count generated using the TrypRNAseq pipeline (<https://github.com/klprint/TrypRNAseq>). Briefly, FastQC and *T. brucei* cutadapt were used to

check the quality of the raw sequences generated and to remove sequencing primers respectively. Bowtie2 was used to align the data generated to the TREU97 reference genome indexed using samtools. Python scripts was used to generate the read counts and gene annotations were manually inserted using the TritypDB. Correlation between the biological replicates were assessed using the Pearson R correlation coefficient.

3.7 Gene expression analysis using RT q-PCR

Both treated and untreated cells were harvested and total RNA was extracted using the ZymoQuick-RNA MiniPrep Plus Mini kit according to the manufacturer's instructions. RT-qPCR experiment was conducted using the Luna Universal One-Step RT-qPCRkit (New England Biolabs) following the manufacturer's instructions. Data generated was analyzed using the Quant Studio™ 3 and 5 Real-Time PCR Systems (ThermoFisher Scientific, Singapore). Primers used targeted cyclin 2 gene, ribonucleotide reductase and transferrin receptor genes. Histone H2A was used as the endogenous control.

3.8 Iron content analysis

3.8.1 Protein reagent preparation and protein assay

The protein reagent was prepared as described by Grintzalis *et al.* (2015). Coomassie Brilliant Blue G-250 (Sigma-Aldrich, Germany) was dissolved in 100 ml of 2 N HCl by stirring for 40 minutes. The residues were removed and filter sterilized. The reagent was diluted into a 1:1 molar ratio before use. One milligram per milliliter (1mg/ml) of bovine serum albumin (BSA) was prepared and diluted into different concentrations (1-40) µg BSA/ml and these concentrations were used to plot the standard curve. Two hundred microliters (200 µl) of the protein concentrations

were aliquoted into a microtiter plate (Costar®) and mixed with 50 µl of the protein reagent. A reagent blank (200 µl of sterile water) was used. The set-up was incubated at room temperature for 5-10 minutes. The absorbances were read at 610 nm and 470 nm using the Varioskan Lux Elisa plate reader (ThermoFisher Scientific, USA). A standard curve was plotted using the ratio of the absorbance against the different protein concentrations. This curve was used to estimate the protein concentrations of the test samples.

3.8.2 Iron content determination

The iron content was estimated for deferoxamine and gallic acid treated cells as described by Dragset *et al.* (2015). Briefly, approximately 5×10^6 cells were harvested and suspended in 100 µl of sterile water out of which 10 µl was used for protein content determination. Hundred microliters (100 µl) of spent media (media collected after harvesting the cells) was analyzed for iron. Both media and cells were digested with 200 µl of 100% nitric acid at 80 °C for 1 hour and incubated at 20 °C overnight. After digestion, 60 µl of 30% hydrogen peroxide was added to stop the reaction. Sterile water was added to give a final volume of 2 ml. The iron content was measured by atomic absorption spectroscopy using Analyst 300 spectrophotometer (PerkinElmer, USA).

3.9 Cytotoxicity against macrophages (Raw 264.7)

Toxicity of compounds to Raw 264.7 cells was evaluated. Briefly, the cells were seeded into 96-well plates at $\sim 1 \times 10^5$ cells/ml in DMEM supplemented with 10% FBS, 100X penicillin/streptomycin, 2 g/l NaHCO₃ and incubated at 37 °C for 24 hours in 5 % CO₂. The Raw 264.7 cells were treated with varying concentrations of the compounds for 48 hours at 37 °C in 5 % CO₂. Cell viability was estimated by MTT reagent and absorbance was measured at 570 nm

using the Varioskan Lux plate reader (ThermoFisher Scientific, USA). Cytotoxic Concentration (50%), CC_{50} , values were estimated using the Graphpad prism 6 software and the selectivity index (SI) was calculated as the ratio of the CC_{50} to IC_{50} values.

CHAPTER FOUR

4.0 RESULTS

4.1 Cell viability

4.1.1 Growth rate of *Trypanosoma brucei brucei*

A steady growth was observed and the cells exhibited a 10-12 fold increase every 24 hours. This validates the viability of the cells as shown in the cumulative growth curve (Figure 6).

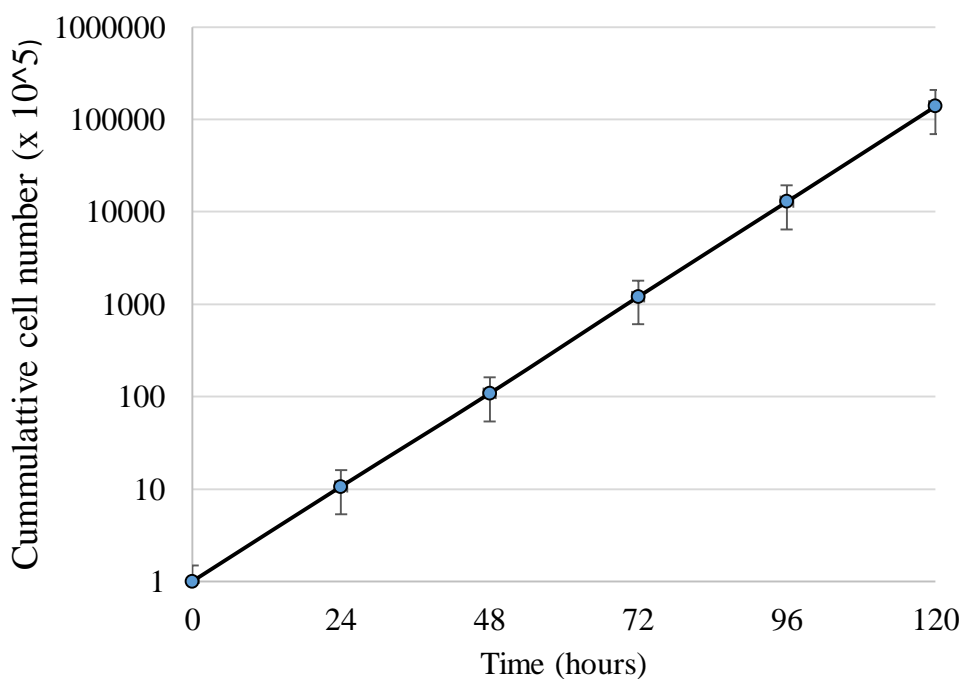


Figure 6: Cumulative growth rate of bloodstream forms of *Trypanosoma brucei brucei* (GUtat 3.1). The mean values are expressed as mean \pm SD (n=3). A 10-12-fold increase in cell number was observed after every 24 hours.

4.2 Trypanocidal activity of iron chelators against *Trypanosoma brucei brucei* in vitro

The anti-trypanosomal effect of the different iron chelators using the alamarBlue assay and the IC₅₀ for each iron chelator was determined. Diminazene aceturate, deferoxamine and gallic acid exhibited the most anti-trypanosomal activity with IC₅₀ of 0.1 μM, 3.3 μM and 14.2 μM respectively (Table 1). Rosmarinic showed moderate trypanocidal activity while caffeic acid, chlorogenic acid, ferulic acid, quinic acid and protocatechuic acid showed negligible antitrypanosomal activity with IC₅₀ >100 μM (Table 1). Dose responses of the most potent compounds are shown (Figure 7).

