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Modulation of haemolysis induced by plasmodium falciparum-derived haem products

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MODULATION OF HAEMOLYSIS INDUCED BY *PLASMODIUM FALCIPARUM*-DERIVED HAEM PRODUCTS

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A Research Report submitted to the Faculty of Health Sciences, University of Witwatersrand, in fulfillment of the requirements for the degree of Master of Science in Medicine.

September 2016

DECLARATION

I, Mogini Nadasan, declare that this research report is my own work. It is being submitted for the degree of Master of Science of Medicine at the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signature.....

.....02nd.....day of .....September..... 2016

DEDICATION

My research is dedicated to my Parents Ganas and Vijiyaluxmi Nadasan
and to Dr Thilendran and Rayona Nadasan for always being the ultimate pillars of strength and support, and for your guidance and love.
Thank you!!!

ACKNOWLEDGEMENTS

Thank You to Professor Robyn Van Zyl for your mentoring, knowledge sharing, commitment to my studies and the resulting invaluable lessons learnt under your guidance. This was truly an enriching academic experience due to you.

Thank You to Natasha Jansen Van Vuuren, Chien-Teng Chen and Nelusha Shunmoogam-Gounden for your assistance in the lab and for your time spent teaching and supporting me. All your help and willingness to guide is really appreciated. I am extremely grateful to you all.

Thank You to Leanne Wentworth (Director Regulatory Affairs, Mylan) and Christine White (Regulatory Affairs Manager, Mylan) for your consistent support, interest, motivation and encouragement throughout my studies. Deeply appreciated.

Thank You to my family and friends for your encouragement.

ABSTRACT

The degradation of the host's haemoglobin by the malaria parasite releases the toxic molecules free haem (ferriprotoporphyrin IX (FPIX) and hydrogen peroxide, which may cause significant membrane lipid peroxidation. This disrupts the oxidative defence of the RBC milieu. Scavengers of reactive oxygen species (ROS) or iron-induced lipid peroxidation may offer protection. Metal chelators are known to inhibit the latter reactions. As such eleven 8-hydroxyquinoline derivatives which possess anti-oxidant properties were selected to determine if they can modulate the degree of haemolysis induced by haem products. Incubation of RBC's with FPIX (under varying conditions of concentration, time, in presence of albumin, Fe(III)-citrate and with/without 8-hydroxyquinoline compounds) were investigated. The anti-oxidant activity was clarified using various *in vitro* assays to evaluate the free radical scavenging activity, ferrous ion chelating activity, ferric ion reducing ability and lipid peroxidation inhibiting/stimulating ability. The amino-substitutions displayed similar free radical scavenging activity to the positive controls, ascorbic acid and Trolox™, with 5-amino-8-hydroxyquinoline having the strongest scavenging activity (IC_{50} value: 12.12 ± 1.36 µM). Whilst 2-methyl-8-hydroxyquinoline (Fe(II)C_{50} value: 5.67 ± 1.45 µM) was a more potent iron chelator than EDTA (Fe(II)C_{50} value: 24.85 ± 3.12 µM). All compounds displayed strong Fe^{3+} reducing ability when compared to the standard Trolox™. Ten of the eleven 8-hydroxyquinoline derivatives inhibited lipid peroxidation activity except for 5-amino-8-hydroxyquinoline. Albumin, Fe(III)citrate and EDTA decreased lysis induced by 5 µM FPIX, whilst Trolox™ displayed a dose response effect inducing more lysis than FPIX alone. According to the present study, the 8-hydroxyquinoline derivatives, particularly 5-amino-8-hydroxyquinoine and 2-amino-8-quinolinol derivatives had effective *in vitro* anti-oxidant activity and ability to inhibit oxidative degeneration as a mechanism to protect the RBCs from free radical chain reaction damage which could lead to haemolytic anaemia.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abs: absorbance

ACD-vacutainers: acid citrate dextrose vacutainers

ATP: adenosine triphosphate

CM: culture media

CNS: central nervous system

°C: degrees celcius

DPPH: 1,1-diphenyl-2-picryl-hydrazyl

DMSO: dimethyl sulfoxide

EDTA: ethylenediaminetetraacetic acid

FPIX: ferriprotoporphyrin IX

FZ: ferrozine [3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine]

g: grams

g/l: grams per litre

GSH: glutathione

Heme Fe(III)PPIX: ferriprotoporphyrin IX

Hpi: hours post-invasion

IC_{50}: 50% inhibitory concentration (concentration that reduced the measured effect by 50%)

mg: milligram

ml: millilitre

mm: millimetre

mM: millimolar

MW: molecular weight

PBS: phosphate buffered saline

PV: parasitophorous vacuole

rpm: revolutions per minute

RBC: red blood cell

ROS: reactive oxygen species

TCA: trichloroacetic acid

TEAC: Trolox™ equivalent anti-oxidant capacity

TVN: tubovesicular network

Tween-20: polyoxyethylenesorbitan monolaurate

µl: microlitre

µM: micromolar

WHO: World Health Organisation

CHAPTER 1: GENERAL INTRODUCTION

1.1 Malaria

"A powerful and co-ordinated global response together with continued investment in research and development will rid entire continents of the disease and eventually eradicate malaria from the world."
(WHO Sixty-eighth World Health Assembly, 2015)

An alarming malaria fact, as presented by the WHO and specific to our continent:
"Most deaths occur among children living in Africa where a child dies every minute from malaria."
(WHO Fact Sheet No. 94, 2014)

The latest data confirms approximately 214 million cases of malaria worldwide and an estimated 438 000 deaths in 2015 (WHO Fact Sheet No. 94, 2016). Of all associated deaths, 90% occurred in Sub-Saharan Africa. Children under 5 are particularly susceptible, accounting for more than 70% of all malaria deaths. Close to half of the world's population is at risk of malaria, therefore universal access to malaria prevention and treatment is key in order to minimize the impact of the disease and is therefore the focus of an aggressive global technical strategy post 2015 (WHO Fact Sheet No. 94, 2016).

Malaria is caused by protozoa (single-celled parasites) in the genus *Plasmodium,* namely *P. vivax, P. ovale, P. malariae, P. knowlesi* and *P. falciparum,* with the latter being the most pathogenic (Singh and Daneshvar, 2013). The life cycle of *P. falciparum* is complex as it involves invasive, trophic and replicative forms in two hosts – a human and a female *Anopheles* mosquito (Figure 1).

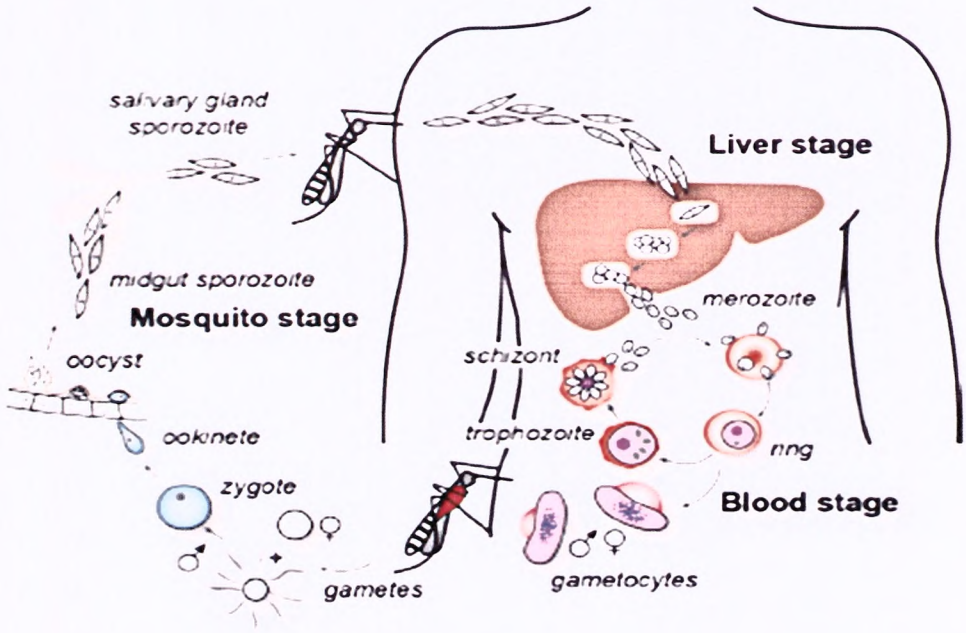


Figure 1: The life cycle of *P. falciparum* (Cowman *et al.*, 2010).

Transmission between both hosts involves several morphological transformations (Hanssen *et al.,* 2010). The first stage – the asexual reproductive cycle – begins with transference of motile sporozoites into the host's blood stream during mosquito feeding i.e. during blood feeding when an infected female *Anopheles* mosquito injects plasmodial sporozoites from its salivary gland into the human bloodstream (WHO, 2011). These are immediately carried to and invade hepatic parenchymal cells where they multiply.

Up to 30,000 exoerythrocytic daughter merozoites are produced from a single sporozoite in a hepatocyte, and these are released into the bloodstream (Caraballo, 2014). Once released the merozoites are free to quickly invade the host's red blood cells (RBCs) which are in circulation – the beginning of the blood phase of the infection (Cowman *et al.*, 2012; Braunwald *et al.*, 2005).

The development of the parasite continues through the ring, trophozoite and schizont progression stages whilst intra-erythrocyte (Hanssen *et al.*, 2010), and due to these

continuous growth and replication activities the host cell is irreversibly changed. The release of the merozoites has been identified to be the result of a swelling phase and automatic curvature of the tensed infected RBC membrane induced by the parasite – the smaller the RBC, the greater the speed with which the first merozoite is released (Figure 2). The curled and buckled membrane releases all remaining merozoites by exposure to blood flow (Abkarian *et al.*, 2011). Gametocytes which are also formed from the asexual blood stage are then ingested by a feeding mosquito. Maturation into male and female gametes occurs in the mosquito mid-gut. The zygote is fertilized and development from an ookinete to an oocyst to sporozoites occur which then migrate to the mosquitoes salivary glands (Cowman *et al.*, 2012).

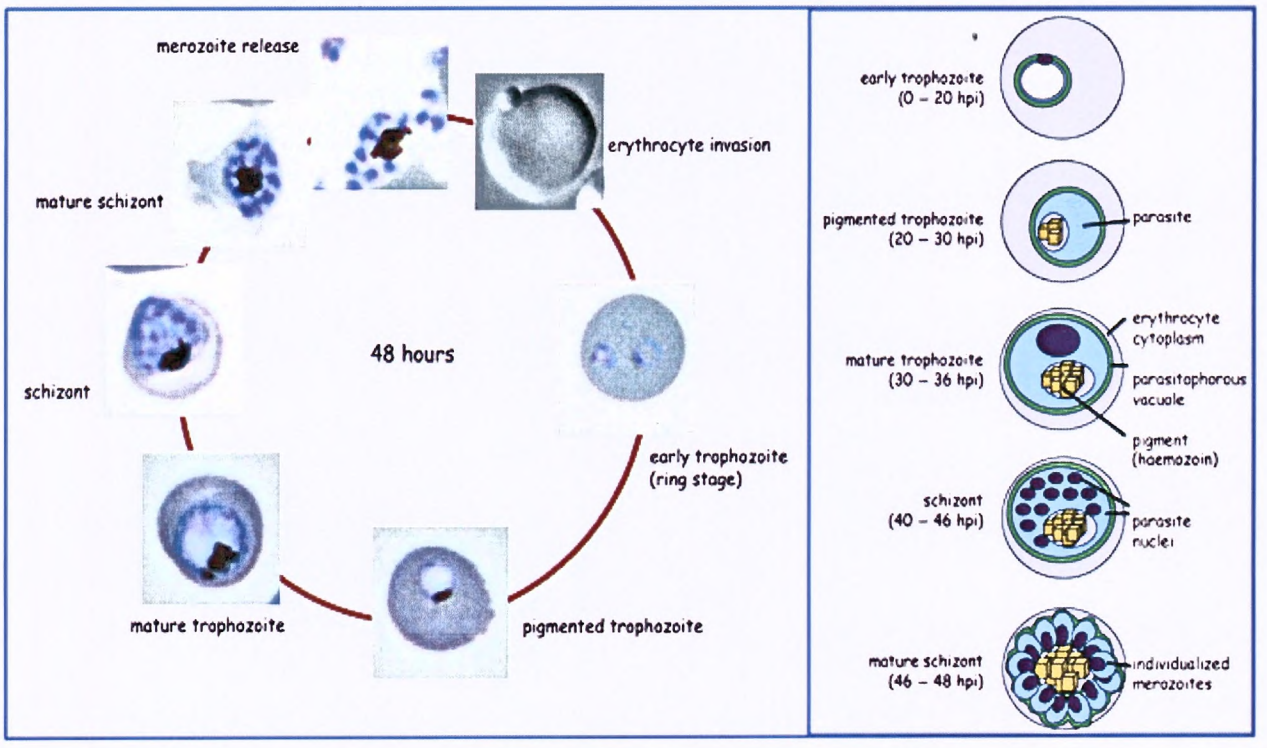


Figure 2: The 48 hour intra-erythrocytic development of *P. falciparum* (Mbengue *et al.*, 2012). The development through the different stages of parasite from trophozoite to merozoite release are shown by snap-shots of live-imaging of Giemsa-stained infected RBC (left panel) and as schemes (right panel). Hpi, hours post-invasion.

1.2 Malaria parasite haemoglobin digestion

1.2.1 Haemoglobin

Haemoglobin which gives blood its red colour, is perhaps the most recognized oxygen-carrying protein in nature. It is responsible for capturing atmospheric oxygen and the delivery thereof to cells and tissues for respiration, thereby sustaining the cells energy requirements and maintaining cellular homeostasis (Pishchany and Skaar, 2012).

In healthy adults, haemoglobin amounts to one-third of total erythrocyte mass and approximately 15% of the whole blood mass. It is a molecule of high importance during infection as many microbes (e.g. Gram-negative and Gram-positive bacteria) rely on haemoglobin as a generous source of the essential nutrient, iron, to grow within their hosts, and thus utilize elaborate mechanisms in order to acquire it. *Plasmodium* on the other hand, assimilates the protein, using it as a source of amino acids (Pishchany and Skaar, 2012).

1.2.2 Haemoglobin ingestion

Whilst within the erythrocyte, the RBCs protect the malaria parasite from the host's immune system, however difficulties also exist in accessing nutrients for growth and disposing of waste products during this stage of the lifecycle (Klonis *et al.*, 2007). The parasite transports, endocytoses and digests the host haemoglobin within the digestive vacoule as a food source to support parasite growth and asexual replication (Lazaruz, 2008), thereby causing biochemical changes to the host RBCs (Figure 2*)*. Mature human RBCs contain 310-350 mg/ml (± 20 mM) haemoglobin (Klonis *et al.*, 2007). Between 60 to 80% of the host haemoglobin present in the RBC is digested in the malaria parasitic acidic food vacuole, to provide a source of amino acids (which are incorporated into malarial proteins) and osmolytes. This also occurs to create adequate space for growth and division (Klonis *et al.*, 2007). The breakdown of haemoglobin in malaria organisms into simpler forms is proposed to be a metabolic pathway and not a random process (Goldberg *et al.*, 1990).