Table 1: Anti-trypanosomal activity of phenolic acids on *Trypanosoma brucei brucei*

Test compounds	IC ₅₀ (μM)±SD
Diminazene Aceturate (DA) ^a	0.1 ± 0.02
Deferoxamine (DFO)	3.3 ± 0.26
Gallic Acid (GA)	14.2 ± 1.5
Rosmarinic Acid	17.31± 0.08
Caffeic Acid	>100
Ferulic Acid	>100
Chlorogenic Acid	>100
Quinic acid	>100
Protocatechuic Acid	>100

^a Standard anti-trypanosomal drug

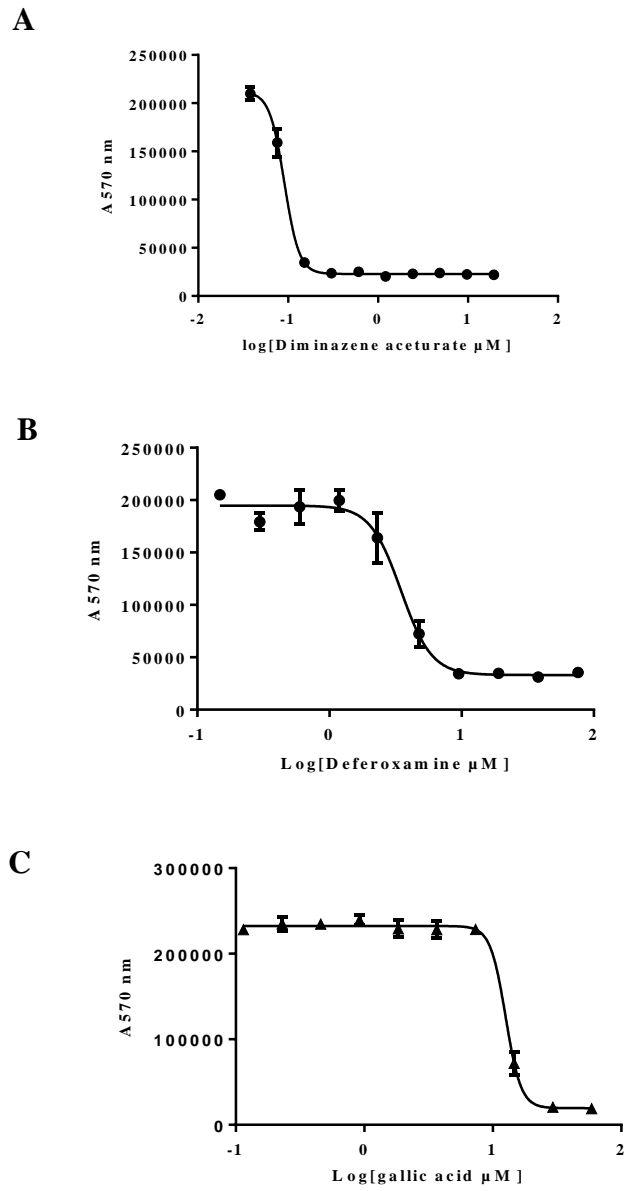


Figure 7: Dose response curve of (A) diminazene aceturate, (B) deferoxamine and (C) gallic acid on *Trypanosoma brucei brucei*. Plots were obtained after three independent experiments.

4.3 Effect of chelators on the growth rate of *Trypanosoma brucei brucei*

4.3.1 Iron chelators inhibited the growth of parasites *in vitro*

To further assess the effect of the active compounds on the growth rate of the parasite, a growth assay was done using different concentrations of the chelators. Different concentrations of diminazene aceturate used were 0.05 μM and 0.1 μM . The concentrations of deferoxamine used were 3.3 μM , 6.6 μM , 14.2 μM while that of gallic acid were 3 μM , 7 μM , 14 μM . (Figure 8). A reduction in cell density was observed for diminazene aceturate treated cells after 48 hours at both concentrations (Figure 8A). No detectable growth inhibition was observed when cells were treated with deferoxamine (3.3 μM), however there was an inhibition growth at 6.6 μM and 13.2 μM (Figure 8B). Growth was inhibited after 48 hours with the gallic acid at the lowest concentration of 3 μM and a drastic reduction of growth was observed at increasing concentrations (Figure 8C).

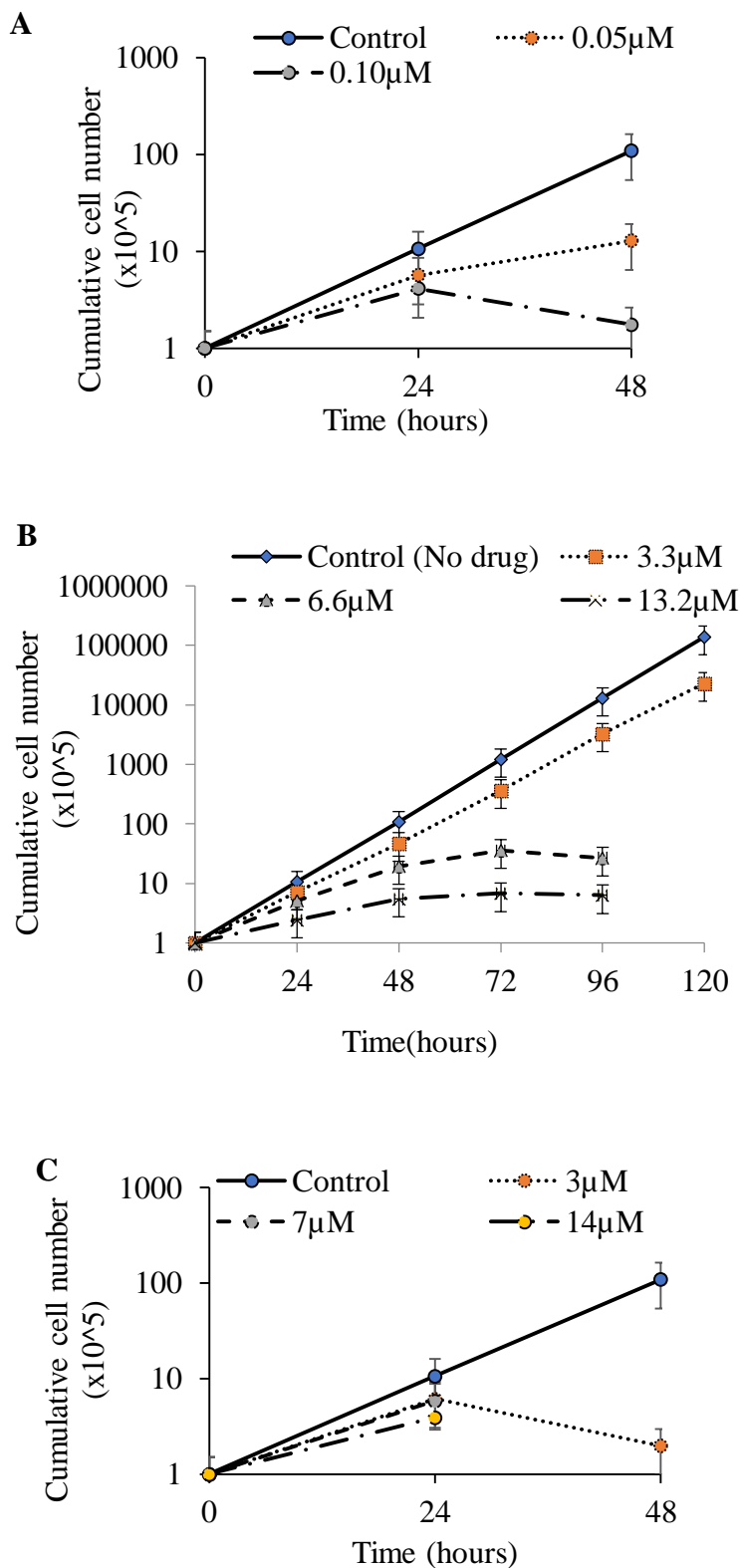


Figure 8. Dose dependent effect of compounds on the growth of *Trypanosoma brucei brucei*. Cumulative growth curve analysis of cells in the presence of different concentrations of chelators. (A) diminazene aceturate (B) deferoxamine (C) gallic acid.

4.4 Effect of chelators on the cell morphology and DNA synthesis

4.4.1 Treatment with iron chelators leads to loss of kinetoplast

To characterize the effect of the chelators on parasite morphology, the cells were stained with DAPI and viewed under the fluorescent microscope. Most of the control cells had normal morphology and also contained at least a nucleus and a kinetoplast (Figure 9A). Eighty-two percent (82%) of diminazene aceturate lost their kinetoplast (Figure 9B). The percentage of cells that lost their kinetoplast in deferoxamine and gallic acid treated cells were 26% and 22% respectively (Figures 9C and 9D).