1.2.3 Haemoglobin degradation

The degradation of haemoglobin releases the main by-product free haem (ferriprotoporphyrin IX, FPIX) – a hydrophobic iron-protoporphyrin complex (four pyyrole rings linked by –CH group) (Figure 3). The complex is called ferroprotoporphyrin or haem when the iron atom is in the ferrous state, and is called ferriprotoporphyrin or haemin in the ferric state (Kumar and Banyopadhyay, 2005). To prevent the toxic effects of this haemoglobin degradation byproduct (Klonis *et al.*, 2007), the parasite polymerises the FPIX units together in a crystalline lattice (Sullivan *et al.*, 1996) to form haemozoin or malaria pigment – an inert, highly insoluble and compact crystal (as a detoxification by polymerization mechanism) (Sullivan *et al.*, 1996). These brown crystals as formed by biomineralization are seen in the vacuole of the parasite in advanced and the later mature stages of *P. falciparum*-invaded human RBC's (Park *et al.*, 2008)

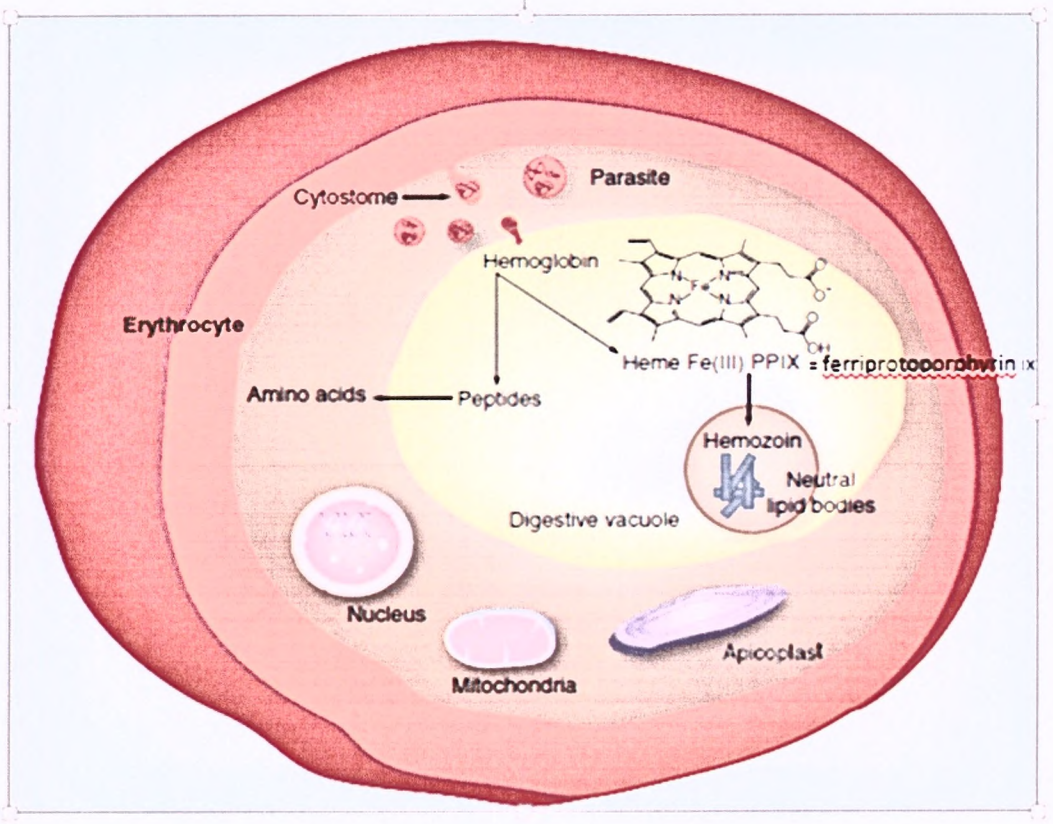


Figure 3: Intra-erythrocyte haemozoin formation (Fong and Wright, 2013).

1.2.4 Haem removal and detoxification

High levels of free haem leads to toxicity affecting and injuring organs, tissues and cells as described in detail in Figure 4. The following strategies exist for haemoglobin and haem removal in the human body to limit the potential associated toxicity, to ensure iron homeostasis and to prevent microbial growth:

1) mature erythrocytes move and flow continuously in the blood for approximately three months, and are thereafter removed from the blood stream by macrophages, haemoglobin from such erythrocytes are digested to facilitate the recycling of haem-iron;

2) should erythrocyte lysis occur, liberated haemoglobin is captured by the plasma protein haptoglobin (forming a haptoglobin-haemoglobin complex which is recognized by macrophages and removed from the plasma) to prevent oxidative damage inflicted by haemoglobin; and

3) the plasma protein called haemopexin rapidly binds any free haem that is released from haemoglobin extracellulary (Pishchany and Skaar, 2012).

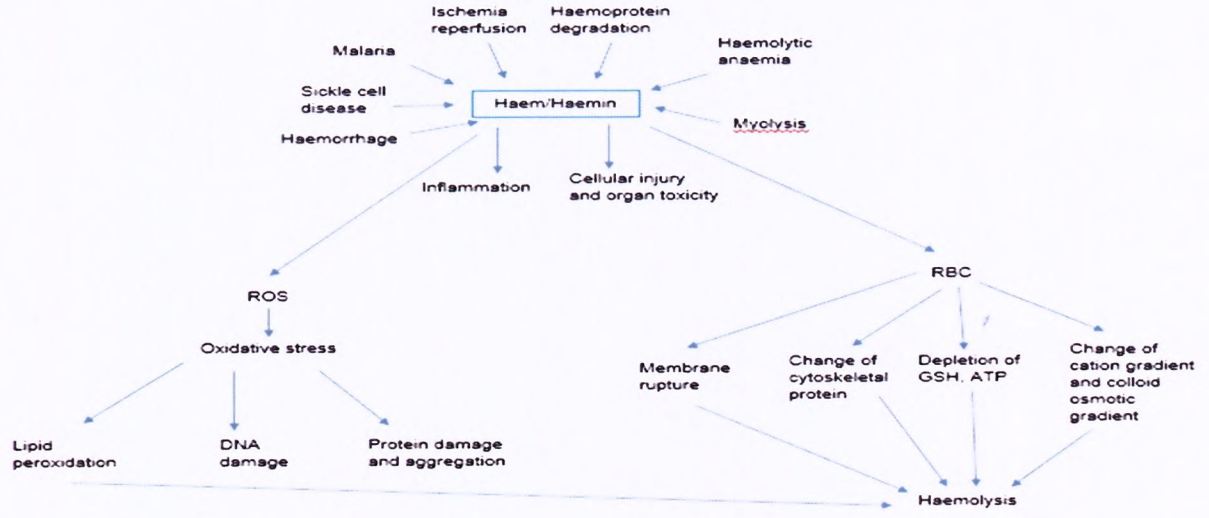


Figure 4. The toxic effects of free haem/haemin (Kumar and Banyopadhyay, 2005).

The above haem removal/detoxification mechanisms can however become overwhelmed by high levels of lysis within the malarial life cycle (i.e. free haem up to 20 μM). The result is haem catalyzed oxidative reactions and non-specific haem uptake –

haem's toxic and damaging effects as outlined in Figure 4. There are four factors that are involved (Kumar and Bandyopadhyay, 2005):

1. ROS: iron-derived ROS leads to oxidative stress which in turn causes lipid, DNA and protein damage (Letarte *et al.*, 1993).

1.1 During lipid peroxidation: aqueous haemin accumulates in membranes, increasing oxidation of the components thereby causing permeability and structural disorders, cell lysis and ultimately death (Schmitt *et al.*, 1993).

1.2 DNA damage occurs when: haemin ferric iron is reduced to ferrous iron which combines with oxygen to produce the following reactions: nicking and DNA degradation into progressingly smaller pieces with increased time of exposure to iron, damage of mitochondrial DNA of hepatocytes (Suliman *et al.*, 2002) and deletion of coding region on exposure of liver to haemin, uncoiling of circular plasmid DNA to open linear forms (Aft and Mueller, 1983).

1.3 Protein damage and aggregation results when: haemin catalyses the degradation of protein to small peptide fragments (particularly the protein HBP93) (Mueller and Aft, 1984); oxidises lipoproteins and forms conjugates/reactive substances (Camejo *et al.*, 1984); causes covalent cross-linking of lipoproteins resulting in formation of protein aggregates (Miller and Shaklai, 1994). Free haem enhances the change of low-density lipoproteins to cytotoxic oxidised products (Jeney *et al.*, 2002).

1.4 Vascular disorders e.g. artherosclerosis, haemolytic anaemia, vasculitis, reperfusion injury etc. result from oxidative stress (Wagener *et al.*, 1997).

2. Inflammation: both haem and haemin are pro-inflammatory agents. Haem stimulates intracellular, vascular and endothelial leukocyte adhesion molecules (Lavrovsky *et al.*, 1994) which recruit leukocytes – this leads to endothelial cell injury, inflammatory lesions and vascular disorders (Wagener *et al.*, 2001). Haemin activates neutrophils during haemolysis (Kumar and Bandyopadhyay, 2005).

3. Cellular injury and organ toxicity: when endothelial cells are exposed to haem, polymorphonuclear leukocyte-mediated killing is greatly increased (Balla *et al.*, 1991,

2003) which affect kidney, heart, liver, neuron, leukocyte functions. Kidney function is affected due to the resultant renal vasodilation, diuresis, increased natriuresis, renal blood flow and urine volume, local inflammation, all resulting in renal failure (Rodriguez *et al.*, 2003). Renal epithelial cells are also affected as their growth is decreased Gonzalez-Michaca *et al.*, 2004). Loss of cardiac cell integrity results as it damages sarcolemma and causes the release of cytosolic enzymes (Bhoite-Solomon *et al.*, 1993). Oxidative stress is induced by haemin through its ability to increase lipid peroxidation and decrease glutathione content (Nath *et al.*, 1995).

4. RBC changes: Extensive cell and tissue damage occurs – being hydrophobic, haem easily enters cell membranes therefore the cell is more susceptible to oxidant-mediated killing and lipid-lipid, lipid-protein and protein-protein associations are severely disrupted. Haem-induced haemolysis occurs in two phases – in the first phase, large amounts of potassium are lost followed by a decrease in glutathione and adenosine triphosphate (ATP); in the second phase, large amounts of haemoglobin is lost (Chou and Fitch, 1981). Changes of cytoskeletal protein occurs due to oxidation, spectrin and protein 4.1 cross-linking occurs making the membrane skeleton unstable. In patients with inherited conditions such as sickle cell anaemia and β-thalassemia oxidative membrane damage and permeability disorders (membrane rupture) occur can be aggravated by released haem (Liu *et al.*, 1985). Glutathione (GSH) and ATP are depleted - glutathione degrades haem and thereby protects erythrocytes from oxidative stress (Atamna *et al.*, 1995); (also refer to RBC – haem-induced haemolysis phases described above). In the presence of haem, erythrocyte cation gradients and colloid osmotic systems gradients are changed which also contribute to haemolysis (i.e. lysis of cells and death (when haemin promotes oxidation and aggregates in membranes)). This stimulates potassium loss and swelling (Chou and Fitch, 1981).

The following are disease states characterised by severe haemolysis whereby there are high concentrations of free haem:

1. Haemorrhage: haem is neurotoxic and its release follows central nervous system (CNS) haemorrhage; it may also be present in intracranial haematomas (Goldstein *et al.*, 2003).

2. Hemoprotein degradation: during pathological states, toxicity effects are caused from the release of haem from hemoproteins. Hemoproteins inhibit the haemin-induced lipid peroxidation and are therefore effective anti-oxidants. When bound with protein i.e. hemopexin, to form a complex, haem becomes less or non-toxic (Delanghe and Langlois, 2001). Hemoproteins transport haem to the liver for degradation in the reticuloendothelial system (Muller-Eberhard and Fraig, 1993).

3. Myolysis: is the degeneration of muscular tissue due possibly to parasitic presence/infection, enzymatic activity, tissue hypoxia, endotoxins as well as parasitized red-cell sequestration in the capillaries and the resultant vessel occlusion in muscle tissue (Autino *et al.*, 2012; Keltz *et al.*, 2013). Acute toxicity and tissue injury occurs on exposure to large amounts of haem proteins – free haem is markedly injurious to tissues (Donnell *et al.*, 2005; Nath *et al.*, 2000).

4. Hemolytic anaemia: ROS cause many vascular disorders; ROS also alter uninfected RBC membranes thereby becoming targets of auto-antibodies (Autino *et al.*, 2012).

5. Sickle cell disease: as sickle erythrocytes release exorbitant amounts of free haem (Uzunova *et al.*, 2010); Malaria, as discussed in Section 1.5; and Ischemia reperfusion: restoration of blood supply to oxygen-deprived tissue in turn increases the extent of injury – when aerobic metabolism is interrupted, metabolic intermediates accumulate. Once oxygen is again available, aerobic metabolism also resumes with the accompanying generation of oxygen free radicals. The anti-oxidant system becomes overwhelmed leading to oxidative cell damage including lipid peroxidation (Dugan and Choi, 1999).

1.3 Antimalarial agents and haemozoin formation

The *Plasmodium* species produce haemozoin and many current antimalarials therefore target the inhibition of this biological pathway such as the quinolone and xanthone groups.

The haemozoin inhibitor, chloroquine, formerly the mainstay of antimalarial chemotherapy, as used for over 40 years since the 1940's, acts on haemoglobin metabolism in the parasitic food vacuole (Sullivan *et al.*, 1996). It blocks the formation of

the inert haemozoin crystals by binding to free haem units thus retaining their toxic lytic properties (Egan, 2008) and thereby competing with haemozoin polymers for this molecule. It has also been shown to bind to haemozoin (Fitch and Kanjananggulpan, 1987) preventing polymer prolongation by the incorporation of chloroquine molecules (Sullivan *et al.*, 1996). The result of the above mechanisms is buildup of toxic, lytic, free haem in the food vacuole (Orjih *et al.*, 1981) or haem-chloroquine complexes (Fitch *et al.*, 1982).

The xanthone family of compounds was found to have a similar mechanism of action to the chloroquine, by the formation of haem-xanthone complexes *in vitro* thereby preventing larger aggregates from forming, proving inhibition of haem biomineralization and parasite growth (Fong *et al.*, 2013).

Artemisinin is highly potent against haemoglobin-digesting stages of malaria parasites, it decreases haemoglobin uptake, leading to a build-up of undigested haemoglobin in the parasite. Interestingly, haemoglobin uptake and subsequent hydrolysis is required to support artemisinin activity i.e. a haemoglobin degradation product is needed for the antimalarial activity of artemisinin (Klonis *et al.*, 2011).

A rapidly acting artemisinin derivative is combined with a longer acting slowly eliminated partner drug, the aim being to cure the patient of the disease i.e. eliminate all parasites from the body, as quickly as possible, prevent progression, transmission and resistance. Current WHO ACT (artemisinin-based combination therapy) treatment guidelines for adults (excluding pregnant women in their first trimester) and children presenting with uncomplicated *P. falciparum* malaria, are artemether + lumefantrine, dihydroartemisinin + piperaquine, or artesunate in combination with amodiaquine, mefloquine or sulfadoxine–pyrimethamine.

The diverse biological and therapeutic activities of 8-hydroxyquinoline (8-OH) have contributed to it being noted as a compound for further investigation. Due to its lipophilic and metal chelating properties, it helps to restore metal balance/homeostasis when metal overload/deficiency caused by abnormal metal absorption/metabolism (dysregulation) occur. Further 8-hydroxyquinoline properties are diverse and include

being antineurodegenerative, anticancer, anti-oxidant, antimicrobial, anti-inflammatory and anti-diabetic (Prachayasittikul *et al.*, 2013).

It is proposed that the use of anti-oxidants will decrease pathogen burden, as molecules which act to deplete ROS, and may therefore open a new avenue for the use of anti-oxidants against malaria (Paiva and Bozza, 2014).

1.4 RBC structure and pathology of haemolysis

Healthy RBCs are biconcave and disc-shaped, with diameters of approximately 7.5 to 8.7 μm and 1.7 to 2.2 μm in thickness (Figure 5).

They possess reversible elastic deformable properties due to their flexible membrane structure which allows repeated passage through small capillaries (of 2 to 3 μm in diameter) during microcirculation (Diez-Silva *et al.*, 2010).

The membrane regulates the volume of cytosol in the intracellular RBC fluid. It has a high surface-to-volume ratio and consists of a phospholipid bilayer which is connected by integral and peripheral proteins (binding known as vertical interactions) to an underlying spectrin network (binding known as horizontal interactions). If the vertical or horizontal interactions are disrupted in any way the spectrin network density is affected, resulting in cell changes (Diez-Silva *et al.*, 2010).

Following the lysis of erythrocytes, haemoglobin is easily oxidized. The following sequence occurs: oxidation from ferrous (Fe^{2+}) to ferric (Fe^{3+}) haemoglobin (methemoglobin) which then readily releases haem. Free haem, a ROS, due to its extreme hydrophobic nature becomes especially dangerous when it is no longer contained within its original physiological site (i.e. within the RBC) – as free haem can readily enter uninfected RBC membranes, disrupting cell structure and function. This increases the susceptibility of the cell to oxidant-mediated injury and killing and microvascular flow impairment (Omodeo-Sale *et al.*, 2005). The haem ring can be spilt by hydrogen peroxide by different sources such as activated leucocytes which cause the release of "free" redox-active iron which can act as a catalyst increasing the production of ROS inside the cell. The result is lipid, protein and DNA damage leading

to eventual cell death (Belcher *et al.*, 2009). The survival of non-infected erythrocytes has been found to be reduced for several weeks after the clearance of *P. falciparum* parasites (Warrel and Gilles, 2002), thus further potentiating the haematological pathology of malaria (Kumar and Bandyopadhyay, 2005; Omodeo-Sale *et al.*, 2005).

The common characteristics amongst intravascular haemolytic diseases are: myoglobin/haemoglobin escape from within the cell, haem-iron-induced-oxidative stress, vascular inflammation, and leukocytes recruitment to the site of vascular injury (Belcher *et al.*, 2009).

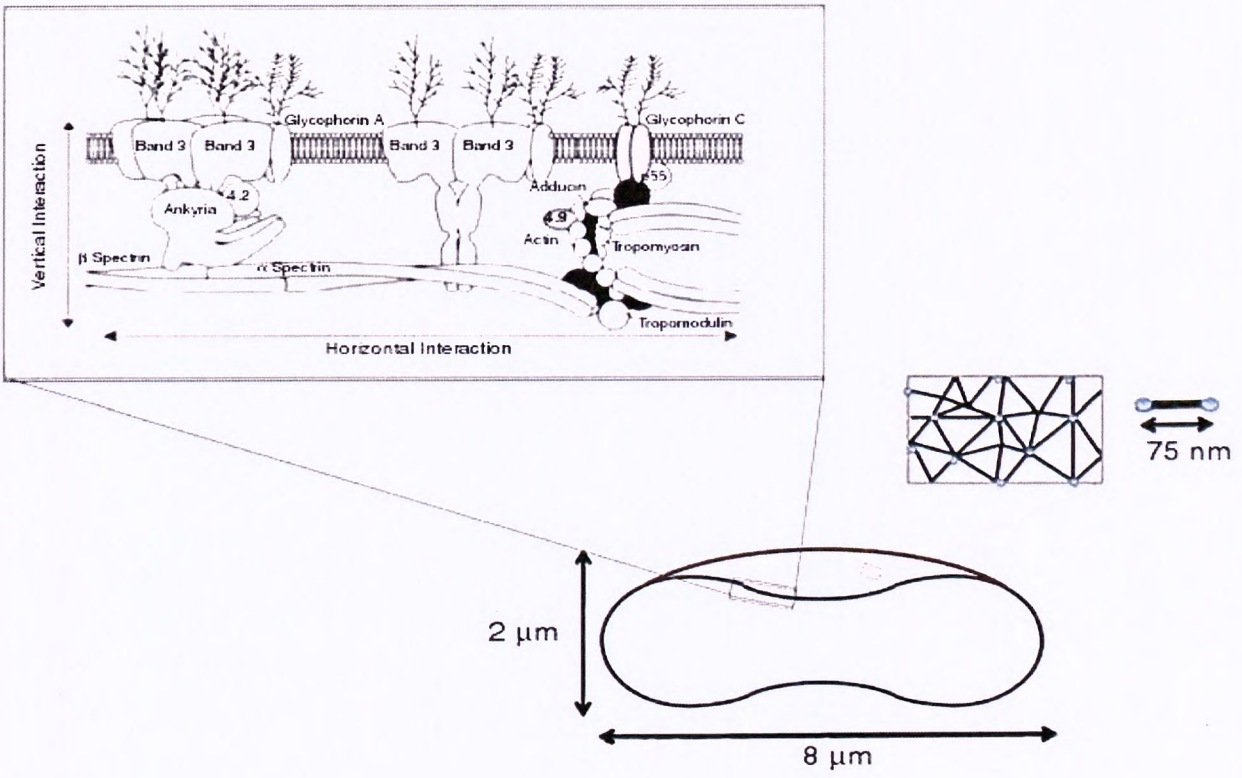


Figure 5: Healthy RBC membrane showing discocyte shape, cross-sectional view (upper left diagram) and two-dimensional connectivity of the spectrin network (upper right diagram) (Diez-Silva *et al.*, 2010).

1.5 Oxidative stress

The generation of ROS plays a crucial role in the development of systemic complications of malaria. Sources of oxidation during malaria infection include:

- Direct reactive species production by *P. falciparum* infected erythrocytes compared to uninfected erythrocytes of twice the amount of $OH^{\bullet}$ radicals and H_2O_2. The intra-erythrocytic developmental stages of the malaria parasite thrive in a highly micro-aerobic environment thereby generating oxidants which constantly challenge and disrupt the oxidative defense of the RBC milieu (Srivastava, 1999). To protect itself from an increased oxidative environment, the parasite imports essential enzymes (catalase, superoxide dismutase) from the erythrocyte (D'Souza *et al.*, 2009).
- Activation of the host defense inflammatory process i.e. phagocytes such as macrophages and neutrophils, which in turn form large amounts of ROS
- Generation of hydroxyl radicals ($OH^{\bullet}$) in the liver
- Transition metal catalysis as during haemoglobin degradation – the malaria parasite requires amino acids for the synthesis of its proteins. With haemoglobin being an abundant protein in the erythrocyte cytoplasm, the organism ingests the host haemoglobin and it therefore serves as a major source of amino acids for the parasite. Haemoglobin is broken down into peptides and amino acids within a vacuolar compartment called the food/digestive vacuole (Goldberg *et al.*, 2005), A large amount of free iron is released during the process of haemoglobin digestion. The iron is oxidized from Fe (II) to Fe (III), with the production of a super-oxide anion radical ($O_2^{\bullet-}$) that is rapidly converted to H_2O_2 (Klonis *et al.*, 2011). Both free FPIX and H_2O_2 are toxic molecules that the parasite needs to destroy or neutralise as free haem may interact with H_2O_2 or iron to form Fe(III)FPIX which can potentially cause significant membrane lipid peroxidation (Egan, 2008). The latter can be defined as the oxidative deterioration of lipids and process whereby free radicals 'steal' electrons from cell membranes, resulting in cell damage and increased production of free radicals (Andreoli *et al.*, 2003). The latter ultimately kill the parasite, but also releases a vast quantity of toxic FPIX into the bloodstream. The formation and accumulation of haemozoin during chronic malaria infection leads to blackening of the spleen and liver (Pishchany and Skaar, 2012).

- The occurrence of ischemia-reperfusion syndrome which produces additional free radicals, resulting from cytoadherance processes (whereby malaria-infected cells adhere to vascular endothelium which prevents removal by the spleen) and anaemia triggered by infection.
- Antimalarials such as chloroquine, primaquine and the artemisinin derivatives as these are all inducers of free radical species (Percario *et al.*, 2012).

As described above, plasmodial infections result in greatly increased oxidative stress and damage, ultimately decreasing the anti-oxidant defence of the host (Srivastava, 1999). Metal chelating agents and anti-oxidants are well-known scavengers of ROS and are able to disrupt the Fenton reaction (Kumar and Bandyopadhyay, 2005; Nimse and Pal, 2015): $H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$.

Due to the emergence of drug resistance to all classes of antimalarial drugs, the use of anti-oxidants as adjunctive treatment to protect against the increased oxidative stress which then perpetuates disease progression and anaemia has increasingly gained attention (Sandhu *et al.*, 2010). As such 8-hydroxyquinoline derivatives which have been shown to possess some anti-oxidant and antimalarial properties have been selected to determine if they can modulate the degree of haemolysis induced by haem products.

1.6 Compounds: 8-Hydroxyquinoline derivatives

8-Hydroxyquinoline is acquired from both plants and chemical synthesis and is currently used as a fungicide in agriculture, preservative in the production of textiles, wood and paper and is also widely utilised for analytical purposes and separation of metals and most importantly, metal chelation, which forms the basis of its bioactive properties. They are also used medicinally as they possess antiseptic properties and are used in gynaecological preparations for the treatment of vaginal trichomoniasis (Strobl *et al.*, 2009).

Metal (ion) homeostasis in the body needs to be maintained as metal imbalance (excess or loss of metal) is the reason for the development and progression of many diseases. 8-Hydroxyquinoline and its derivatives, due to their strong chelating ability, help restore metal balance and treat such diseases (Prachayasittikul *et al.*, 2013).

The 8-hydroxyquinoline group of compounds has therefore been classified as 'privileged structures' (i.e. display favourable drug-like properties for exploration and further development) as they have proven broad-ranging potential pharmacological effects and applications, which include (Prachayasittikul *et al.*, 2013; Song *et al.*, 2015):

- Neuroprotection agents (as chelators to certain metals e.g. copper, iron and zinc) for the treatment of neurodegenerative diseases and gradually developing neurological deterioration due to metal-iron dysregulation, and oxidative stress by reduction of ROS e.g. in Alzheimer's and Parkinson's diseases (Albrecht *et al.*, 2013).

- Anticancer agents as numerous studies have shown diverse anticancer activity and potential as anticancer agents, as the presence of copper, zinc and iron are considered risk factors (Stevens and Kalkwarf, 2013).

- Inhibitors of 2-oxoglutarate and iron-dependent enzymes which are biotargets for numerous human diseases (Leonarz and Schofield, 2008).

- Chelators of metalloproteins (with miscellaneous therapeutic applications) due also to ability to bind with many metalloproteins (metal-chelating bioactivities) (Jacobsen *et al.*, 2011).

- Anti-viral anti-HIV agents for targeting HIV-1 integrase (Mekouar *et al.*, 1998).

- Antibacterial agents with inhibitory properties against a variety of micro-organisms including *Mycobacterium tuberculosis* (without toxicity to mammalian cells), *Echerichia coli*, *Clostridium perfringens, Clostridium difficile* and other human intestinal bacteria (Jeon *et al.*, 2009; Darby and Nathan, 2010).

- Antifungal agents having equivalent and stronger activity than fluconazole (Musiol *et al.*, 2006).
- Antiparasitic activity has been noted between the inhibition of *P. falciparum* multiplication and the potent chelating ability of the 8-hydroxyquinoline's where the sulfhydryl and amino groups of these compounds interact with the metal enzymes of the parasite. The metalloprotein oxidase enzymes are inhibited, stunting glycolysis and parasite growth (Scheibel and Adler, 1980).
- Antileishmanial agents – having displayed potent inhibition of *Leishmania donovani* (Pandey *et al.*, 2013).
- Antischistosomal agents – having shown inhibitory activities i.e. decrease laying of eggs of adult worms and stop egg deposition (Eweas *et al.*, 2013).
- Botulinum neurotoxin BoNT/A inhibitors – as shown through screening (Dickerson *et al.*, 2014).
- Anti-inflammatory activity by inhibiting nitic oxide production (Kim *et al.*, 2005).
- Miscellaneous activities – as glyoxal/methylglyoxal scavengers, as advanced glycation endproducts (AGE) inhibitors, as radio-protective agents and chemo-sensors, for example (Song *et al.*, 2015; Prachayasittikul *et al.*, 2013).

1.7 Aim

The aim of this project was to determine whether the 8-hydroxyquinoline derivatives can protect the uninfected RBC from haemolysis induced by *P. falciparum* haem products, namely haemin.

1.8 Objectives of the study were to

1) Determine the haemolytic effect of FPIX on fresh human RBC at different concentrations and at varying time intervals.

2) Determine the effect of 8-hydroxyquinoline derivatives on RBC integrity.
3) Evaluate the effect of 8-hydroxyquinoline derivatives on FPIX-induced haemolysis.
4) Determine the ability of 8-hydroxyquinoline derivative to interrupt the oxidative stress pathway as a possible mechanism of action.

CHAPTER 2: METHODOLOGY

2.1 8-Hydroxyquinoline derivatives

Eleven commercially available 8-hydroxyquinoline derivatives were purchased from Sigma-Aldrich (Table 1) and were investigated in comparison to appropriate controls as outlined in the method for each assay. Stock solutions of the derivatives (10 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C until required. All derivatives were screened at two set screening concentrations (50 or 200 µM) and those compounds which elicited >60% effect at either concentration, were further evaluated to determine the concentration of the derivative (3.125 - 200 µM) where 50% of the evaluated activity was inhibited (IC_{50} value).

2.2 Haemolysis assays

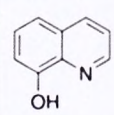
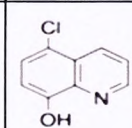
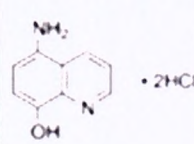
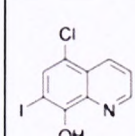
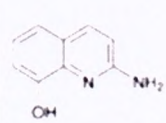
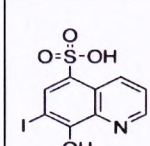
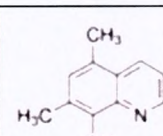
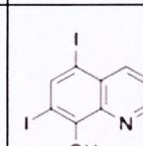
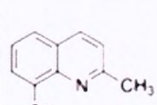
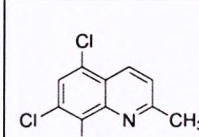
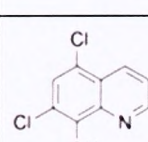
2.2.1 RBC preparation

Whole blood was collected from healthy volunteers (Human Research Ethics Committee clearance certificate obtained according to Appendix A - W-CJ-131030-1) in ACD-vacutainers (acid citrate dextrose blood collection tubes) and stored at 4°C. Blood was used within three days of collection to ensure optimal RBC membrane integrity. RBCs were isolated from the whole blood by washing the tube of blood in phosphate buffered saline (PBS; pH 7.4) and centrifuging (Hettich Zentrifugen Rotofix 32A) at 400*g* for 5 min at room temperature. PBS (1 litre) was prepared using 8 g sodium chloride, 0.3 g potassium chloride, 0.73 g di-sodium hydrogen phosphate dihydrate and 0.2 g monopotassium phosphate, made up with18 MOhm water prepared in-house using a Millipore™ water purification system, sterilized by autoclave and stored at 4°C until required. The whole tube was washed i.e. approximately 4 ml of blood. The plasma and buffy coat were discarded and further washed three times by adding 2 ml PBS to ensure only RBCs remain. Whole blood was washed fresh on the day of each experiment. PBS was added to ensure hydration of the washed RBC's.