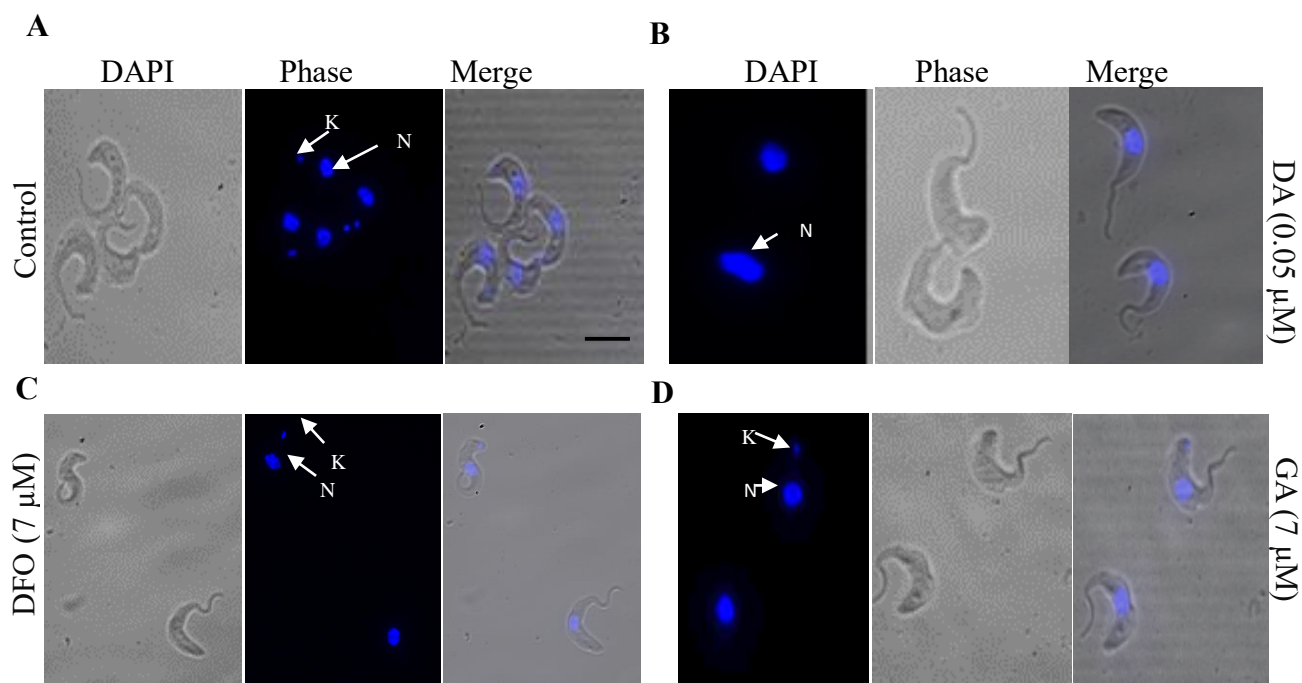


Figure 9: Effects of iron chelators on cell morphology and DNA synthesis: Cells were treated with diminazene aceturate (DA); deferoxamine (DFO) and gallic acid (GA) for 24 hours stained with DAPI for DNA (blue) and cell morphology visualized using the fluorescent microscope (100x). The nucleus is indicated as N and the kinetoplast as K. The concentrations used were the $\frac{1}{2}$ IC₅₀ for diminazene aceturate and gallic acid and 2X IC₅₀ for deferoxamine. Scale bar=10 μ m

4.5. Effects of iron chelators on mitochondrion membrane potential and cell morphology

4.5.1 Treatment with iron chelators affects the mitochondrion membrane potential and parasites' morphology

The effect of the iron chelators on the mitochondrial membrane potential was assessed using the MitoTracker dye[®]. The dye makes use of negative membrane potential by binding to the thiol groups in the mitochondria. Cells with an intact membrane potential are able to retain the dye while those that have defect in the mitochondria membrane will not fully retain the dye leading to the presence of red spots in the cytoplasm or low fluorescence intensity. The test compounds showed a dose dependent effect on the mitochondrial membrane potential as well as the morphology (Figures 10 and 11). About 90% of diminazene aceturate treated cells had a defect in the mitochondrial membrane (Figure 10). Increasing concentration of deferoxamine (7.0 μ M, 14.0 μ M and 30.0 μ M) resulted in the aggregation of dye in the cytoplasm of 70%, 97% and 98% of cells respectively (Figure 11). Cells treated with gallic acid at the respective concentrations: 7 μ M, 14 μ M and 30 μ M had 83%, 87% and 86% of cells respectively showing a loss in their membrane integrity. All treated cells had an altered or abnormal morphology which includes the rounding up of cells, detached cell body and an elongated flagellum. (Figures 10 and 11).

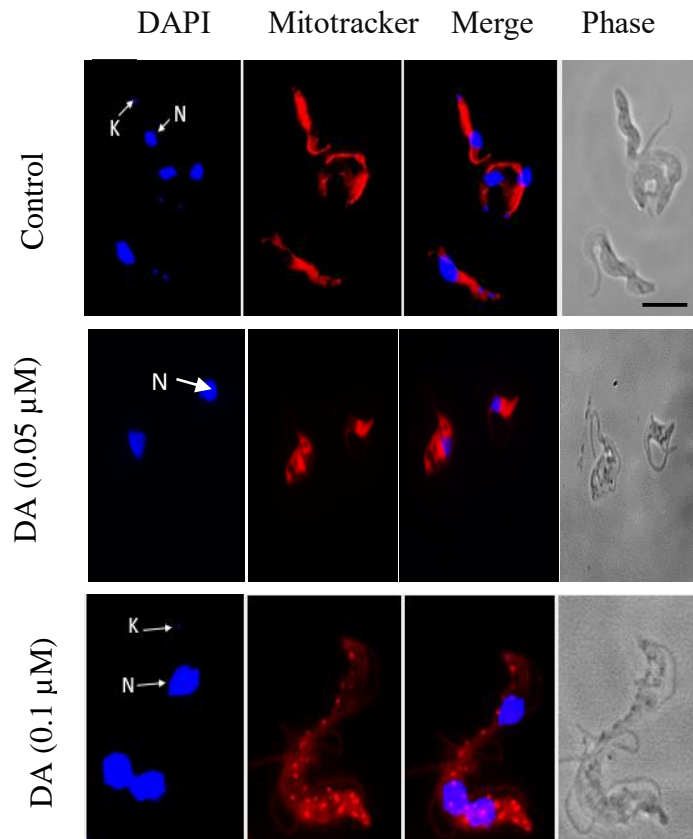


Figure 10. Effects of diminazene aceturate on the mitochondrial membrane potential and cell morphology. Cells were treated with diminazene aceturate (DA) [0.05 μM; 0.1 μM], labeled for mitochondria with Mitotracker red dye (red), counterstained with DAPI for DNA (blue) and cell morphology visualized using the fluorescent microscope (100x). The concentrations used were $\frac{1}{2}$ IC₅₀, IC₅₀. Scale bar= 10 μm

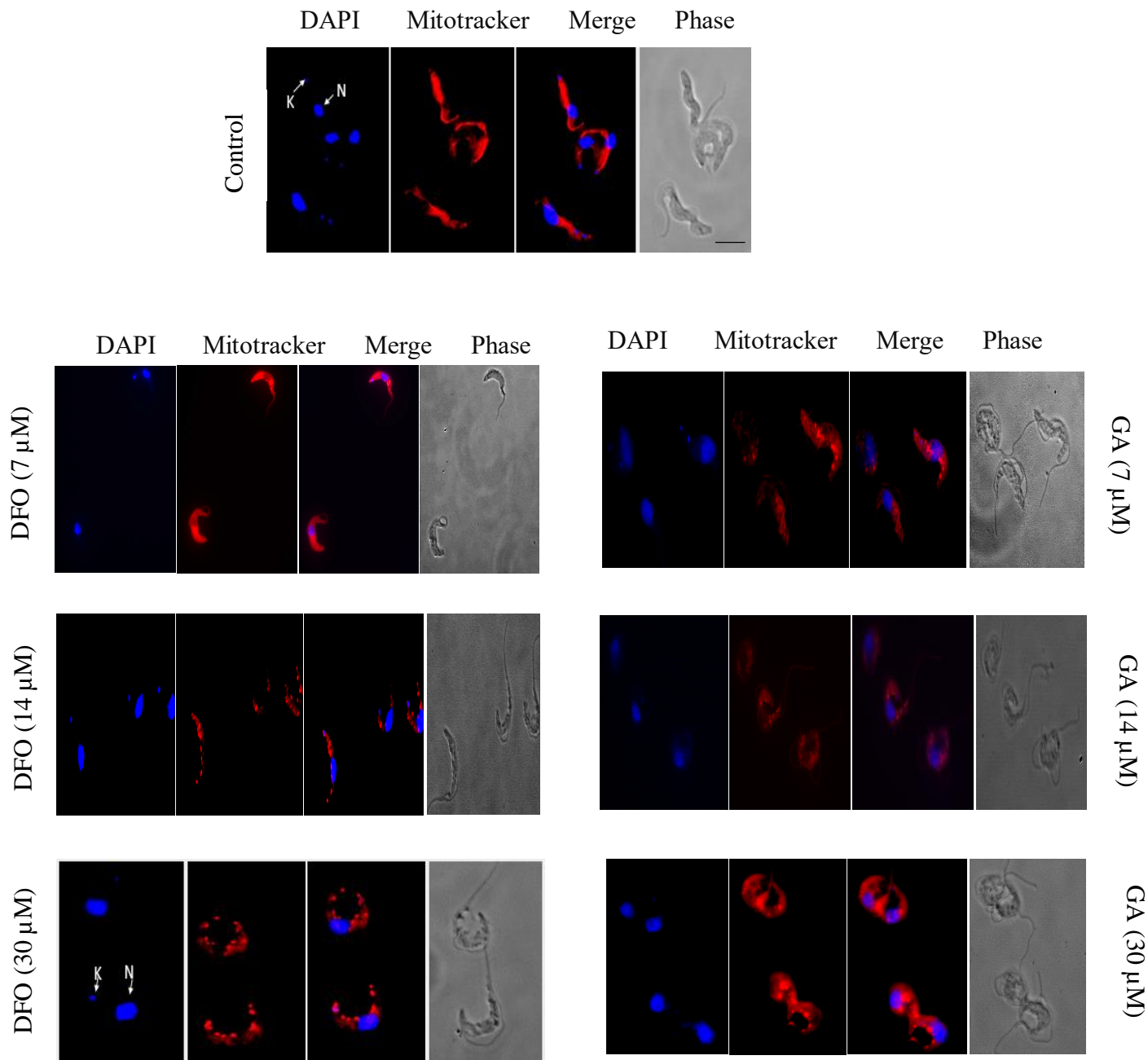


Figure 11. Effects of deferoxamine and gallic acid on the mitochondrial membrane potential and cell morphology. Cells were treated with deferoxamine and gallic acid (DFO and GA) [7 μ M; 14 μ M; 30 μ M] labeled for mitochondria with Mitotracker red dye (red), counterstained with DAPI for DNA (blue) and cell morphology visualized using the fluorescent microscope (100x). The concentrations used were 2x IC₅₀, 4x IC₅₀, 9xIC₅₀. and 1/2 IC₅₀, IC₅₀ and 4x IC₅₀ for DFO and GA respectively. Scale bar = 10 μ m

4.6 Effects of chelators on the cell cycle

4.6.1 Iron chelators cause a reduction in cells in G₀/G₁ and accumulation of cells in the S phase

The effect of the iron chelators as well as the standard anti-trypanosomal drug on the cell cycle phases was evaluated. Cells were treated with different concentrations of compounds (diminazene aceturate, 0.05 μ M; deferoxamine, 7 μ M; gallic acid, 7 μ M) for 24 hours and analysed by flow cytometry (Figures 12 A-D). The untreated cells showed 53.4% \pm 1.10, 14.7% \pm 0.55 and 37.2% \pm 4.54 of cells at the G₀-G₁, S and G₂-M phases respectively. Deferoxamine and gallic acid caused a reduction in cells in G₀/G₁ and accumulation of cells in the S phase. The treated cells had lower percentage cell count at the G₀-G₁ phase relative to the control (diminazene aceturate, 37.6% \pm 5.76; p value = 0.0096, deferoxamine, 37.0 % \pm 8.0, p value = 0.0247; and gallic acid, 32.4% \pm 13.37, p value= 0.0538) There was no significant change in the percentage cell count in the G₂-M phase for all compounds tested (Figure 12E). However, deferoxamine and gallic acid treated cells showed an increase in the percentage cell count at the S phase relative to the control (deferoxamine, 20.8% \pm 1.0; p value = 0.0008 and gallic acid, 20.9% \pm 2.46; p value = 0.013).

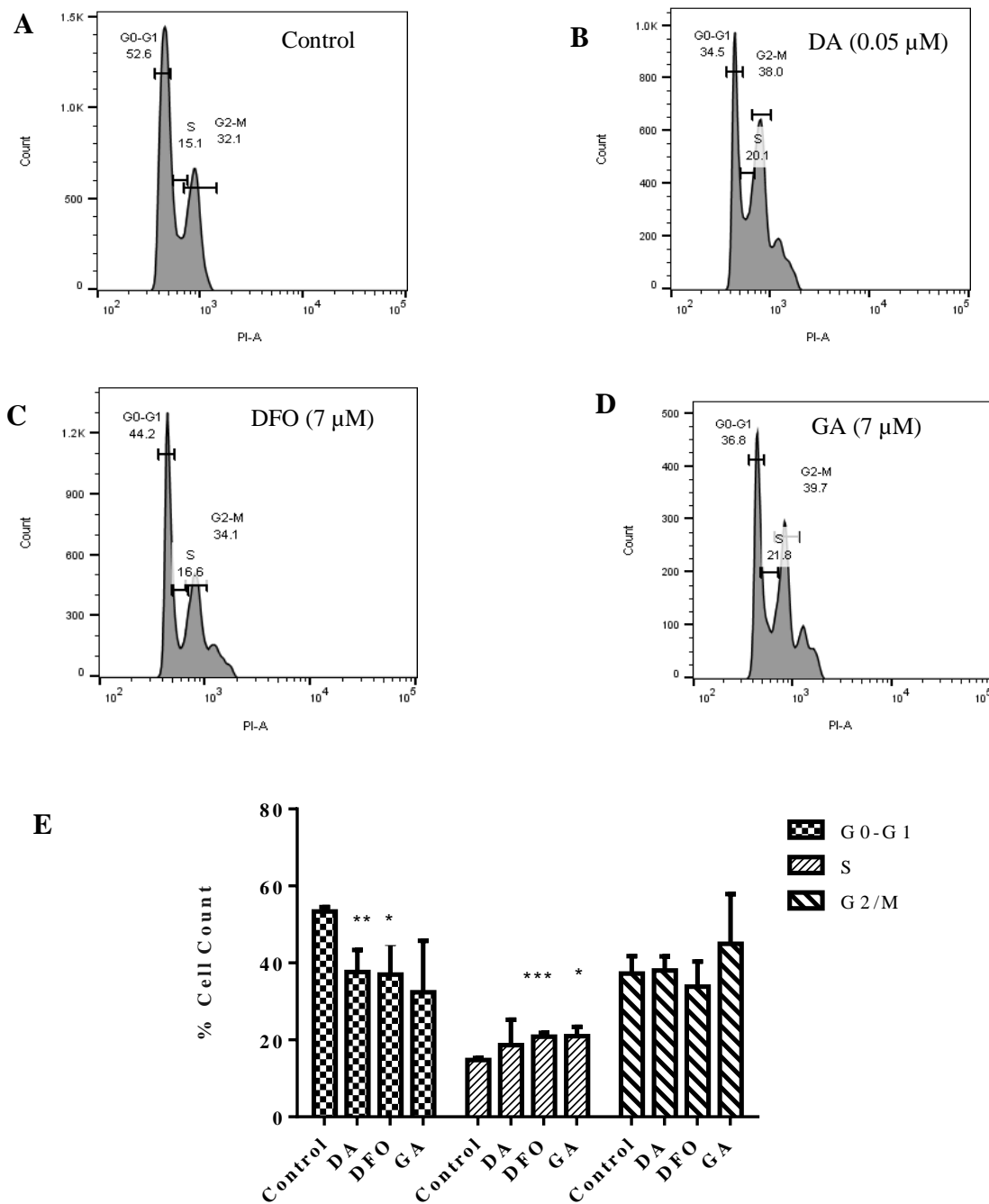


Figure 12: Effect of iron chelators on the cell cycle: (A, B, C, D, Representative histograms) (A) control (untreated); (B) diminazene aceturate (0.05 μM); (C) deferoxamine (7 μM); (D) gallic acid (7 μM). E (Summarized flow cytometry data). Results are represented as average ±SD. Statistical significance was determined using unpaired t test comparing the treated and the control for each phase, p<0.05, n=3