Incomplete culture media was made using 10.4 g RPMI-1640, HEPES 5.9 g, glucose 4 g, hypoxanthine 44 mg, and 1 ml of 50 mg/ml gentamycin 10% (v/v) dissolved in 1 litre autoclaved MilliQ-water. Solution was sterile filtered through 0.22 µM filter and stored at

4°C. For each experiment a 1% haematocrit (HCt) was prepared in incomplete culture media either with or without 10% plasma i.e. 3.6 ml RBC in 221.4 ml complete culture media. Complete culture media was prepared by mixing 10 ml human plasma, 4.2 ml sodium bicarbonate and 85.8 ml incomplete culture media. Plasma free media was used for those experiments where the ability of albumin to maintain RBC shape and prevent lysis was to be evaluated.

Table 1: The chemical names, chemical structures and the abbreviations of the tested 8-hydroxyquinoline derivatives.

Test compound, molecular mass and logP value	**Structure**	**Test compound, molecular mass and logP value**	**Structure**
8-Hydroxyquinoline 8-OH 145.15 g/mol XlogP$_3$ = 2		**5-Chloro-8-hydroxyquinoline** 5-Cl-8OH 179.60 g/mol XlogP$_3$ = 2.9	
5-Amino-8-hydroxyquinoline (dihydrochloride) 5-NH$_3$-8OH-DiHCl 160.17 g/mol XlogP$_3$ = 1.6		**5-Chloro-7-iodo-8-hydroxyquinoline** 5-Cl-7-I-8OH 305.49 g/mol XlogP$_3$ = 3.5	
2-Amino-8-quinolinol 2-NH$_2$-8OH 160.17 g/mol XlogP$_3$ = 1.5		**8-Hydroxy-7-iodoquinoline-5-sulfonic acid** 7-I-5-SO$_3$H-8OH 351.12 g/mol XlogP$_3$ = 1.2	
5,7-Dimethyl-8-hydroxyquinoline 5,7-DiCH$_3$-8OH 173.21 g/mol XlogP$_3$ = 2.7		**5,7-Diiodo-8-hydroxyquinoline** 5,7-Dii-8OH 396.96 g/mol XlogP$_3$ = 3.1	
2-Methyl-8-hydroxyquinoline 2-CH$_3$-8OH 159.18 g/mol XlogP$_3$ = 2.3		**5,7-Dichloro-8-hydroxy-2-methylquinoline** 5,7-DiCl-2-CH$_3$-8OH 215.05 g/mol XlogP$_3$ = 3.5	
5,7-Dichloro-8-quinolinol 5,7-DiCl-8OH 228.07 g/mol XlogP$_3$ = 3.5			

2.2.2 FPIX, albumin and Fe(III)citrate preparation

Stock solutions (10 mM) were prepared: FPIX (1 ml in DMSO), albumin (10 ml in PBS) and Fe(III)citrate (1 ml in NaOH). These solutions were freshly made for each experiment to prevent degradation upon standing. All chemicals used were of analytical grade and obtained from Merck and Sigma-Aldrich.

2.2.3 Effect of haemin on haemolysis

The effect of various concentrations of free FPIX on the RBC lysis was assessed by incubating RBC's concurrently with increasing concentrations of FPIX (5, 50, 100 and 200 µM) to determine if the lysis was concentration dependent. The protocol as described in Section 2.2.5 was followed; except that the absorbance of the supernatant was determined at 540 nm hourly for the first 4 hours, then at 12 and 24 hours of incubation at 37°C in a humidified chamber.

2.2.4 Effect of various factors:

The influence of the following factors on the degree of haemolysis were evaluated and the protocol as described in Section 2.2.5 followed.

2.2.4.1 Presence of albumin

Albumin which is normally present in blood plasma at high concentrations, maintains the biconcave shape of the normal erythrocyte and protects against cell lysis by stabilising the shape. As such the ability of albumin to bind drugs thus preventing FPIX-induced lysis and protect RBC's from lysis was assessed. A dose response curve was generated by incubating 200 µM human albumin in the absence and presence of FPIX at 5, 50, 100 and 200 µM to observe a possible protective effect. All chemicals used were of analytical grade and obtained from Merck.

2.2.4.2 Presence of Fe(III)citrate

To assess the effect on RBC membrane permeability under oxidative stress induced by iron, 200 µM Fe(III)-citrate was incubated with the RBCs.

2.2.4.3 Effect of 8-hydroxyquinoline derivatives on haemolysis

The effects of the 8-hydroxyquinoline derivatives (200 µM) on the degree of haemolysis in the presence of FPIX (5, 50, 100, 200 µM) was determined in the presence of ethylenediaminetetraacetic acid (EDTA) (200 µM), albumin and Fe(III)-citrate, Trolox (5, 50, 100, 200 µM), culture media, 2% Triton X-100 (Triton X-100 in PBS) and 1% HCt in culture media. Triton X-100 was the positive control.

2.2.5 Preparation of experimental plates

To evaluate the effects of the 8-hydroxyquinoline derivatives on haemolysis, 25 µl of each derivative (200 µM) was pipetted into triplicate wells along with 25 µl of FPIX (200 µM), 25 µl of culture media and 200 µl 1% HCt RBC suspension. The positive control 2% (v/v) Triton X-100 was used to induce 100% lysis and culture media was used as the negative control to induce 0% lysis. Plates were then incubated (Prolab incubator) at 37°C for the predetermined time period of 24 hours. Plates were thereafter tapped gently to disrupt pellets and centrifuged for 5 minutes at 350 g (Thermo Scientific Heraeus Megafuge 40 Centrifuge). Of the supernatant, 50 µl was removed and pipetted into a non-sterile plate with 150 µl Millipore™ water. The plate was gently tapped to remove all bubbles and read at 412 nm (porphyrin) and 540 nm (iron) in a plate reader (Labsystems iEMS Reader MF V2 9-0) linked to a computer programme (Ascent Software version 2.6) (which was also used for all assays). The % haemolysis was calculated as follows:

$$\%\ Haemolysis = \frac{Abs\ (test\ compound) - Abs\ (0\%\ lysis\ control)}{Abs\ (100\ \%\ lysis\ control) - Abs\ (0\ \%\ lysis\ control)} \times 100$$

2.3 Anti-oxidant assays

2.3.1 Free radical scavenging activity

The 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\bullet}$) assay was used to estimate the free radical scavenging activity of the test compounds. $DPPH^{\bullet}$ (violet) is a stable free radical (has an unpaired valence electron) and when mixed with a free radical scavenging anti-oxidant (i.e. hydrogen donor), the $DPPH^{\bullet}$ absorbance was reduced (yellow). Therefore, in this

assay, the ability of the test compound to act as a donor of hydrogen or electrons in the transformation of DPPH$^{\bullet}$ into its reduced form (DPPH-H) was measured. Lower IC_{50} values indicated higher anti-oxidant activity (Kadri *et. al.*, 2011). Ascorbic acid and Trolox™ were used as positive controls.

DPPH$^{\bullet}$ solution (96.2 µM) was made to volume required with HPLC grade methanol, thereafter covered with foil, stirred with a magnetic stirrer for 15 minutes and stored at 4°C overnight prior to use. The test derivatives were incubated in the dark for 30 minutes at room temperature with DPPH$^{\bullet}$ solution in non-sterile flat-bottom plates, with initial screening at 200 µM in triplicate wells (Gülcin, 2009). Negative controls - (0% controls) contained 200 µl of methanol and 200 µl of DMSO; and the 100% controls wells contained 200 µl of DPPH$^{\bullet}$ and 50 µl of DMSO. Each further well contained 200 µl of DPPH$^{\bullet}$ and 50 µl of 200 µM compound solution. After the 30 min period, the absorbance of each well was read at 540 nm. The calculation to determine the DPPH$^{\bullet}$ scavenging effect (percentage decolourisation) was as follows (Gülcin, 2009):

$$\% \text{ Free radical scavenging} = \frac{\text{Abs (test compound)} - \text{Abs (colour control)}}{\text{Abs (untreated control)} - \text{Abs (colour control)}} \times 100$$

2.3.2 Iron chelation activity

The chelating activity on ferrous irons (Fe^{2+}) was measured by incubating the compounds with Fe^{2+} and thereafter measuring if the purple coloured Fe^{2+}-ferrozine complex formation was inhibited, resulting in a reduction of the intensity of the purple colour of the complex. The positive control was EDTA at the same starting concentration as test compounds (200 µM). DMSO was added as the control for 100% Fe^{2+}-ferrozine complex formation.

Each compound (20 µl) was added to triplicate wells in non-sterile flat bottom 96 well plate (screened at 200 µM). DMSO/solvent (20 µl) was added as the 100% ferrozine-chelation control (A_0). $FeCl_2$ (30 µl) was added to all wells and the side of the plate was gently tapped to mix reagents. $FeCl_2$ (2 mM) was freshly prepared for each experiment, to prevent degradation upon standing, by dissolving 4.772 mg $FeCl_2$ (measured using

the micro-electronic scale which is accurate to 1 mg with a five decimal point) in 12 ml 5% (w/v) ammonium acetate). The plate was left to stand in the dark at room temperature for 5 minutes. Ferrozine (1.2 mM; 100 µl) was added to all wells. The plate was incubated at room temperature in the dark for 10 minutes. Absorbance was then read at 540 nm. The percentage iron chelation was determined using the following equation (Gülcin, 2009) and compared to that of EDTA which served as the positive control. The concentration required to chelate 50% Fe(II) ($Fe(II)C_{50}$) was determined for those compounds that chelated more than 60% of the added iron.

Iron chelating activity (%) was calculated using the formula for bound ferrous ions:

$$\% \text{ Iron chelation} = \frac{(Abs_{100\%} - Abs_{Test}) \times 100}{Abs_{100\%}}$$

where Abs control = mean absorbance for 100% Fe^{2+} - ferrozine complex formation.

2.3.3 Iron reducing activity

The capability of the compounds to reduce ferric ions [$Fe^{3+}(CN^-)_6$ to $Fe^{2+}(CN^-)_6$] was measured using the method as described by Gülcin (2009). The higher absorbance of the reaction mixture indicated greater reducing power. The positive control was Trolox™ with the 10 mM stock prepared in DMSO and stored at –20°C. Trolox used as it was the control in the whole cell assay which would allow intracellular action due to lipophilicity. Depending on the reducing power of the compounds, the yellow colour of the test solution changes to various shades of green or blue. The solutions were freshly made for each experiment to prevent degradation upon standing.

In microfuge tubes, the following were added, 100 µl of 200 µM compound (prepared in triplicate), 250 µl $NaPO_4$ buffer (pH 6.6) and 250 µl (1% w/v) $K_3(Fe(CN)_6$. The microfuge tubes were incubated at 50°C for 20 minutes. Thereafter 250 µl 10% (v/v) trichloracetic acid was added and centrifuged for 10 minutes at 450g. Thereafter the following were added to a new microfuge tube: 250 µl 'upper layer' of the previous reaction, 250 µl water and 75 µl $FeCl_3$ (0.1% w/v). A volume of 150 µl of the latter was transferred to

triplicate wells in a non-sterile flat bottom 96-well plate and the absorbance read at 690 nm (Gülcin, 2009).

In order to calculate the Trolox™ equivalent anti-oxidant capacity (TEAC), a standard curve of Trolox™ was constructed by incubating a range of Trolox™ concentrations in the above reaction to generate a linear regression curve (r^2 = 0.9938) (Figure 6) from which the equation y=mx+c was determined using GraphPad Prism and then used to calculate the TEAC value for each 8-hydroxyquinoline derivative.

2.3.4 Lipid peroxidation activity

The ferric thiocyanate method was used to evaluate the effect of the compounds on preventing peroxidation of a linoleic acid emulsion – the percentage inhibition of lipid peroxidation was calculated. All compounds were screened at a standard concentration of 200 µM and compared to the positive control Trolox™. The linoleic acid mixture without the addition of sample was used as the negative control.

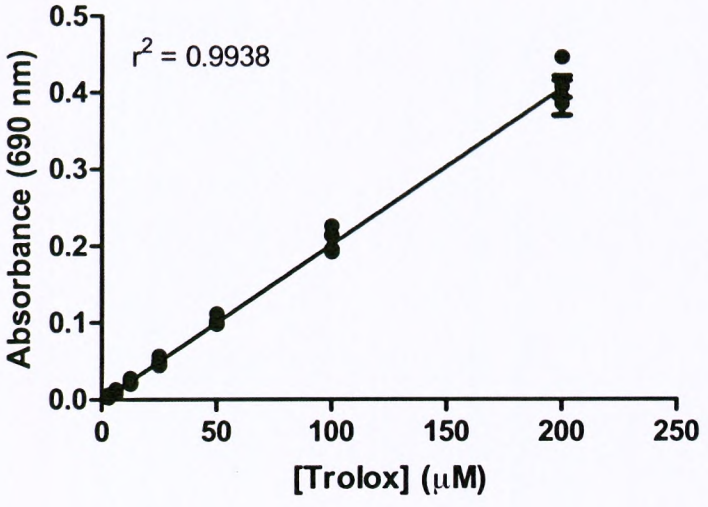


Figure 6: Trolox™ standard curve (iron-reducing activity).

Sodium phosphate buffer (0.2 M, pH 7.0) was prepared by mixing Solution A and Solution B (78 ml of A and 122 ml of B to make a total volume of 200 ml); where solution A consisted of 2.76% (w/v) $NaH_2PO_4.H_2O$ and solution B consisted of 3.56% (w/v) $Na_2HPO_4.2H_2O$. The test compounds (25 µl; 200 µM) were plated in triplicate, with

DMSO as a negative control plated in row H. These were reacted with 125 µl linoleic acid emulsion and 100 µl sodium phosphate buffer in a non-sterile 96 well plate. Linoleic acid emulsion (100 % v/v) was prepared fresh by mixing 51.2 µl linoleic acid and 51.2 µl Tween-20 (emulsified in the Tween) and the volume made up to 10 ml with sodium phosphate buffer. The 0% lipid peroxidation control with 125 µl buffer added and no linoleic acid emulsion. The mixture was shaken in a plate shaker at 1300 rpm for 1 minute and incubated for 37°C for 24 hours. Thereafter, 25 µl of the latter reaction was reacted with 70% ethanol (150 µl), 30 g/L ammonium thiocyanate (40 µl) and 40 µl of 5 mM ferrous chloride (prepared fresh in 3.5% (v/v) HCl). The absorbance was read at 492 nm. If the absorbance was greater than 1, the supernatant was diluted four fold (50 µl reaction solution and 150 µl 70% ethanol). Inhibition of lipid peroxidation was calculated using Trolox™ equivalent anti-oxidant capacity (TEAC). A standard curve of Trolox™ was constructed (r^2 = 0.952) (Figure 7), from which the equation y=mx+c was used to calculate TEAC value for each 8-hydroxyquinoline derivative.