4.7 Effects of iron chelators on gene expression

4.7.1. Treatment with deferoxamine leads to upregulation of iron metabolic genes

To determine the effect of deferoxamine on gene expression in the trypanosome, the total transcriptome of deferoxamine treated cells were analyzed relative to the control using Illumina RNA sequencing. Prior to that, ribosomal RNA was depleted using the RNase H rRNA depletion method. This was done to enhance the efficiency of the sequencing. The depletion of rRNA from the total RNA was confirmed by Northern blot by the absence of the three bands which represents the subunits of the rRNA (Figure 13A). The presence of the beta tubulin blots shows the mRNA transcript was intact even after rRNA depletion (Figure 13B). The read counts generated from the mRNA sequencing were highly correlated across the biological replicates (Pearson's r correlation was 0.9883 and 0.9758 for the untreated and deferoxamine treated respectively) (Figure 14). It was observed that almost all the genes in the trypanosomes were upregulated. Among the iron dependent genes, ribonucleotide reductase was highly expressed with a relative fold increase of 10 while the transferrin receptor had a 4-fold increase. Cyclin 2 gene had a 13-fold increase (Figure 15)

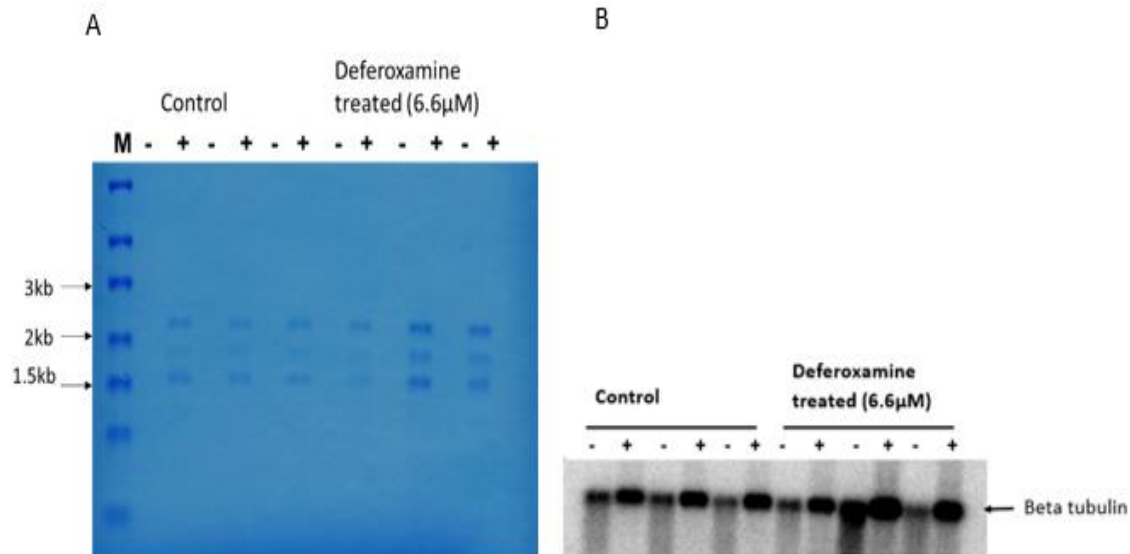


Figure 13: Effect of deferoxamine on mRNA transcripts: (A): Gel electrophoresis after rRNA depletion. (B): Northern blot of beta tubulin mRNA -: rRNA depleted (1.5µg); +: non depleted rRNA(2.0µg). Ribosomal RNA 28S:1.8kb, 28S: 1.5kb 18S: 2.3kb

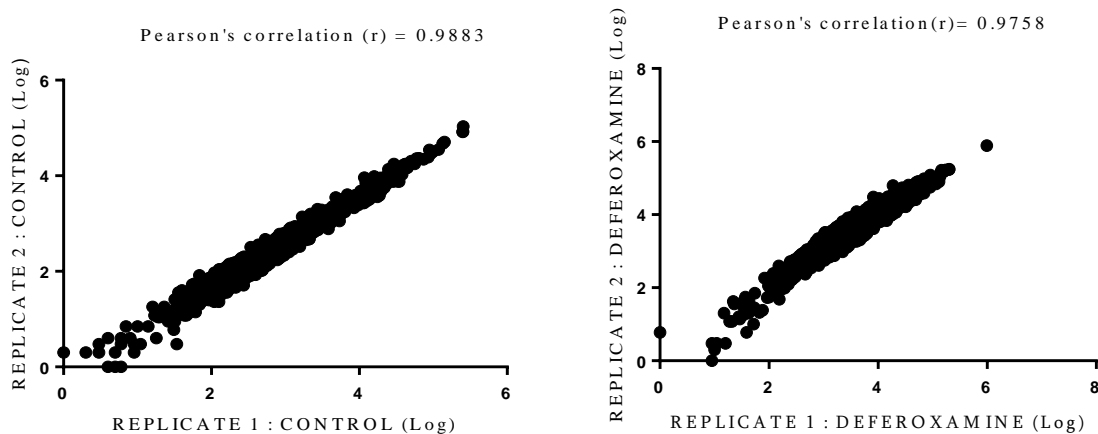


Figure 14: Correlation of the gene expression between biological replicates. The read counts generated from the mRNA sequencing were used and correlation coefficients are indicated inside the plots

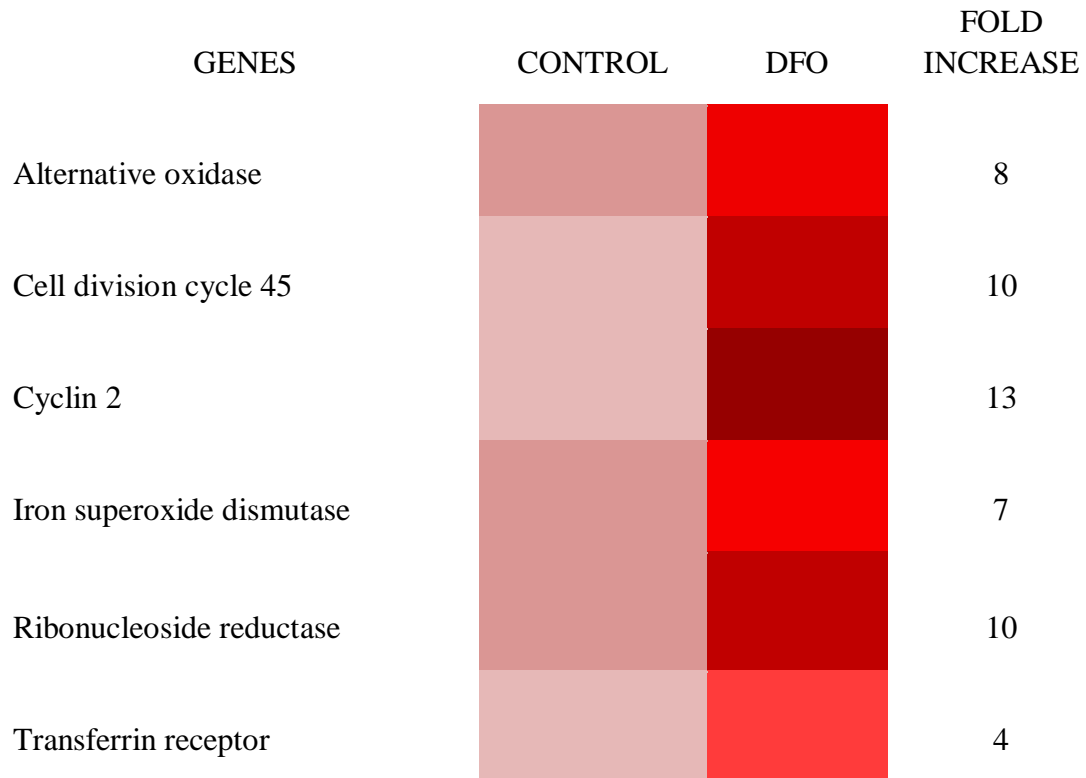




Figure 15: Effect on deferoxamine (DFO) on gene expression. Heat map showing the fold increase of some iron dependent genes. Cells were treated with DFO (6.6 μ M).and incubated for 24hours. Deep red represents the highly expressed genes  while the light red represents the low expressed genes 

4.7.2. Treatment with iron chelators leads to differential expression of iron metabolic genes

RT-qPCR was performed to validate results obtained from the mRNA seq data and to also compare relative gene expression when treated with gallic acid and diminazene aceturate. All the treated cells [diminazene aceturate (0.05 μ M), deferoxamine (7 μ M) and gallic acid (7 μ M)] showed an increase in the cyclin 2 (diminazene aceturate, 5-fold; deferoxamine, 8-fold and gallic acid, 7-fold) (Figure 16B) and ribonucleotide reductase genes (diminazene aceturate, 25-fold; deferoxamine, 52-fold and gallic acid, 50-fold) (Figure 16C). Gallic acid and diminazene aceturate had a 0.6-fold and 0.3-fold decrease in the transferrin receptor respectively (Figure 16A) while deferoxamine had a 1.4-fold increase in the transferrin receptor. The increase in all the selected genes in the deferoxamine treated cells confirms results from the mRNA seq data.

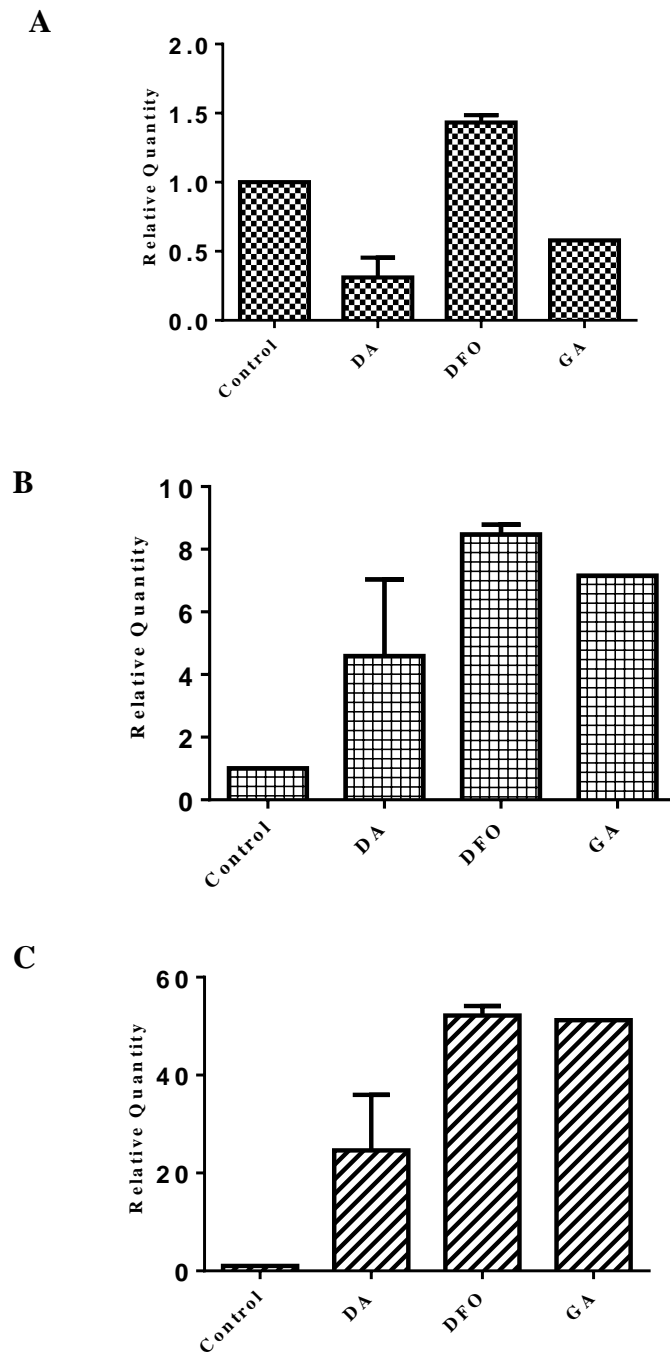


Figure 16: Relative gene expression of iron dependent genes. RT-qPCR analysis of mRNA expression of (A) Transferrin receptor (B) Cyclin 2 (C) Ribonucleotide reductase after 24 hour treatment with diminazene aceturate (DA), deferoxamine (DFO) and gallic acid (GA). The values are expressed as the relative quantity with respect to the control. The data represent the mean of 2 independent experiments.

4.8 Iron content determination

4.8.1 Iron chelators decrease intracellular iron content

The intracellular iron content (amount of iron in the cell) and the extracellular iron content (amount of iron in media) were determined using the atomic absorption spectrometry. There was a reduction in the intracellular iron content in deferoxamine treated cells. Gallic acid significantly reduced the intracellular iron content (deferoxamine, 0.1407 ± 0.008 ; p -values = 0.1695 and gallic acid =, 0.130; p -value = 0.0354) (Figure 17).

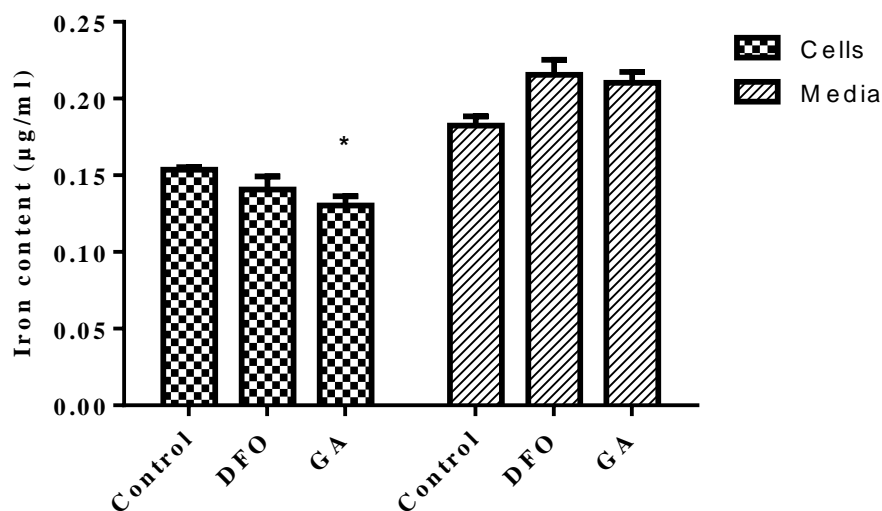


Figure 17: Effect of iron chelators on intracellular and extracellular iron content. Cells were treated with 7 μ M of deferoxamine (DFO) and gallic acid (GA). Significance was determined using the unpaired t test comparing treated and control, $p < 0.05$

4.9 Cytotoxicity of chelators against human macrophages

4.9.1 Iron chelators are moderately toxic to macrophages

To determine the toxicity of the test compounds against macrophages. MTT cell viability assay was done and the CC_{50} values generated were used to calculate the selectivity index (SI). Compounds with selectivity index greater than 10 are considered less toxic to macrophages. All the test compounds were found to be toxic with a lower selectivity index (Table 2).

Table 2: Selectivity index (SI) of test compounds against macrophage cell lines

Test Compounds	$IC_{50}(\mu M)\pm SD$	$CC_{50}(\mu M)\pm SD$	$SI=CC_{50}/IC_{50}$
Deferoxamine	3.3 ± 0.26	26.7 ± 0.32	8.1
Gallic acid	14.2 ± 1.5	36.1 ± 3.33	2.5
Diminazene aceturate	0.1 ± 0.02	0.0014 ± 0.05	0.014

A selectivity index < 10 indicates toxicity to the macrophage relative to the parasites

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION, RECOMMENDATIONS

5.1. Discussion

Phenolic acids (a class of polyphenols) have been shown to have anti-protozoan, anti-bacterial and anti-cancer properties (Maurya and Devasagayam, 2010 and He *et al.*, 2016) but few studies have been done to evaluate their effect on trypanosomes. The aim of this study was to assess the trypanocidal activities of some phenolic acids. Gallic acid showed the highest trypanocidal activity with IC_{50} of 14.2 μ M comparable to the value obtained by Koide *et al.* (1998) and was able to inhibit parasite growth after 24 hours. Gallic acid was also able to inhibit parasite growth even at a lower concentration whereas protocatechuic acid, also a hydroxybenzoic acid, was inactive. The iron binding abilities of these phenolic acids have been linked to the number and positions of the hydroxyl groups attached to the benzene ring (Andjelković *et al.*, 2006). The hydroxyl moieties get deprotonated under physiological pH and bind to iron. The differences in trypanocidal activities observed between gallic acid and protocatechuic acid could be linked to the galloyl structure as in the case of gallic acid with three hydroxyl groups while protocatechuic acid with a catechol structure had two hydroxyl moieties. Hence the hydroxy number could have contributed to the differences in their trypanocidal activities. Hydroxybenzoic acids with the galloyl group are better chelators than those with the catechol groups (Andjelković *et al.*, 2006) This is further supported by studies conducted by Merschjohann and Steverding, (2006) where 2,3-dihydroxybenzoic acid (bearing a catechol group) showed weak trypanocidal activity.