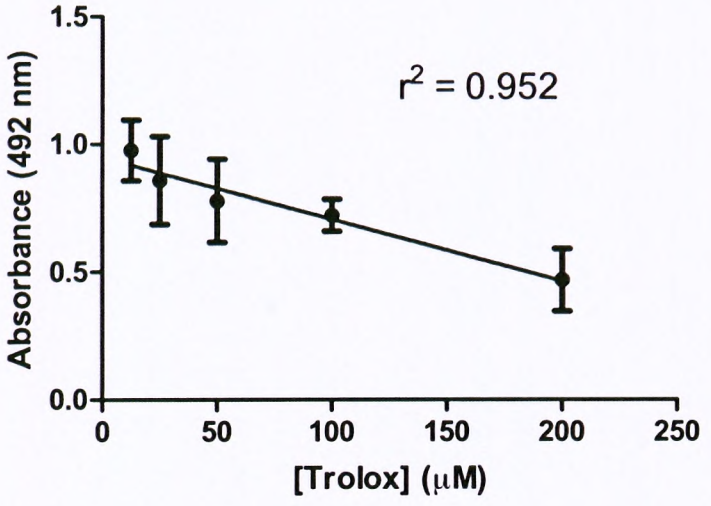


Figure 7: Trolox™ standard curve (lipid peroxidation activity)

2.4 Data analysis

Each derivative was evaluated in triplicate and each assay repeated at least in triplicate from which the mean and standard deviation (s.d.) was calculated. The IC_{50} value (i.e

half maximal inhibitory concentration) was determined using a log sigmoid dose response curve plotted using the Graphpad Prism version 3.0 for all assays

2.5 Human ethics

Human ethics and biosafety clearance was obtained from Wits Medical School Human Research Ethics Committee and Institutional Biosafety Committee in order to work with human blood and plasma. Certification was obtained for the use of human blood (W-CJ-131030-1, M090532, M140669 and 20090503) as well as approval from the Institutional Biosafety Committee (Appendix A and B). Human blood was obtained from healthy individuals not on antibiotics/warfarin/heparin or medication known to affect RBC or haematological status such as aspirin, anticoagulants, chemotherapy medicines, chloramphenicol, hydantoins and quinidine.

CHAPTER 3: RESULTS

3.1 Haemolysis assays

3.1.1 Haemin-induced RBC lysis activity

The plate layout for the RBC lysis assays i.e. FPIX at 5, 50, 100 and 200 µM alone, and the positive and negative controls are depicted in Figure 8 below.



Figure 8: Dose dependent lysis of FPIX alone in comparison to positive and negative controls.

Triton X-100 was used to treat the RBC's in the last row of the plate (Figure 8 above) and demonstrated 100% lysis, rapidly as lysis occurred within 5 minutes. Being a commonly used detergent in laboratories, a non-ionic surfactant, it is a proven haemolytic agent and permeabilises the membranes of animal cells. In the presence of Triton X-100 the hydrogen bonding in the cell's lipid bilayer is disrupted, leading to the integrity and tightness of the lipid membrane being compromised and thereby affecting cellular structure (Koley and Bard, 2010). Complete culture media was used to demonstrate 0% lysis, as it is isotonic and would not induce any lysis but would support cell growth by supplying a source of energy and nutrients which regulate the cell cycle (Yang and Xiong, 2012).

The haemin-induced RBC lysis activity was found to be both time and concentration dependent, as shown in Figure 9 and Figure 10. The degree of lysis was confirmed visually in Figure 8 taken at the 24 hour time-point. The greatest percentage of lysis was seen at the highest concentration of 200 µM at 24 hours. FPIX-induced lysis was therefore shown to be slower acting than Triton X-100.

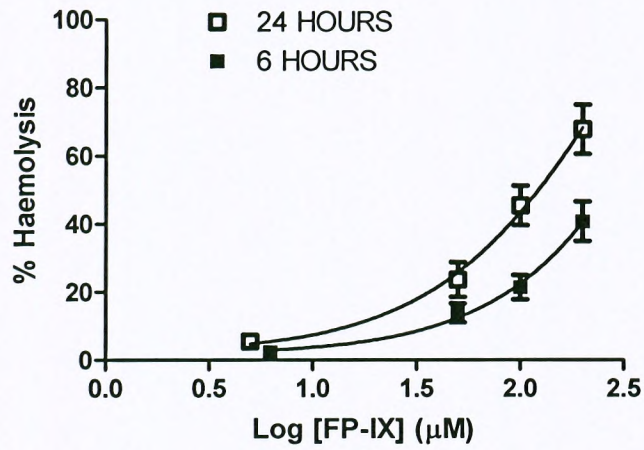


Figure 9: Percentage FPIX-induced haemolysis after 6 hour and 24 hour time periods.

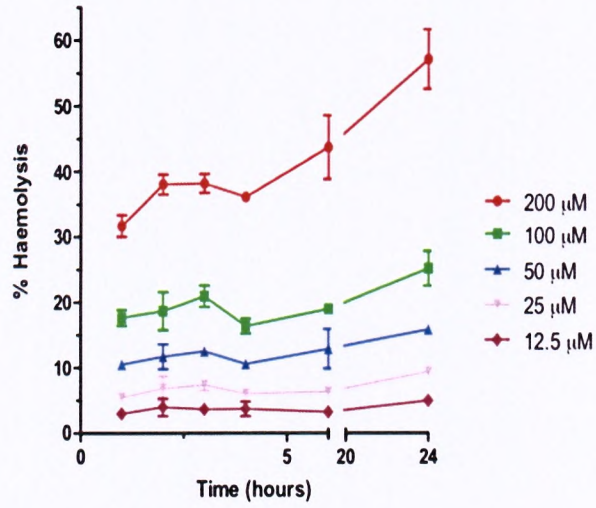


Figure 10: Percentage FPIX-induced haemolysis for varying times (0-24 hours).

At the 24 hour time-point the 12.5, 25 and 50 μM concentrations, the lysis range was between 2.5% and 15%, therefore only a small degree of lysis was induced, with trending following the 12.5 μM graph. The greatest degree of lysis (55%) was shown at the 200 μM after 24 hours incubation.

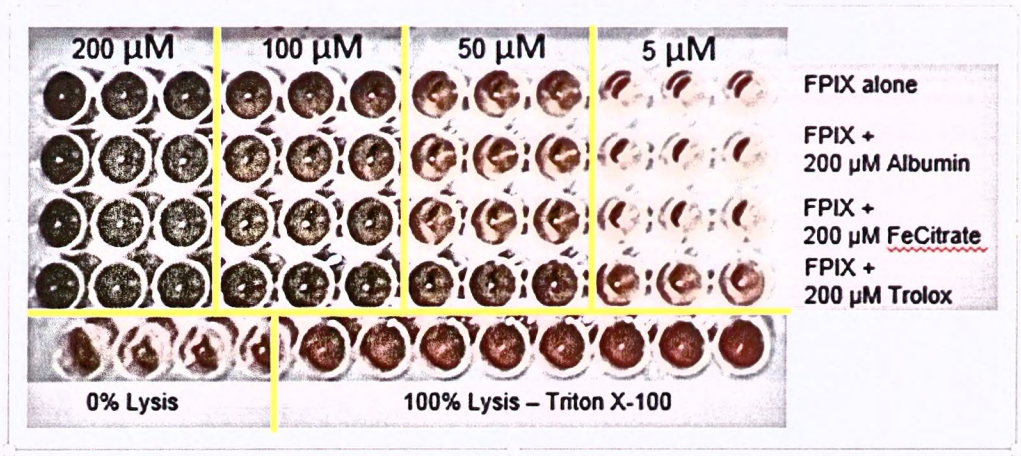


Figure 11: Effect of albumin, iron and Trolox™ on FPIX-induced lysis after 24 hours

The degree of lysis visually observed in Figure 11 with FPIX alone, and combined with albumin, iron citrate and Trolox™ corresponded to the results presented in Figures 12 to 14 below, i.e. the effect of albumin and iron on FPIX-induced RBC lysis was concentration dependent and followed the same profile as FPIX. Trolox™ with FPIX did however induce lysis in a dose dependant manner. Omarova and Antonenko (2014) reported that 3 mM Trolox™ protected against oxidative haemolysis. Thus it is possible that the 200 µM Trolox™ used in this study was insufficient to protect the RBC against the FPIX-induced haemolytic effects and higher concentrations are required to protect the RBC".

3.1.2 Effect of albumin on FPIX-induced RBC lysis

When albumin was added to FPIX, the concentration dependent effect of FPIX was still observed (Figure 11 and 12). The FPIX with albumin lysis results followed the same trend as the FPIX alone results in terms of concentration and percentage lysis, as seen below, with slightly decreased lysis activity noted.

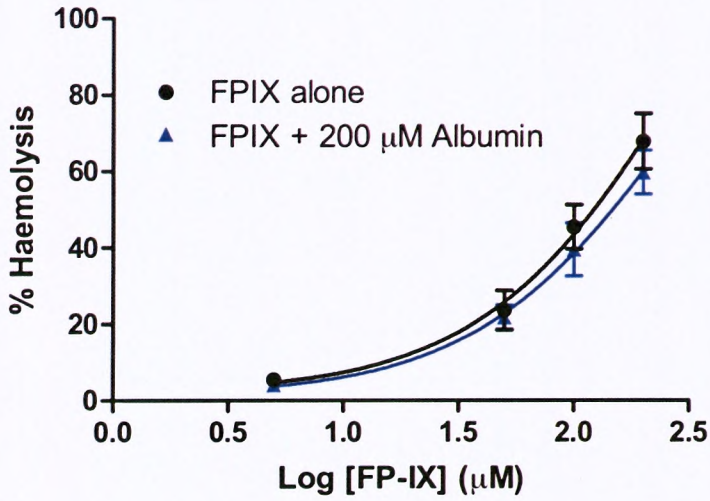


Figure 12: Effect of Albumin on FPIX-induced RBC lysis after 24 hours

3.1.3 Effect of iron on FPIX-induced RBC lysis

A similar trend to the appearance of the dose-response curve for albumin was shown with FPIX and iron (Figure 13). Iron citrate decreased lysis induced by 5 μM FPIX by approximately 2 fold.

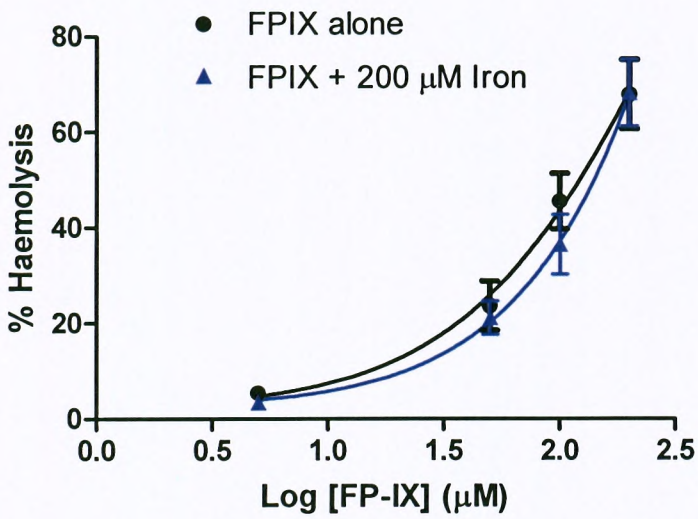


Figure 13: Effect of Fe(III)citrate on FPIX-induced RBC lysis after 24 hours.

3.1.4 Effect of Trolox™ on FPIX-induced RBC lysis

The various concentrations of Trolox™ tested produced a dose response effect as observed for the same concentrations of FPIX alone (Figure 11 and Figure 14). The 200 µM Trolox™ did show a dose response effect when plotted against the changing concentrations of FPIX as seen below, however at the lower concentrations (5 and 10 µM) appeared to have induced more lysis than inhibiting it. The most favourable activity was at 200 µM, where the haemolytic activity was decreased by approximately 30%.

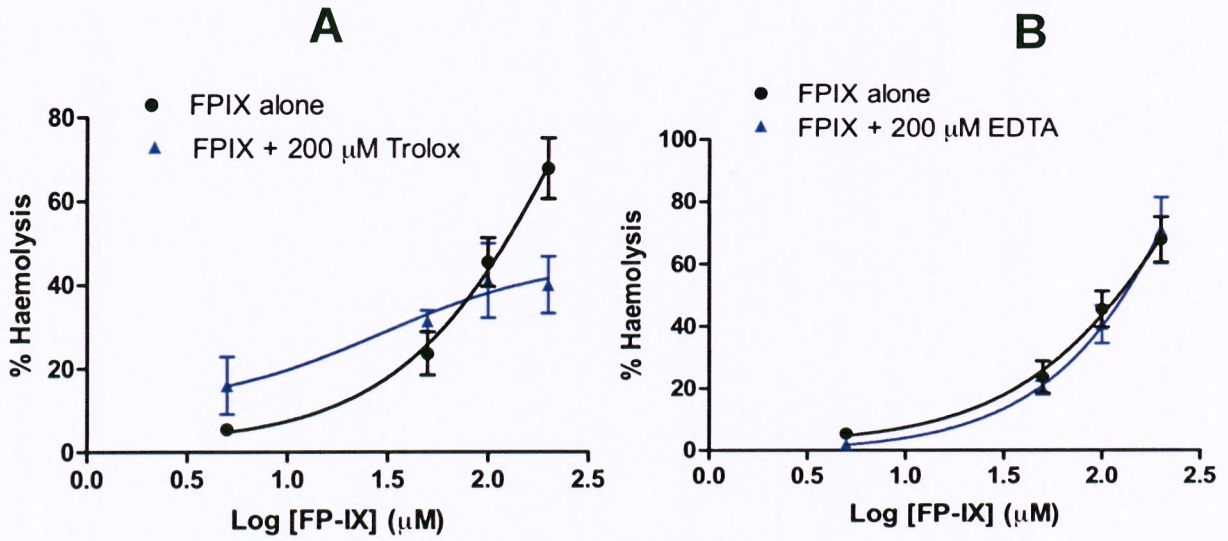


Figure 14: Effect of Trolox™ (A) and EDTA (B) on FPIX-induced RBC lysis after 24 hours

3.1.5 Effect of EDTA on FPIX-induced RBC lysis

EDTA, a non-specific metal chelating agent was found to have a minimal effect in reducing FPIX-induced haemolysis (Figure 14).

3.1.6 Effect of 8-hydroxyquinolines on haemolysis

The eleven 8-hydroxyquinoline compounds were incubated with FPIX at 5, 50, 100 and 200 µM concentrations. The dose response curves for each compound are presented below (Figure 16-26).

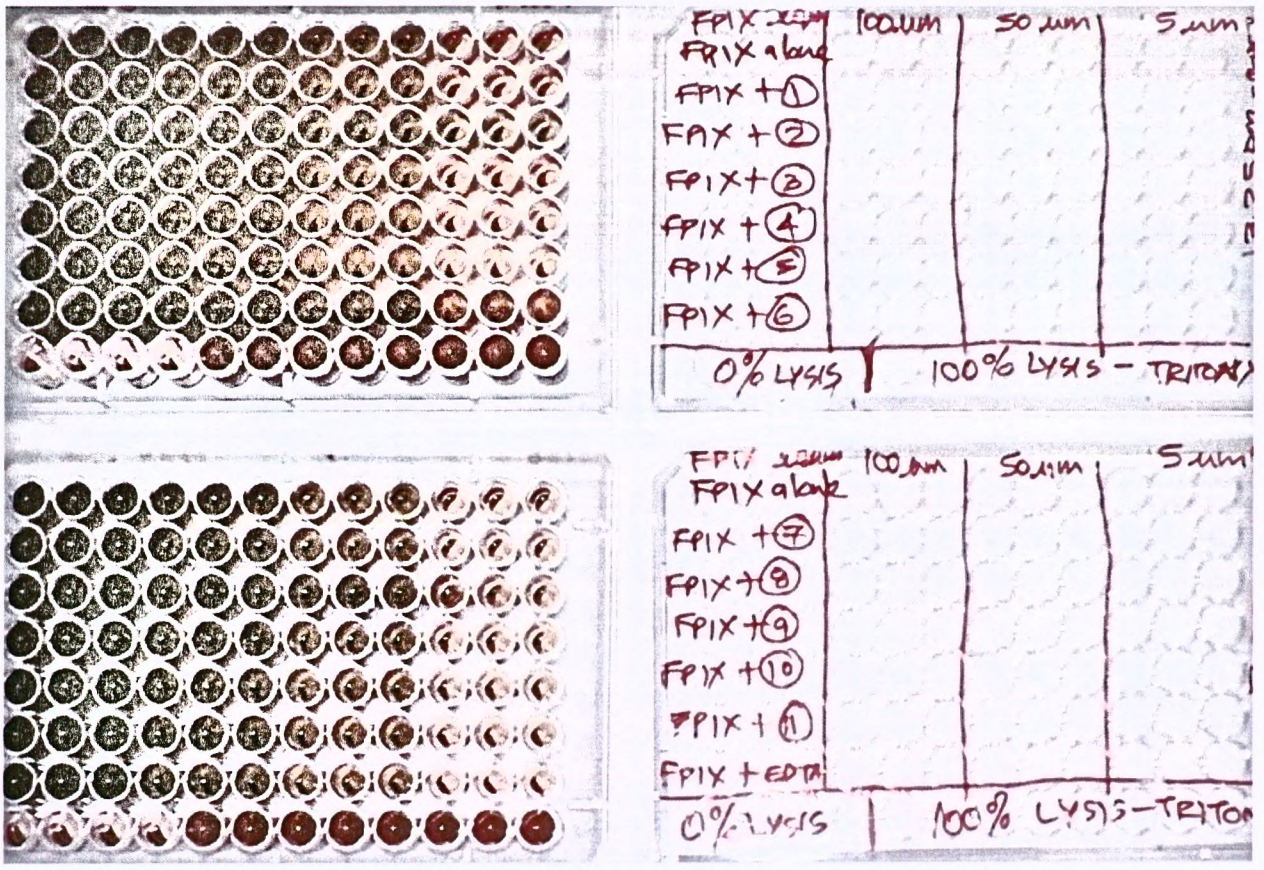
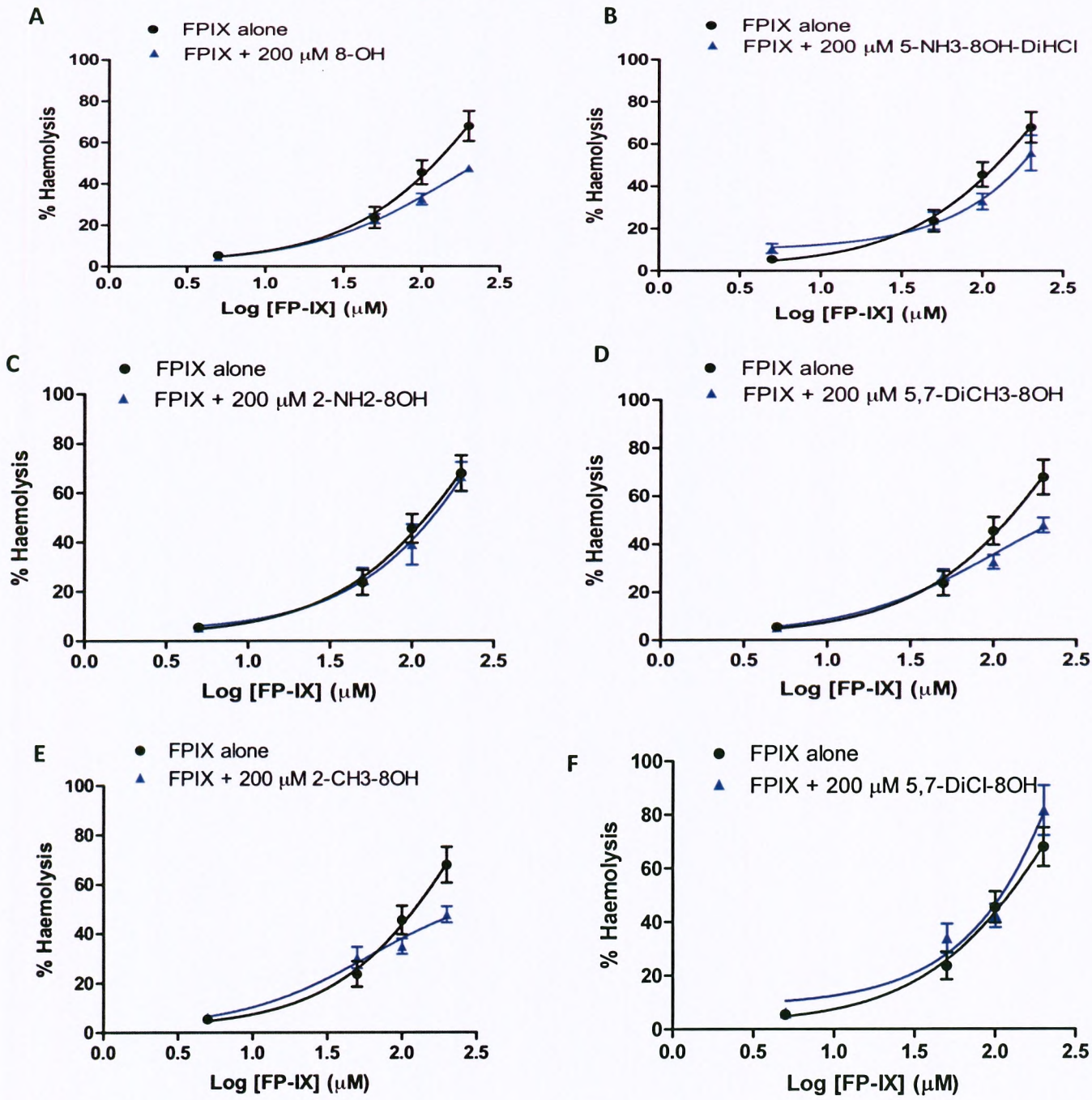


Figure 15: Concentration effect of 8-OH compounds on FPIX-induced haemolysis.

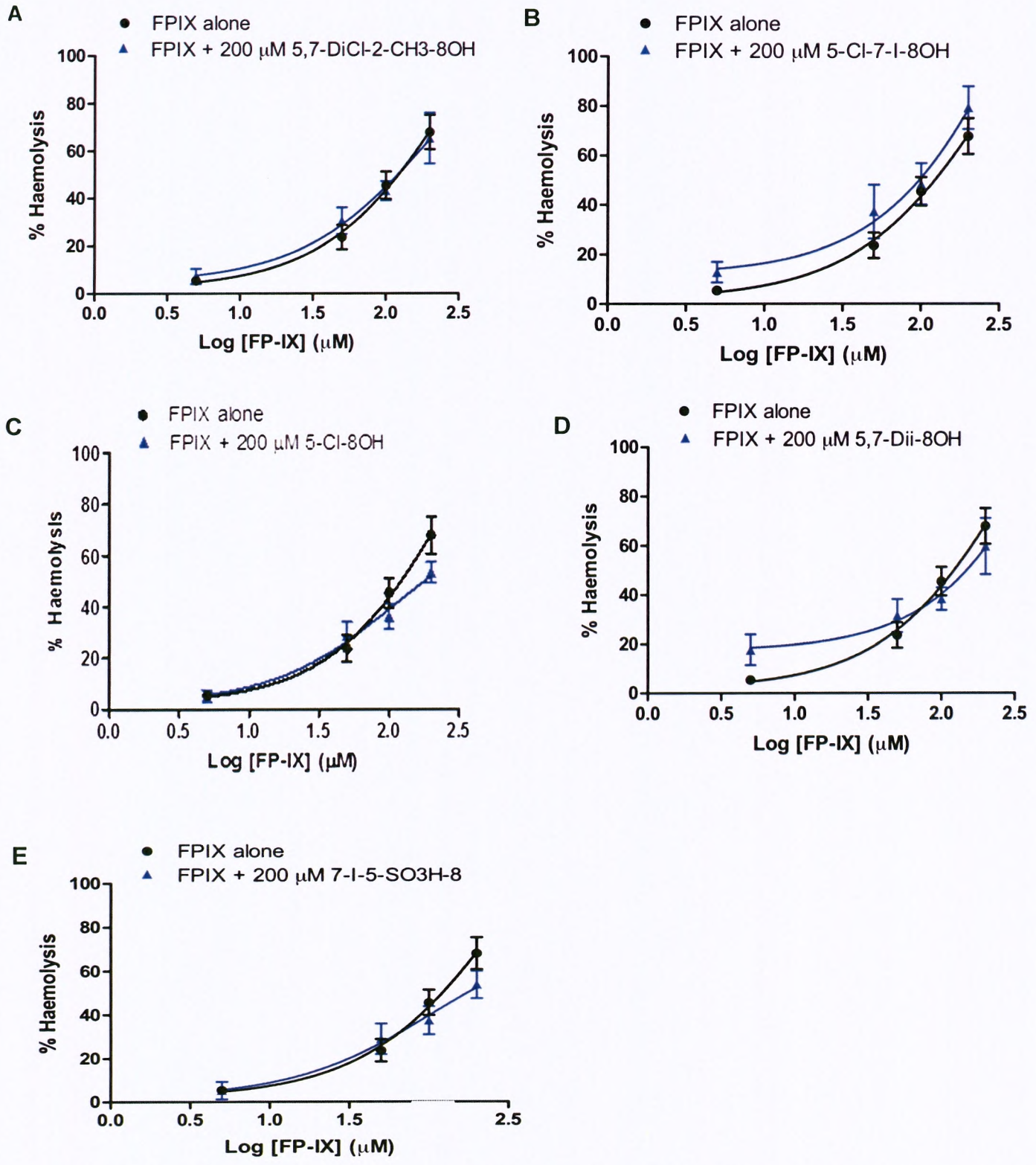
Figures 17B and 17D show that compounds 5-Cl-7-I-8OH and 5,7-Dii-8OH induced a minimal increase in haemolysis compared to FPIX alone. These compounds therefore offered no protective effect to the RBC's. Figures 17C and 17E show that compounds 7-I-5-SO_3H-8OH and 5,7-DiCl-2-CH_3-8OH had a similar effect to FPIX, and neither induced or prevented haemolysis.

At 5 µM FPIX, the effect of the 8OH on the % haemolysis induced was variable with 5,7-DiCl-8-OH inducing significantly more haemolysis than the other compounds. Derivatives, 5-NH_3-8OH-DiHCl, 5-Cl-7-I-8OH and 5,7-Dii-8OH also increased haemolysis in the range of 4.08-10.97% more than that of FPIX alone (Figures 16B,

17B and 17D). Increased haemolysis was also seen at higher FPIX concentrations i.e. at 200 µM FPIX for 5,7-DiCl-8-OH and 5-Cl-7-I-8OH.



Figures 16 A-F: 24 Hour FPIX alone and FPIX with A. 8-OH, B. 5-NH_3-8OH-DiHCl, C. 2-NH_2-8OH, D. 5,7-DiCH_3-8OH, E. 2-CH_3-8OH and F. 5,7-DiCl-8OH respectively.



Figures 17 A-E: 24 Hour FPIX alone and FPIX with A. 5-Cl-8OH, B. 5-Cl-7-I-8OH, C. 7-I-5-SO_3H-8, D. 5,7-Dii-8OH and E. 5,7-DiCl-2-CH_3-8OH respectively.

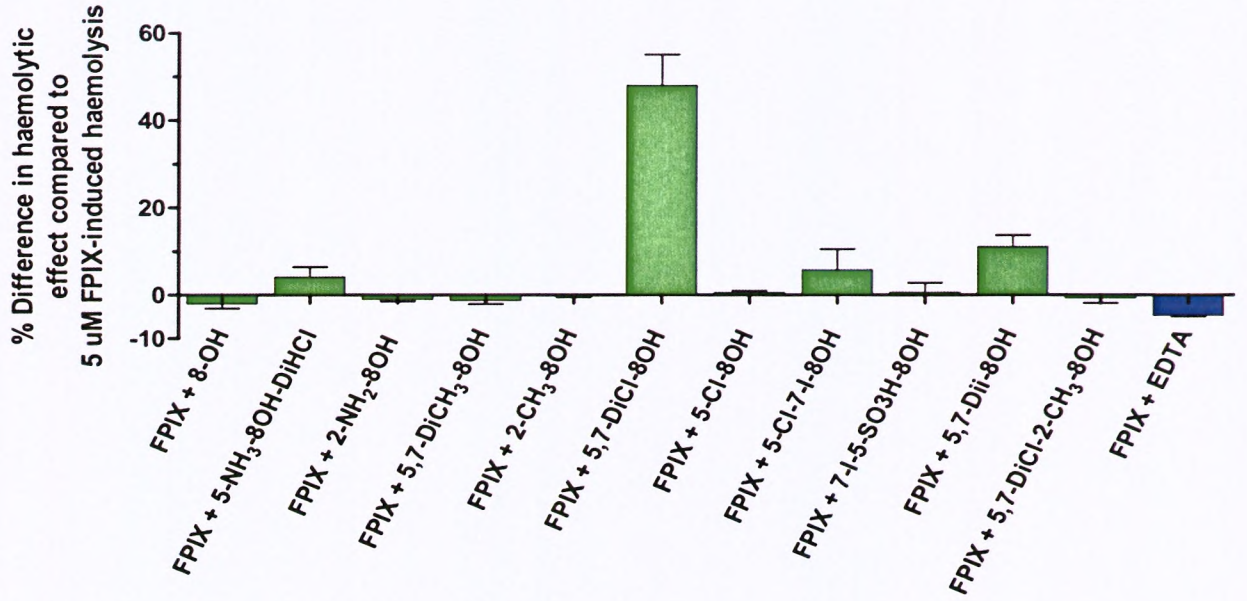


Figure 18: Percentage difference in haemolytic effect compared to 5 μM FPIX-induced haemolysis

3.2 Anti-oxidant assays

3.2.1 Free radical scavenging activity

The compounds which showed the most scavenging activity were 5-amino-8-hydroxyquinoline (IC_{50} value: 12.12 ± 1.36 μM), 5,7-dimethyl-8-hydroxyquinoline (IC_{50} value: 12.85 ± 1.65 μM) and 2-amino-8-quinolinol (IC_{50} value: 14.79 ± 0.94 μM). The IC_{50} value of Trolox and ascorbic acid (100 μM) were shown to be 18.34 ± 1.16 μM and 25.77 ± 3.14 μM respectively (Table 2).