All the hydroxycinnamic acid derivatives used in this study were either moderately active or inactive against the trypanosome. This observation could be due to the low iron binding ability of the hydrocinnamic acid.

Another factor that influences the cytotoxic activity of compounds is the ability of the compound to generate reactive oxygen species (ROS). Iron chelators have antioxidant activities since they are able to mop up free radicals (Adjimani and Asare, 2015) generated during some metabolic process. Paradoxically, the generation of ROS is also another effect of some iron chelators. A balance between their antioxidant activity and their free radical generation has an impact on the extent of trypanocidal activity. For example, quercetin, a metal chelating flavonoid with a good antioxidant activity was found to be moderately active against trypanosomes (Merschjohann and Steverding, 2006) hence its antioxidant activity neutralized its trypanocidal effect. The trypanocidal activity of gallic acid could be as a result of the generation of ROS (Koide *et al.*, 1998). This mechanism also causes gallic acid to induce apoptosis in cells (Chuang *et al.*, 2010; Locatelli *et al.*, 2013). Therefore, the balance between the antioxidant effect and the ROS generation of gallic acid could explain its trypanocidal effect. Interestingly, in other studies, gallic acid was inactive against other kinetoplastids such as *Leishmania donovani* (Nardella *et al.*, 2018) and *Trypanosoma cruzi* (Andréo *et al.*, 2015). This could be due to the intracellular nature of these parasites.

Diminazene aceturate was also trypanocidal but unlike the iron chelators, its activity is linked to the effect on the kinetoplast. The drug acts by binding to the minor groove of the DNA, preventing replication (da Silva Oliveira and de Freitas, 2015) leading to growth inhibition and loss of the

kinetoplast. Being a good iron chelator, the dose dependent trypanocidal activity of deferoxamine was confirmed with IC_{50} values comparable to other studies conducted (Breidbach *et al.*, 2000; Merschjohann and Steverding, 2006). At varying concentrations, deferoxamine was also able to inhibit the growth of the parasites.

Apart from growth inhibition, the chelators appeared to affect the morphology of trypanosomes by altering their shape and organelles such as the kinetoplast and mitochondrion. The loss of kinetoplast in diminazene aceturate treated cells observed could be an effect of its mechanism as described by Kuriakose *et al.* (2012). Eighty-two percent (82%) of the cells lost their kinetoplast and even in the absence of the kinetoplast the cells were still able to proliferate under normal culturing conditions. This observation agrees with suggestions made by Schnauffer *et al.* (2002) that the absence or loss of the kinetoplast were not lethal to trypanosomes as the cells were still able to grow. Similar morphological changes were also observed in deferoxamine and gallic acid treated cells but unlike in diminazene aceturate treated cells, about 26% and 22% of cells were affected respectively. Iron chelators are not known to bind directly to the DNA hence effects observed could be attributed to the activity of the ribonucleotide reductase. Iron chelation does not directly affect this enzyme but could prevent the addition of iron into new ribonucleotide reductase apoproteins (Breidbach *et al.*, 2002). This could make newly synthesized apoproteins enzymatically inactive and thus causing a decrease in the rate of DNA synthesis. A reduction in DNA synthesis could cause a halt in the cell cycle process. A halt or arrest in the cell cycle means an increase or accumulation of cells in one phase relative to the other. During DNA damage, there is an increase in expression of the ribonucleotide reductase in the S phase of the cell cycle (Guarino *et al.*, 2014). A study by Korte *et al.* (2011) revealed that an overexpression of ribonucleotide

reductase caused an increase in the cellular dATP content. In this study, the upregulation of the ribonucleotide reductase gene could have been a compensatory mechanism to increase the cellular DNA content by increasing the expression of the enzyme. The halt in the DNA synthesis through the accumulation of cells in the S phase could be due to two possible mechanisms: the upregulation of the gene could cause an increase in the expression of the ribonucleotide reductase protein, but in an iron depleted environment, the protein is not fully functional or the increase in gene expression is not enough to produce the amount of ribonucleotide reductase needed (poor protein turnover). In most eukaryotes, control of the ribonucleotide reductase involves several factors which include allosteric regulation, proteolysis of the subunits, activity of inhibitors and the regulation of gene expression (Guarino *et al.*, 2014).

Since the kinetoplast encodes essential proteins in the mitochondrion, MitoTracker assay was performed to further investigate the effect of the chelators on the mitochondrion of the trypanosome. The dose dependent effect of deferoxamine and gallic acid on the mitochondrial membrane could be attributed to their effect on the function of the iron superoxide dismutase or the generation of ROS. A fully functional mitochondrion will have an intact membrane potential leading to the internalization and the retention of the dye in the mitochondrion. In a defective cell, the membrane potential will be lost and the dye will aggregate in the cytoplasm as reported by Andréo *et al.* (2015). The generation of reactive oxygen species causes damage to the mitochondrion leading to a loss in its membrane potential (Zuma *et al.*, 2015). Since the machinery for mopping up free radical is iron dependent, the deprivation of iron could cause a dysfunctional iron superoxide dismutase (Fe-SOD) hence cells with a dysfunctional Fe-SOD will be overwhelmed by these reactive oxygen species and this can ultimately affect the mitochondrial

membrane. Some esters of gallic acid have also been shown to affect the mitochondrial membrane of *T. cruzi* (Andréo *et al.*, 2015). The corresponding 13-fold increase in the Fe-SOD gene observed in this study could be the parasite's mechanism against oxidative stress. For intracellular microorganisms such *Leishmania spp.*, oxidative defense becomes important when the parasites have to survive in the host macrophage. The Fe-SOD enzyme is a chemotherapeutic target because any defect could enhance its elimination by the innate immune system (Kim *et al.*, 2012). Overexpression of some key enzymes have been shown to protect cells from oxidative damage. For example, in *Leishmania major*, overexpression of ascorbate peroxidase prevents the cells from apoptosis as result of oxidative stress (Dolai *et al.*, 2009). Also, in *Trypanosoma cruzi*, overexpression of the mitochondrial Fe-SOD has been shown to reduce the parasites sensitivity against oxidative stress (Piacenza *et al.*, 2009). However, Temperton *et al.* (1998) observed that when the *T. cruzi* SOD-B1 enzyme activity is increased to about 8-fold, the parasites become more sensitive to benznidazole (a drug which acts as prooxidant). It was further explained that overexpression of this enzyme could cause an imbalance in the redox homeostasis leading to an increase in parasite's drug sensitivity. However, the results obtained in this study cannot tell whether an upregulation in Fe-SOD gene will cause an overexpression of the enzyme. A similar observation was made for diminazene aceturate treated cells but how this happens is unclear. However, pentamidine (an anti-trypanosomal drug) which is structurally similar to diminazene aceturate have been shown to act by affecting mitochondrial membrane (Andréo *et al.*, 2015). Pentamidine breaks down the membrane leading to a dysregulation in the intra and extra cellular calcium ion content of the cell (Andréo *et al.*, 2015) hence diminazene aceturate could be affecting the membrane potential of the mitochondrial membrane via the same mechanism. The generation of ROS, iron chelation and nitric oxide affects the expression of trypanosome alternative oxidase

(TAO) gene. For example, Tsuda *et al.* (2005) reported that under stressed conditions the upregulation of alternative oxidase could be a mechanism of preventing oxidant induced apoptosis. The addition of the iron chelator, salicylhydroxamic acid (2-hydroxybenzhydroxamic acid, SHAM) and glycerol affected the aerobic and anaerobic processes of the parasite leading to rapid growth inhibition (Manta *et al.*, 2012). The upregulation of the alternative oxidase gene by 8-fold in deferoxamine treated cells could help reduce oxidant induced stress. Studies have also suggested the effect of TAO in preventing programmed cell death. In the area of plant metabolism, nitric oxide which plays a key role in plant stress responses has been shown to upregulate the expression of alternative oxidase in *Chlamydomonas reinhardtii* (green algae) (Zalutskaya *et al.*, 2017).

The effects of the chelators observed in this study confirms the fact that adequate amount of intracellular iron is necessary for essential metabolic processes in the parasite (Nairz *et al.*, 2010). Therefore, iron chelators which are able to selectively reduce the intracellular iron content of the parasite could be very promising drug candidates. From this study both deferoxamine and gallic acid reduced intracellular iron content of the parasites. The results obtained for deferoxamine also agrees with results by Breidbach *et al.* (2002) which reports deferoxamine to act by reducing intracellular iron content. Since the gallic acid also showed effects similar to those observed for deferoxamine, it is most likely that it functions as an intracellular iron chelator. Most pathogens have developed different ways of obtaining iron in an iron limited environment. For example, under iron starved conditions, *Leishmania amazonensis* upregulates its iron transporter (LIT-1) within the phagolysosome (Huynh and Andrews, 2008) to increase its intracellular iron content. The upregulation of the transferrin receptor in the deferoxamine treated cells could be an indication of a compensation mechanism employed by the cells under iron starved conditions in order to

increase their iron intake. The 4-fold increase in the expression of this receptor is comparable to observation made by Beate *et al.* (1999) who reported a 3-fold upregulation of the receptor in deferoxamine treated cells. Studies by Mussmann *et al.* (2004) also reported a 5-fold increase in the transferrin receptor in response to transferrin starvation. The expression of the trypanosome transferrin receptor is via the regulation of the ESAG 6 transcript at the post transcriptional level. Beate *et al.* (1999) reported that in the absence of iron, the expression levels of ESAG 6 increases to about 3-fold and this causes a relative increase in the transferrin receptor but the mechanism of the regulation still remains unclear. In this study however, a low expression of the transferrin receptor in the gallic acid treated cells was observed even though it has reduced intracellular iron content. This could mean its trypanocidal activity could involve other mechanisms in addition to its iron chelation properties.

The general cell proliferation process and the production of normal daughter cells involves a smooth progression of the cell cycle. This process is a combined effect of the cyclins, cyclin dependent kinases and their inhibitors (McKean *et al.*, 2003). The effects of iron chelators on the cell cycle have been shown to result from differential expressions of these genes and their respective protein expressions. Iron chelators are known to cause cell cycle arrest in the S phase (Gazitt *et al.*, 2001; Kicic *et al.* 2001; Le and Richardson 2002). The relative increase in the cyclin 2 gene found in this study could also account for the accumulation of cells in the S phase of the cell cycle emphasizing the importance of this gene in the G1/S phase transition. Upon treatment with iron chelators deferoxamine and aroylhydrazone, Le and Richardson (2002) observed an increase in the mRNA levels of the cyclin dependent inhibitor (p21) which plays an important role in G1 cell cycle arrest but there was however a decrease in its protein levels. This observation was

unique since in other iron depletion experiments, the levels of transcription correlated to the levels of protein expression (Fu and Richardson, 2007). Hence a combined effect of cyclins, their kinases and their inhibitors are essential for smooth progression. An arrest in any of the cell cycle phases generally leads to an inhibition in the growth of the parasites. Most drugs that target the cell cycle are in the clinical trial stage of drug development (Dickson and Schwartz, 2009) hence drugs which are able to affect the cell cycle stages may be promising.

In the search for effective therapeutic agents, one requirement of a potential drug candidate is its relative toxicity against the host cells. An ideal drug must be selectively toxic to the parasites. However, in the absence of a better alternative, some drugs are still administered despite their toxicity. For example, diminazene aceturate is still used as an anti-trypanosomal despite its side effects and toxicity (Kuriakose *et al.*, 2012). Murine macrophages were used in this study because they are comparable to the normal human cell lines and drug efficacy against them could be informative. Iron chelators are generally more toxic to trypanosomes compared to mammalian cells *in vitro* (Breidbach *et al.*, 2000; Merschjohann and Steverding, 2006) and gallic acid has been shown to be toxic against cancer cell lines (Koide *et al.*, 1998). From this study, even though all the chelators were toxic, they were less cytotoxic compared to diminazene aceturate.

5.2 Conclusion

Phenolic acids with iron chelating properties exhibited good trypanocidal activities with gallic acid being the most potent phenolic acid. Iron chelators are able to inhibit growth of *Trypanosoma brucei in vitro*. Iron deprivation through the use of chelators affects the cell morphology, and mitochondrion membrane potential. The chelators also caused loss of kinetoplast. Also the use of iron chelators caused S phase cell cycle arrest while increasing the expression of ribonucleotide reductase and cyclin 2 genes. The potent iron chelators may act by reducing the intracellular iron content of the parasite.

5.3 Recommendations

Further studies should be done on the protein expression levels of these iron dependent enzymes to correlate their protein levels to those of the transcripts. Also the iron chelators should be tested in an *in vivo* system to fully understand their mechanisms of action and relative toxicities.

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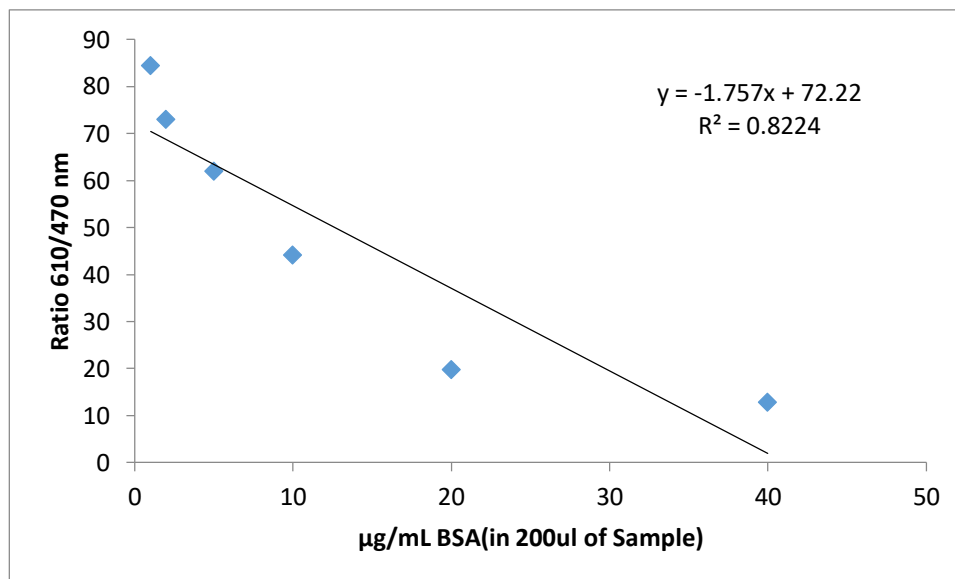
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7.0 APPENDIX

(I) Standard curve for BSA



(II): Sequences of primers used of RT-qPCR

GENE ID	GENE NAME	SEQUENCES
Tb927.7.2820	Histone H2A (FW)	AGTGAAGAAGGCATCGAAGG
	Histone H2A (RV)	CACGGATAGCTCCAGCAGTT
Tb927.9.15680	Transferrin receptor subunit (FW)	GATCGTGGGTGTTGACCTCT
	Transferrin receptor subunit (RV)	CAGATATGTTTGCGGGGACT
Tb927.11.14080	Cyclin 2 (FW)	GTGCTCACCAGAATGCTTCA
	Cyclin 2 (RV)	GCCACCAATACCTGCAAAGT
Tb927.11.12790	Ribonucleoside-diphosphate reductase small chain (FW)	CGTCATTGCAACTCGAAGAA
	Ribonucleoside-diphosphate reductase small chain (RV)	CTGCGTCCAGAGAGAAAACC

(III) Schematic Diagram of RNase based rRNA depletion method (Src: AG Clayton, University of Heidelberg, Germany)

RNase H based RNA depletion method