3.2.2 Iron chelation activity

The measurement of the absorbance of the ferrous ion-ferrozine complex displayed the chelating activity of the test compounds, and was compared to that of EDTA. The Fe(II)C_{50} value of the same concentration (200 μM) of EDTA was shown to be 24.85 ± 3.12 μM (Figure 19). Fe(II)C_{50} values for compounds 5,7-dimethyl-8-hydroxyquinoline, 5,7-dichloro-8-quinolinol, 5-chloro-7-iodo-8-hydroxyquinoline, 8-hydroxy-7-iodoquinoline

-5-sulfonic acid, 5,7-diiodo-8-hydroxyquinoline and 5,7-dichloro-8-hydroxy-2-methylquinoline were found to be lower chelating activity, whereas 8-hydroxyquinoline, 5-amino-8-hydroxyquinoline dihydrochloride and 2-amino-8-quinolinol showed very similar activity. The compound with more potent chelating activity than EDTA was 2-methyl-8-hydroxyquinoline ($Fe(II)C_{50}$ value: 5.67 ± 1.45 µM). A lower $Fe(II)C_{50}$ value shows considerable higher chelating activity.

Table 2: Free radical scavenging activity of 8-hydroxyquinoline derivatives compared to Ascorbic acid and Trolox™.

Compound (200 µM)	% Free radical scavenged at 200 µM		$Fe(II)C_{50}$ (µM)	
	Average	SD	Average	SD
8-hydroxyquinoline	82.83	12.54		
5-amino-8-hydroxyquinoline	34.19	9.73	12.12	1.36
2-amino-8-quinolinol	30.87	7.24	14.79	0.94
5,7-dimethyl-8-hydroxyquinoline	20.66	5.13	12.85	1.65
2-methyl-8-hydroxyquinoline	77.91	6.58		
5,7-dichloro-8-quinolinol	83.97	0.46		
5-chloro-8-hydroxyquinoline	91.36	2.86		
5-Chloro-7-iodo-8-hydroxyquinoline	78.62	9.20		
8-hydroxy-7-iodoquinoline-5-sulfonic acid	87.20	4.23		
5,7-diiodo-8-hydroxyquinoline	86.63	1.96		
5,7-dichloro-8-hydroxy-2-methylquinoline	73.27	6.25		
Trolox™ (100 µM)	18.54	5.65	18.34	1.16
Ascorbic acid (100 µM)	18.34	4.77	25.77	3.14

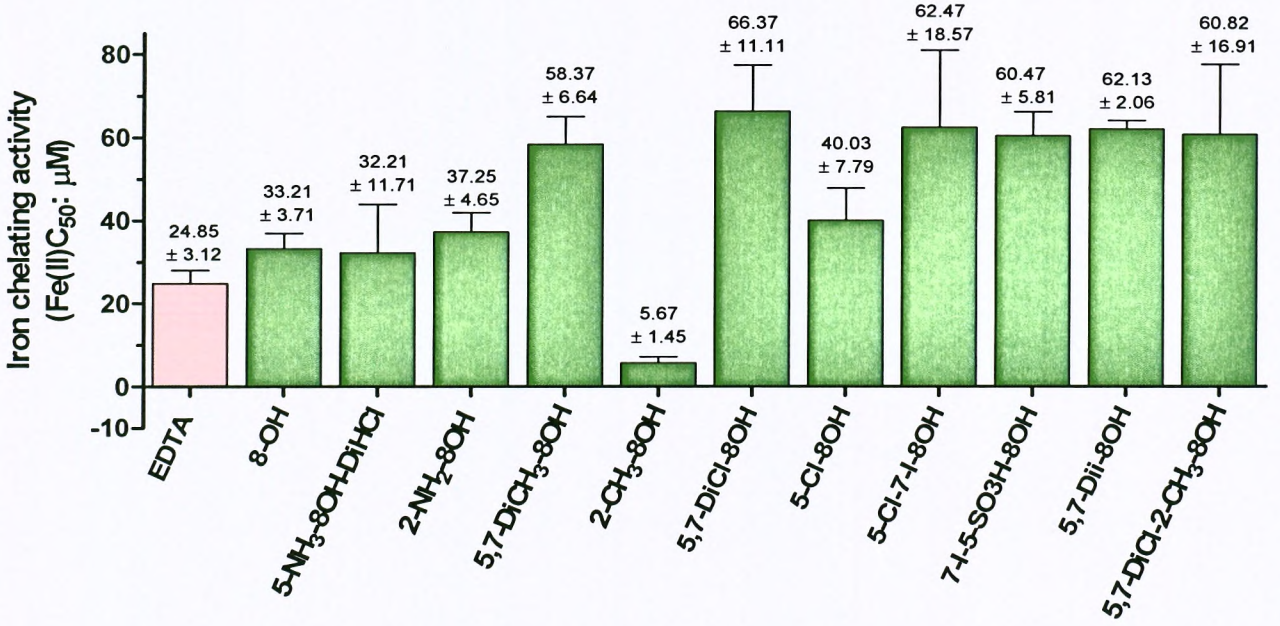


Figure 19: Iron chelating effect of 8-hydroxyquinoline derivatives compared to EDTA.

3.2.3 Iron reducing activity

The activity of each compound was assessed by the following – the greater the absorbance of the reaction mixture, the greater the iron reduction capability. As can be seen from Figure 20 below, all compounds displayed strong Fe^{3+} reducing ability when compared to the standard Trolox™ and therefore showed the potential to stop the destructive effects of radical chain reactions.

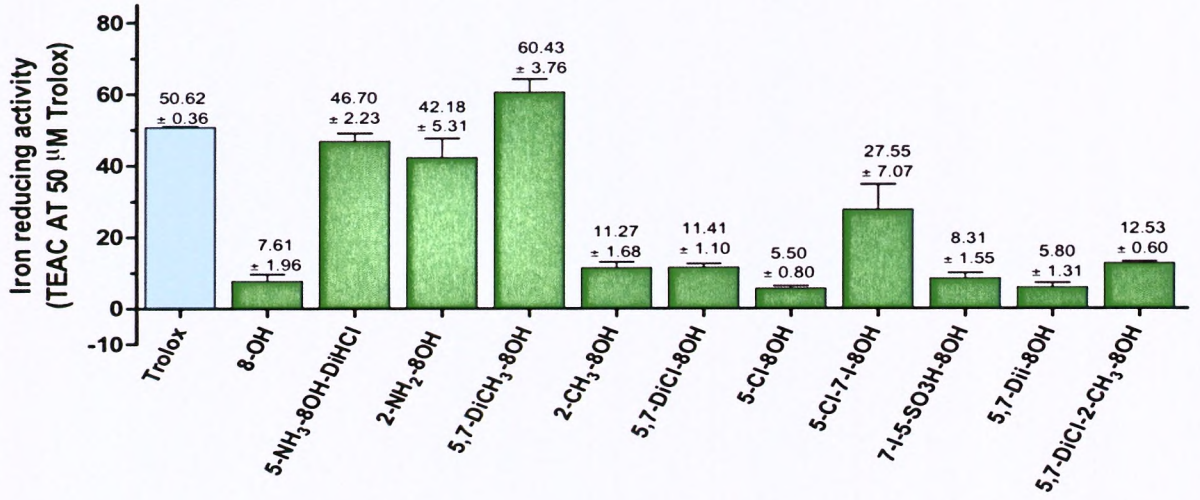


Figure 20: Iron reducing effect of 8-hydroxyquinoline derivatives compared to Trolox™.

3.2.4 Lipid peroxidation activity

Lipid peroxidation is a marker of oxidative cell damage (Gutteridge, 1995). The ability of the 8-hydroxyquinoline compounds to inhibit lipid peroxidation (i.e. inhibit the amount of peroxide produced during oxidation) was assessed. The percentage lipid peroxidation effect of 200 µM of the derivatives is shown in Figure 21. All the 8-hydroxyquinoline derivatives inhibited lipid peroxidation activity except 5-amino-8-hydroxyquinoline.

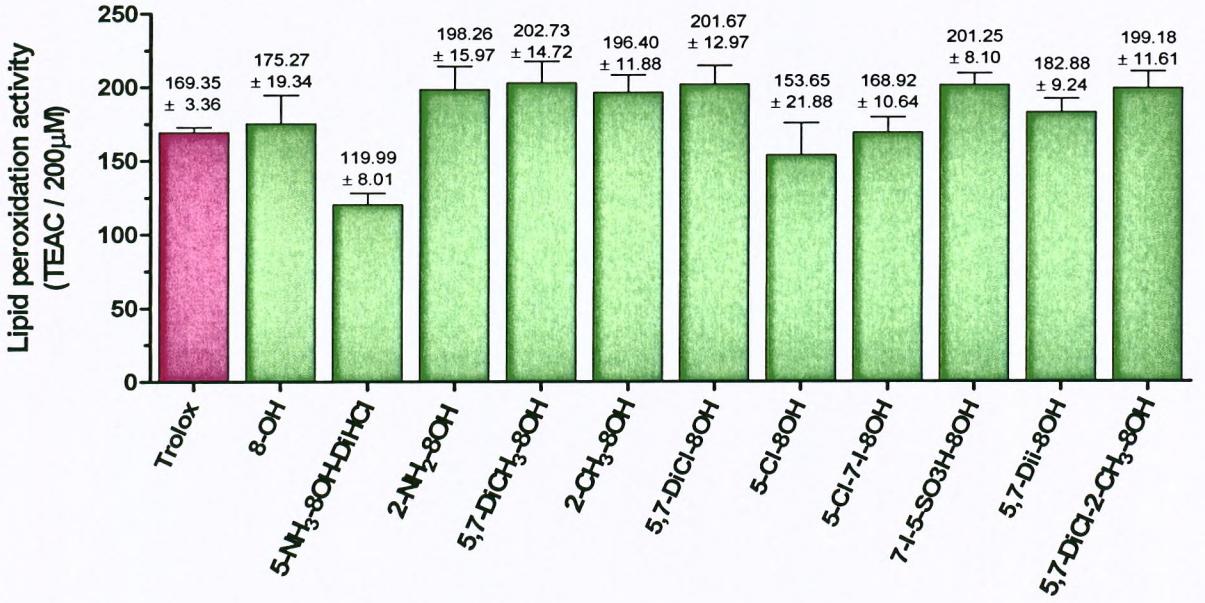


Figure 21: Lipid peroxidation effect of 8-hydroxyquinoline derivatives compared to Trolox™ (i.e. concentration of peroxidation product detected after treating with a constant concentration of 8-hydroxyquinoline derivative)

CHAPTER 4: DISCUSSION

The development of the intra-erythrocytic parasite continues through the ring, trophozoite and schizont stages (Hanssen *et al.*, 2010), and due to these continuous growth and replication activities the host cell is irreversibly changed. When haemoglobin is released from infected RBC's due to haemolysis, it is no longer guarded by anti-oxidants normally contained within the RBC, the vasculature and exposed tissues are under threat of damage. Attempts by specialised plasma scavenger proteins and other molecules/detoxifying systems to neutralize and impound the free haemoglobin and haem, may work; however may also become overwhelmed by severe haemolysis, leading to severe clinical effects, as experienced in malaria (Schaer *et al.*, 2013).

The aim of this study was therefore to assess the potential lysis prevention activity and anti-oxidant properties (free radical scavenging, iron chelation, iron reduction and lipid peroxidation inhibiting activity) of several 8-hydroxyquinoline derivatives (Section 1.7).

ROS (hydrogen peroxide, superoxide anions and hydroxyl radicals) are formed due to aerobic processes biologically and also exogenously by medication, ionizing radiation and ultra violet light. Erythrocytes are highly susceptibility to oxidation, due to having a high membrane concentration of polyunsaturated fatty acids and the function of O_2 transport of haemoglobin molecules, which is associated with increased ROS. On exposure of free haemoglobin to H_2O_2, haem degradation occurs and iron ions are released, which initiate free radicals and lipid peroxidation leading to structural damage that in turn indicates oxidative damage. Various mechanisms protect and assist in increasing the resistance of erythrocytes to oxidative stress endogenously, such as anti-oxidant enzymes (Ajila and Rao, 2008). Despite the existence of extensive anti-oxidant systems in RBC membranes, there are limitations to the protective effect of the cytosolic cellular anti-oxidant system – H_2O_2 generated by the membrane bound haemoglobin is not accessible to the cytosolic anti-oxidants and therefore form degradation products. This is then released from the RBCs exposing other tissues. The oxidative damage to the membrane itself alters the erythrocyte function and leads to its removal by

macrophages and the spleen. Exogenous defence mechanisms such as anti-oxidant compounds (ascorbic acid) which aim to limit bodily harm, are also utilised for protection and therefore have a place in order to further enhance the existing internal defence system (Nagababu *et al.*, 2010). The anti-oxidant potential of both food and medicinal products are therefore being explored and fully researched to determine their pharmacological properties in order to evaluate their protective effect against oxidative damage in erythrocytes, as in this study on the properties of the 8-hydroxyquinolines (Ajila and Rao, 2008).

Compounds which contain the quinoline structure have been widely used for malarial treatment; however due to the evidence of drug resistance there is a focus on researching novel compounds within this group to circumvent this problem. The malarial anti-parasitic activity of the 8-hydroxyquinoline derivatives is based on its lipophilicity as indicated by its high lipid-water logP and particularly by its metal binding constants (Scheibel and Adler, 1982). Its chelating ability is also linked to its anti-oxidant activity by the formation of mixed ligand metal complexes within biological fluids. 8-Hydroxyquinoline acts on the intracellular malaria parasite, blocking glycolysis and growth (Scheibel and Adler, 1980). Its chelating and lipid solubility ability was shown to increase when substitutions with chlorine group was made at the C-5 or at both the C-5 and C-7 positions. This however resulted in little improvement to its antimalarial activity (Prachayasittikul, *et al.* 2013).

It is important to note that FPIX is haemolytic on its own as well as when it is in a complex with the antimalarial chloroquine (the toxicity of this combination may explain the antimalarial activity) as chloroquine potentiates the haemolytic activity of FPIX (Orjih *et al.*, 1988).

The mechanism of haemolysis by FPIX and effect on erythrocytes has been a topic of interest as presented in research literature (Koslova *et al.*, 2014; Schaer *et al.*, 2013; Ganji *et al.*, 2012). Measurements of FPIX-induced haemolysis, erythrocyte swelling, osmotic fragility, potassium loss, lipid peroxidation, and change in ATP and glutathione concentrations have been conducted (Chou and Fitch, 1981). The results obtained by

Chou and Fitch (1981) are in agreement with previous investigations on mouse erythrocytes (which are proven to be similarly affected as human erythrocytes) – the time-course of haemolysis exists in two phases whereby 5 µM FPIX showed a lag phase of approximately 1 hour (caused 50% lysis of a 2% (v/v) mouse RBC suspension during 2.5 hr incubation at 37°C), and when the concentration of FPIX was increased the phase was shortened. Haemolysis was temperature dependent when using mouse RBC, and stalled at temperatures lower than 29°C. In the initial phase, at 5 µM an extremely high degree of potassium loss was noted (due to FPIX-induced leakage by the erythrocyte membrane), reaching 90% of the total present within 30 minutes of incubation, and reaching a maximum within 5 minutes, with concurrent swelling of erythrocytes and display of osmotic fragility (due to the osmotic gradient created by haemoglobin and the resulting entry of water). ATP concentrations were initially stable during the first 10 minutes of exposure to FPIX and fell rapidly thereafter. The effect of FPIX on the erythrocyte membrane was evaluated by measuring the presence of malonyldialdehyde (which is one of the many major products of lipid peroxidation and which is used as an index for lipid peroxidation and a marker for oxidative stress) (Ajila and Rao, 2008), however no increase was noticed (Chou and Fitch, 1981). At high (threshold) concentrations of FPIX the RBC shape and its membrane transforms and becomes defective, distortions of the membrane nanostructure and cell morphology appear as domains with grain-like structures ", which lead to the formation of echinocytes and sphero-echinocytes (Koslova *et al.*, 2014).

According to studies done by Dutta and Fitch (1983), erythrocytes on exposure to haem and prior to lysis, display massive potassium loss and increased osmotic fragility. Their assays showed that haem at 5 µM caused very little haemolysis in human erythrocytes at 37°C, whereas at higher concentrations of 10 µM or greater (with little effect of temperature, as low as 20°C) haemolysis commenced and progressed immediately to maximum within a short period of time i.e. 20 to 30 minutes (Dutta and Fitch, 1983). In the current study a similar trend at 37°C with minimal haemolysis at 5 µM concentration was seen; whereas noticeable increases in haemolysis was seen following the 4 hour

time period, particularly at the 50 µM, 100 µM and 200 µM concentrations (Figure 9 and 10).

The destructive effect of FPIX on the erythrocyte skeleton is as follows: it releases band 4.1 (the link between the cell transmembrane glycoproteins and the skeletal protein network) by the important skeletal protein spectrin, causes spectrin dimerization followed by the complete dissociation of skeletal interactions. It also hydrophobically associates with phospholipid bilayers (Wyse and Butterfield, 1988). The oxidation and aggregation of proteins is considered the final cumulative point of oxidative degradation of the erythrocyte membrane (Akoev *et al.*, 1998).

The DPPH$^{\bullet}$ scavenging assessment is a proven and commonly used analytical technique for determining the ability of a compound to react with a free radical and donate a hydrogen to it. The anti-oxidant assays were therefore conducted to determine the free radical scavenging activity (% inhibition) of the initial screening concentrations (200 µM) of the 8-hydroxyquinoline compounds (Table 2). Only the amino- and dimethyl-substitutions displayed similar anti-oxidant activity to the positive controls, ascorbic acid and Trolox™ (Table 2), with 5-amino-8-hydroxyquinoline having the strongest scavenging activity (IC_{50} value: 12.12 ± 1.36 µM), followed by 5,7-dimethyl-8-hydroxyquinoline (IC_{50} value: 12.85 ± 1.65 µM) and 2-amino-8-quinolinol (IC_{50} value: 14.79 ± 0.94 µM). Similar results were confirmed by DPPH$^{\bullet}$ assays conducted by Chen and Van Zyl (2015). Other similar study reports have shown that various free radical scavengers investigated, including EDTA, showed no protective effect on FPIX-induced haemolysis. In one study, only cysteine, dithiothreitol and mercaptoethanol were proven to offer protection (Chou and Fitch, 1981).

Iron is highly reactive *in vivo* (as it participates in Fenton reactions and can initiate biological damage). In the presence of free ferrous ions, H_2O_2 which normally has low reactivity, forms hydroxyl radicals (Mohammadpour *et al.*, 2012) and iron is therefore considered the most important lipid oxidation pro-oxidant (Gülcin, 2009). Ferrous iron chelating agents offer effective anti-oxidant ability by binding to Fe^{2+}, reducing its redox potential and stabilizing the oxidative form. Substances which can chelate iron,

therefore supress oxidative reactions to alleviate the destroying effects of free radicals (Himmi *et al.*, 2016). The chelating effect of the test compounds on ferrous ions was observed (Figure 29) and compared to EDTA. The chelating activity of EDTA was found to be 24.85 ± 3.12 µM ($Fe(II)C_{50}$ value) and the most potent test compounds, showing very similar activity were calculated to be 37.25 ± 4.65 µM for 2-amino-8-quinolinol, 33.21 ± 3.71 µM for 8-OH and 32.21 ± 11.71 µM for 5-amino-8-hydroxyquinoline dihydrochloride, with 2-methyl-8-hydroxyquinoline displaying the strongest chelating potential ($Fe(II)C_{50}$ value: 5.67 ± 1.45 µM). These test compounds are effective ferrous ion chelators and therefore could be cytoprotective *in vivo*, by restoring metal balance and reducing toxicity.

It has been proposed that new antimalarial treatment strategies should include the addition of iron chelating agents (such as desferrioxamine) to current antimalarial regimens (chloroquine, pyrimethamine and quinine), particularly in cases of drug resistance, as this combination successfully inhibited *in-vitro* growth of late stages of the malaria parasite (Van Zyl *et al.,* 1992). This implies that trace metals fulfil vital roles in malarial biology (Ginsburg *et al.*, 1986). The intracellular copper chelator, neocuprine, have also been shown to inhibit parasites and also causes a decrease in Cu/Zn SOD (cuprozinc superoxide dismutase), the copper-containing enzyme in erythrocytes, in both *Plasmodium*-parasite-infected erythrocytes and saponin-purified parasites. The parasites have been shown to export copper in order to minimize its toxicity implying an efflux mechanism (Rasoloson *et al.*, 2004).

The iron reducing power of the 8-hydroxyquinolines was compared to the standard Trolox™ - the results showed effective reductive ability (Figure 20). The compounds with the strongest reducing power i.e. ability to terminate the very damaging radical chain reactions, were 5-chloro-8-hydroxyquinoline (IC_{50} value: 5.50 ± 0.80 µM) and 5,7-diiodo-8-hydroxyquinoline (IC_{50} value: 5.80 ± 1.31 µM) and the compound with the weakest power was 5,7-dimethyl-8-hydroxyquinoline (IC_{50} value: 60.43 ± 3.76 µM) as compared to Trolox™ (IC_{50} value: 50.62 ± 0.36 µM). The iron reducing potential of the compounds has not been reported in literature.

Lipid peroxidation inhibiting or stimulating activity was assessed by the effect of the 8-hydroxyquinolines on oxidation of linoleic acid (Figure 21). It has been proposed that intra-parasitic iron activates the cleavage of the endoperoxide bridge, causing free radicals to form within the parasite which then continue to alkylate haem and proteins, causing cell death (Fong *et al.*, 2013). In addition, as outlined in Section 1.2.1, free haem has various negative effects which include causing the combining of lipid bilayers, cell and lipid lysis, decreased enzyme activity and catalysis of chain reaction lipid peroxidation, which results in erythrocyte, protein and DNA damage. This is caused by the generation of ROS and cell-toxic aldehydes such as 4-hydroxyl nonenal and malonyldialdehyde (Gülcin, 2009). Erythrocytes do have various anti-oxidant enzymes which serve as protection against ROS. Plasma ascorbic acid protects the lipids from ROS in *P. falciparum* infection, however can be rapidly oxidized – levels decrease and the metabolite which is produced i.e malonyldialdehyde increases. Lipid peroxidation starts when all ascorbic acid is depleted, and therefore this supports the proposal of anti-oxidant administration as a lipid protection mechanism (for detoxification of lipid hyperperoxides), particularly in order to decrease malaria-associated anaemia (Raza *et al.*, 2013) to protect against the cellular damage of oxidative stress. Ten of the eleven 8-hydroxyquinoline derivatives inhibited lipid peroxidation activity except for 5-amino-8-hydroxyquinoline (Figure 21).

Albumin decreased lysis induced by 5 μM FPIX by approximately 1 fold (Figure 12). Serum albumin is known to offer protection against free haem accumulation in the plasma and resultant oxidative damage. It scavenges free haem, providing a D-shaped cavity, stabilising the interaction by two salt bridges and also supplies a donor oxygen to the iron atom of haem, in order to ensure it remains in a reduced state (Ferreira *et al.*, 2013). Albumin further protects erythrocytes from haemolysis by sulfhydryl blocking agents, when it binds to FPIX and removes it from the erythrocyte surface (Chou and Fitch, 1981).

EDTA is able to form complexes with various metals (Blake *et al.*, 1996). A higher concentration of EDTA is proposed to be used as EDTA chelates all metals not only Fe and Cu which can induce lipid peroxidation; but others such as Ca that can protect RBC from lysis. Stability constants for the different metals forming complexes with EDTA are as follows Na (1.7), Li (2.8), Ba (7.8), Sr (8.6), Mg (8.7), Ca (10.6), Mn (13.4), Fe (14.4), Co (16.1), Zn (16.1), Cd (16.4), Pb (18.3) and Ni (18.4). The metal with the higher stability constant competes for the chelating agent with a metal which has a lower stability value and is able to replace it. Other factors also play a role, such as structure, pH (Flora and Pachauri, 2010). The haemolytic activity of FPIX may not be due solely to metal involvement.

FPIX has however been proven to cause DNA strand scission in the presence of oxygen and the reducing agent, 2-mercaptoethanol, whereby circular supercoiled DNA is transformed to open circle or linear forms. It has been shown that Mg^{2+} and Ca^{2+} inhibit the DNA scission whereas Co^{2+}, Zn^{2+}, Ni^{2+} Cu^{2+} stimulate the destruction (Aft and Mueller, 1983).

In summary, the following 8-hydroxyquinoline derivatives displayed varying effects. The 5-amino-8-hydroxyquinoline, 2-amino-8-quinolinol and 5,7-dimethyl-8-hydroxyquinoline derivatives showed the strongest free radical scavenging activity. The 2-methyl-8-hydroxyquinoline, 5-amino-8-hydroxyquinoline, 8-hydroxyquinoline and 2-amino-8-quinolinol showed the strongest iron chelation activity and the 5-chloro-8-hydroxyquinoline and 5,7-diiodo-8-hydroxyquinoline showed iron reducing powers. Ten of the eleven 8-hydroxyquinoline derivatives inhibited lipid peroxidation activity except for 5-amino-8-hydroxyquinoline. The compounds which showed the best inhibition of FPIX-induced lysis were 8-hydroxyquinoline, 2-amino-8-quinolinol and 5,7-dimethyl-8-hydroxyquinoline and this activity could be due to their iron chelating and free radical scavenging activity respectively (Table 2 and Figure 19), i.e due to chelating iron ions with the formation of inert iron complexes unable to initiate lipid peroxidation and possible ability to suppress free radical processes at three stages: the formation of superoxide ion, the generation of hydroxyl radicals in the Fenton reaction and the formation of lipid peroxy radicals (Afanas'ev, IB *et al.*, 1989).

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

The results of the anti-oxidant activities, i.e. inhibition of oxidative degradation, as evaluated by the different methods of assessment has led to the conclusion that the 8-hydroxyquinolines offer varying but effective anti-oxidant protective activity.

Further to this, it has been published that the 8-hydroxyquinoline derivatives have the potential to inhibit the malaria parasite directly (Prachayasittikul *et al.*, 2013), however the exact mechanism of action still needs to be determined. As all the derivatives displayed iron chelating properties, the question remains whether the chelation of metals would be harmful or beneficial to the infected patient.

The 5-amino-8-hydroxyquinoline and 2-amino-8-quinolinol derivatives have shown the best anti-oxidant properties from the assay results and warrant further investigation.

It is hoped that this report will prompt studies to further determine the anti-oxidant abilities of the 8-hydroxyquinolines to inhibit oxidative degeneration as a mechanism to protect erythrocytes from free radical chain reaction damage and lysis caused by *P. falciparum* and by-products produced during infection.

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APPENDIX A: Human research ethics committee clearance certificate

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH 10005, 10th floor, Tel +27 (0)11-717-1252
Medical School Secretariat: Medical School Room 10M07, 10th Floor, Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za Fax +27 (0)11-717-1265

Ref: W-CJ-131030-1 30/10/2013

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Prof R van Zyl.

Project title: The chemotherapeutic properties of novel synthetics and natural compounds.

Reason: This is a laboratory study in which human blood and plasma for the in vitro maintenance of *Plasmodium falciparum* for experimental purposes such as drug sensitivity and toxicity studies. There are no human participants.

2013 -10- 30

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy - HREC(Medical) Secretariat: Anisa Keshav, Zanele Ndlovu.

R14/49 Dr Robyn L van Zyl

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140669

NAME: (Principal Investigator)	Dr Robyn L van Zyl
DEPARTMENT:	Pharmacy & Pharmacology Medical School
PROJECT TITLE:	The Chemotherapeutic Properties of Novel Synthetic and Natural Compounds (Renewal previously M090532)
DATE CONSIDERED:	29/05/2009 (Initial Approval) 26/06/2014 (Renewal)
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	26/06/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/We fully understand the conditions under which I am/We are authorized to carry out the above-mentioned research and I/We undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/We undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

Principal Investigator Signature — Date: 21-07-2014

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX B: Institutional biosafety committee clearance certificate

Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE
(R 14/16)

CLEARANCE CERTIFICATE - RENEWAL **PROTOCOL NUMBER: 20090503**

BRIEF DESCRIPTION OF APPLICATION:

Chemotherapeutic properties of novel synthetic and natural compounds

APPLICANT: Dr R van Zyl

SCHOOL/DEPARTMENT : Pharmacy/Pharmacology

DATE CONSIDERED: 20090528 and 20140327

DECISION OF COMMITTEE:

Approved unconditionally

1. This clearance certificate expires on 20190402 and may be renewed on application
2. An annual report must be provided on the anniversary date of this certificate, for as long as the project continues
3. Notification of any proposed modifications must be submitted on the attached form

DATE: 20140403 **CHAIRPERSON** _____
(Professor A Capovilla)

DECLARATION BY APPLICANT:

To be completed in duplicate and **one copy** returned to the Secretary, Room 10004, 10th floor, Senate House, University.

1. I have read, understood and accepted the approval conditions above
2. I agree to submit a yearly progress report to the Committee and to submit an interim report on the form provided, in the event of any significant unforeseen event, *e.g.* suspension of a drug trial, temporary closure or relocation of my laboratory, etc
3. I note that the University Safety Officer, or his/her representative, may at any reasonable time inspect my laboratory or trial site to ensure compliance with current Health and Safety legislation. I undertake to offer my full co-operation in any such inspection.
4. I have read, understood and will comply with the *recommended standard operating procedures for the handling of biohazardous materials* posted at http://web.wits.ac.za/Academic/Research/Biosafety.htm
5. I declare (delete as appropriate) that:
    a. I have all the approvals required by statute or regulation and by the funding agencies supporting this work, or
    b. that I will not begin work until such approvals are obtained

Signed: _____ **Date:** _____

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PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES