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The effect of novel Quinoxaline derivatives on the life cycle of malaria parasite and vector

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THE EFFECT OF NOVEL QUINOXALINE DERIVATIVES ON THE LIFE CYCLE OF MALARIA PARASITE AND VECTOR

Teboho Malimabe

WITS UNIVERSITY

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in the fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg, South Africa

2019

DECLARATION

I, Teboho Malimabe, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at this or any other University.

……

Teboho Malimabe

Signed at…… on the…… day of……2019.

DEDICATION

To my loving and supportive parents; Mokete and ‘Mateboho Malimabe

To my dear younger brothers; Tumelo and Lerato Malimabe

“*I am because you are”* African Proverb

PUBLICATIONS AND CONGRESS ATTENDANCE

Publications:

Sewpersad, S., Malimabe, T., Oliver, S., Koorbanally, N and Van Zyl, R.L. The antimalarial and insecticidal effect of novel quinoxaline-quinoline derivatives. In preparation.

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Malimabe, T., Sewpersad, S., Oliver, S., Koorbanally, N and Van Zyl, R.L. The antimalarial and insecticidal effect of novel quinoxaline-quinoline derivatives. Conference of Biomedical and Natural Sciences and Therapeutics (CoBNest). Stellenbosch, South Africa. 7-10 October 2018 (Oral presentation).

ABSTRACT

Malaria is one of the major global health problems especially in the sub-Saharan Africa. While the current treatments are effective, there is a growing concern about the emergence of resistant strains of *Plasmodium* to antimalarial drugs and the *Anopheles* vector to insecticides. Therefore, there is a need to develop novel compounds. As such, thirty-five novel quinoxaline-based hybrid compounds consisting of quinoxaline-chalcone-phenyl (**QCP)**, quinoxaline-pyrazoline-phenyl(**QPP**), quinoxaline-chalcone-quinoline(**QCQ**) and quinoxaline-pyrazoline-quinoline (**QPQ**) compounds were synthesized with the aim to evaluate their effect on the life cycle of *Plasmodium* and *Anopheles*. Twenty-four compounds exhibited antimalarial activity against the asexual stages of the NF54 strain of *P. falciparum* using the parasite lactate dehydrogenase (pLDH) assay*;* with eleven compounds displaying promising activity with IC_{50} values less than 10 µM. Compound **QCQ3** was the most active (IC_{50} value: 4.19 ± 0.52 µM); but was less active than dihydroartemisinin (DHA) (IC_{50} value: 0.006 ± 0.01 µM) and quinine (IC_{50} value: 0.11 ± 0.01 µM). Two of the active compounds, **QCP7** and **QCP8** slowed ring and trophozoite development affecting the haemozoin structure and both displayed a synergistic interaction with quinine. Seven compounds displayed minimal ovicidal activity (1-15%) against *Anopheles arabiensis* (KWAG), while twelve possessed low larvicidal activity (mortality range: 0 - 23%) compared to DDT. None of the compounds displayed anti-oxidant activity by chelating ferrous ions or scavenging the free radicals of DPPH$^{\bullet}$. The preliminary toxicity profile of the compounds indicated that thirteen compounds were toxic to human kidney epithelial cells (IC_{50} values range: 13.66 ± 0.46 to 36.77 ± 0.89 µM); whilst only **QPQ8** was toxic to *Artemia franciscana* (1.05 ± 0.78 µM). Eighteen compounds had anticancer activity against chronic myelogenous leukaemia cells, with **QCQ10** the most active at 4.00 ± 0.50 µM. The selectivity index ranged between 1.27 and 7.71 compared to the human kidney epithelial cells. *In silico* predictions indicated that the compounds displayed favourable drug-like properties with good ionisation and absorption. Although none of the compounds induced haemolysis, these quinoxaline compounds have been predicted *in silico* to have a high risk of being toxic as other *in vivo* tested quinoxaline derivatives. These findings indicated that compound **QCP6**, **QCP7**, **QCP8**, **QCP9**, **QCP10** may have potential as antimalarial and anti-leukaemic agents and warrant further investigation.

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LIST OF ABBREVIATIONS

ADME	Absorption, distribution, metabolism and elimination
ACD-B	Acid citrate dextrose solution-B
ACT	Artemisinin-based combination therapy
APAD	3-Acetylpyridine adenine dinucleotide
APADH	3-Acetylpyridine adenine dinucleotide hydrogen
ATP	Adenosine triphosphate
CDC	Centers for Disease Control and Prevention
°C	Degree Celsius
DDT	Dichlorodiphenyltrichloroethane
DHA	Dihydroartemisinin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
∑FIC	Sum of Fractional inhibitory concentration
g	Gram
g	Gravitational constant/ G-force
g/mol	Grams per mole
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
HEPES	N-2-Hydroxyelthlypiperazine-N'-2-ethane-sulfonic acid
IC_{50}	Concentration required to inhibit 50% of parasite/cell growth
IRS	Indoor residual spraying
L	Litre
LC_{50}	Lethal concentration required to kill 50% larvae/nauplii
LDH	Lactate dehydrogenase
LLINs	Long-lasting insecticide-treated nets
m	Metre
mg	Milligram
mL	Millilitre
mM	Millimolar
µL	Microlitre

μM	Micromolar
M	Molar
mol	Mole
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n.d	Not determined
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NBT	Nitroblue tetrazolium
%	Percentage
PBS	Phosphate buffered saline
PES	Phenazine ethosulfate
pH	Potential of hydrogen
pLDH	Parasite lactate dehydrogenase
RBCs	Red blood cells
Ro5	Rule of Five (5)
rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institution number 1640
SA-DOH	South Africa Department of Health
s.d.	Standard deviation
S.I.	Safety index
$S.I_{nc}$	Safety index (normal:cancer)
$S.I_{pf}$	Safety index (*Plasmodium falciparum*)
TRIS	Trisaminomethane
UV-VIS	Ultraviolet-visible
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization

CHAPTER ONE - INTRODUCTION

1.0 Malaria

Mosquitoes are one of the most important vectors of human disease since they carry and transmit pathogens of serious public health concern globally. Mosquito-borne diseases such as malaria, dengue, yellow fever and Japanese encephalitis kill millions of people every year and thousands suffer from infections transmitted by *Anopheles* mosquitoes (WHO, 2017). Among these diseases, malaria remains one of the most prevalent infectious disease causing morbidity and mortality in the world, especially in sub-Saharan Africa (WHO, 2018). According to latest estimates from the World Health Organization (WHO), there were 219 million global cases of malaria in 2017. This number increased by 2 million cases compared to 2016 and the number of deaths reported reached 435,000 in 2017. Of the 90 countries with reported malaria (Figure 1.1), Africa accounts for 92% of the malaria cases and 93% of the deaths, with at least one child dying every two minutes (WHO, 2018). Despite several treatment and control measures used to prevent and reduce malaria transmission, half of the world's population (approximately 3.2 billion people) remain at risk of malaria and death. The population groups that are at higher risk of complicated malaria are children under age of five, pregnant women (causing severe morbidity such as low birth weight of the child, placental infections, maternal anaemia as well as neurological problems), immunocompromised patients and the elderly (CDC, 2017; Reddy *et al*., 2017; WHO, 2018).

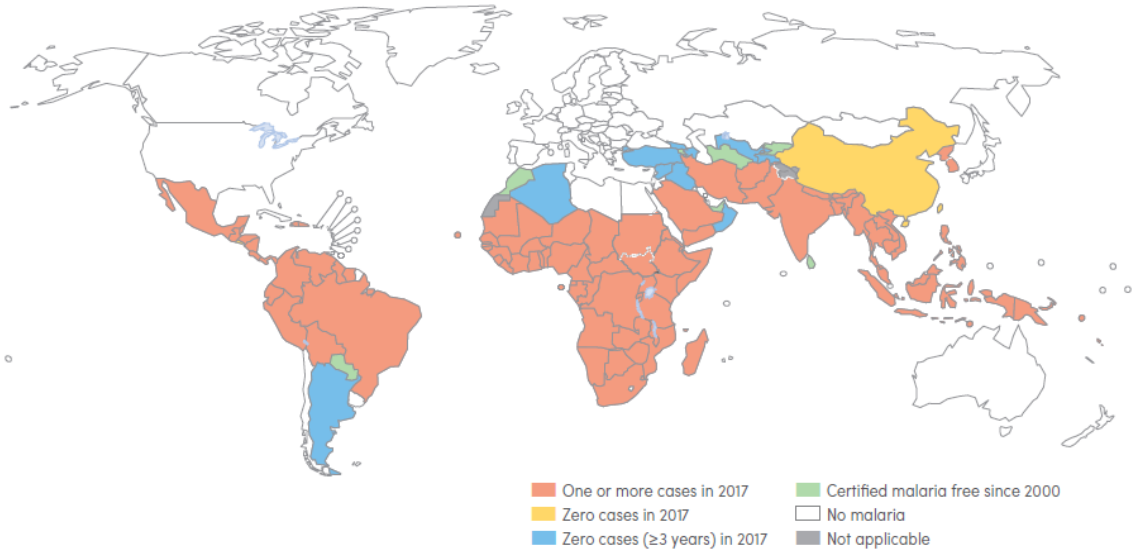


Figure 1.1: Global distribution of malaria (WHO, 2018).

The African continent has socio-economic and climatic conditions favourable to sustain *Anopheles* propagation that directly contributes to the transmission and clinical cases of malaria (Githeko *et al*., 2000; Kakmeni *et al*., 2018). In South Africa, malaria transmission is seasonal, peaking during wet summer months between September to May, but the highest transmission is between January and April (SA-DOH, 2018). There was an increase in malaria cases in South Africa during the 2016/2017 peak season when compared to the 2015/2016 season. A total of 9,478 malaria cases and 76 deaths were reported by March 2017 as compared to 6,385 cases and 58 deaths in 2015/2016 season (SA-DOH, 2017). Some of the factors that led to the increased cases/fatalities were increased rainfall, temperature and humidity; as well as the reduced use of indoor residual spraying (IRS) in areas where malaria cases had declined in recent seasons. The endemic areas in South Africa are KwaZulu-Natal, Mpumalanga and Limpopo provinces (SA-DOH, 2017).

While malaria is preventable and curable, the current interventions are not adequate for its elimination and eradication due to development of antimalarial drug and insecticide resistance by malaria parasite and vector, respectively (Maurya *et al*., 2017; WHO, 2018). Therefore, an ongoing understanding of malaria pathogenesis is needed in order to develop new and improved management strategies.

1.1 Malaria pathogenesis

Malaria is a life-threatening mosquito-borne infectious disease caused by single-celled parasitic protozoa of the genus *Plasmodium*. It is transmitted through the bite of an infected female *Anopheles* mosquito from one host to another (Clark *et al*., 2004; WHO, 2013). There are approximately 156 *Plasmodium* species known to cause malaria infections in different vertebrate species, but only five are known as human malaria parasites; *Plasmodium falciparum*, *P. ovale*, *P. vivax*, *P. malariae* and *P. knowlesi* (Table 1.1) (WHO, 2015).

Plasmodium falciparum causes the most severe and fatal form of malaria and is more prevalent in sub-Saharan African countries. Approximately 95% of all malaria infections in South Africa are due to *P. falciparum* (Hlongwana and Tsoka-Gwegweni, 2016). *Plasmodium vivax* is the second most significant malaria parasite dominant in Latin American and South-East Asian countries (Geleta and Ketema, 2016; WHO, 2018). *Plasmodium ovale* and *P. malariae* only account for a small percentage of malaria infections. *Plasmodium ovale* and *P. vivax* have an added complication of having hypnozoites, which are the dormant parasitic liver stage that can be reactivated weeks to years after the initial infection resulting in another infection (Markus,

2011; Campo *et al*., 2015). Infections with *P. knowlesi* are highly lethal as the parasite has a life-cycle of 24 hours and therefore can reproduce at a very high rate. Infections with *P. knowlesi* have only been reported in South and East Asia (Cox-Singh *et al*., 2010; Pongvongsa *et al*., 2018).

Table 1.1: Characteristics of *Plasmodium* species causing human malaria **(**Antinori *et al*., 2012).

Characteristic	*P. falciparum*	*P. vivax*	*P. malariae*	*P. ovale*	*P. knowlesi*
Erythrocytic cycle (hours)	48	48	72	50	24
Red blood cells affected	All	Reticulocytes	Erythrocytes	Reticulocytes	All
Duration of intra-hepatic phase (days)	5-7	8	15	9	8-9
Merozoites/ infected hepatocyte	30,000	10,000	15,000	15,000	2,000
Hypnozoite	No	Yes	No	Yes	No
Relapses	No	Yes	No	Yes	No
Severe malaria	Yes	Yes	No	No	Yes
Recurrences	Yes	Yes	Yes	No	Yes

1.1.1 Life-cycle of human malaria parasite

Human malaria parasites are highly complex species that require two hosts; female *Anopheles* mosquito vector and human host to multiply and generate new infective parasites (WHO, 2013). For malaria transmission to occur, the parasite must complete its life cycle which involves three stages (Figure 1.2); mosquito stage (sporogonic cycle), liver stage (exo-erythrocytic cycle) and blood stage (erythrocytic cycle) with the latter two stages occurring in the human host (Cowman *et al*., 2012).

1.1.2 *Anopheles*: Malaria vector

Anopheles is a genus of mosquito that consists of approximately 430 species and of these 30-40 are considered as human malaria vectors in nature, while the rest are either unable to sustain the malaria parasites' development or bite human beings infrequently (CDC, 2015). Only infected female *Anopheles* mosquitoes are directly involved in malaria transmission since they

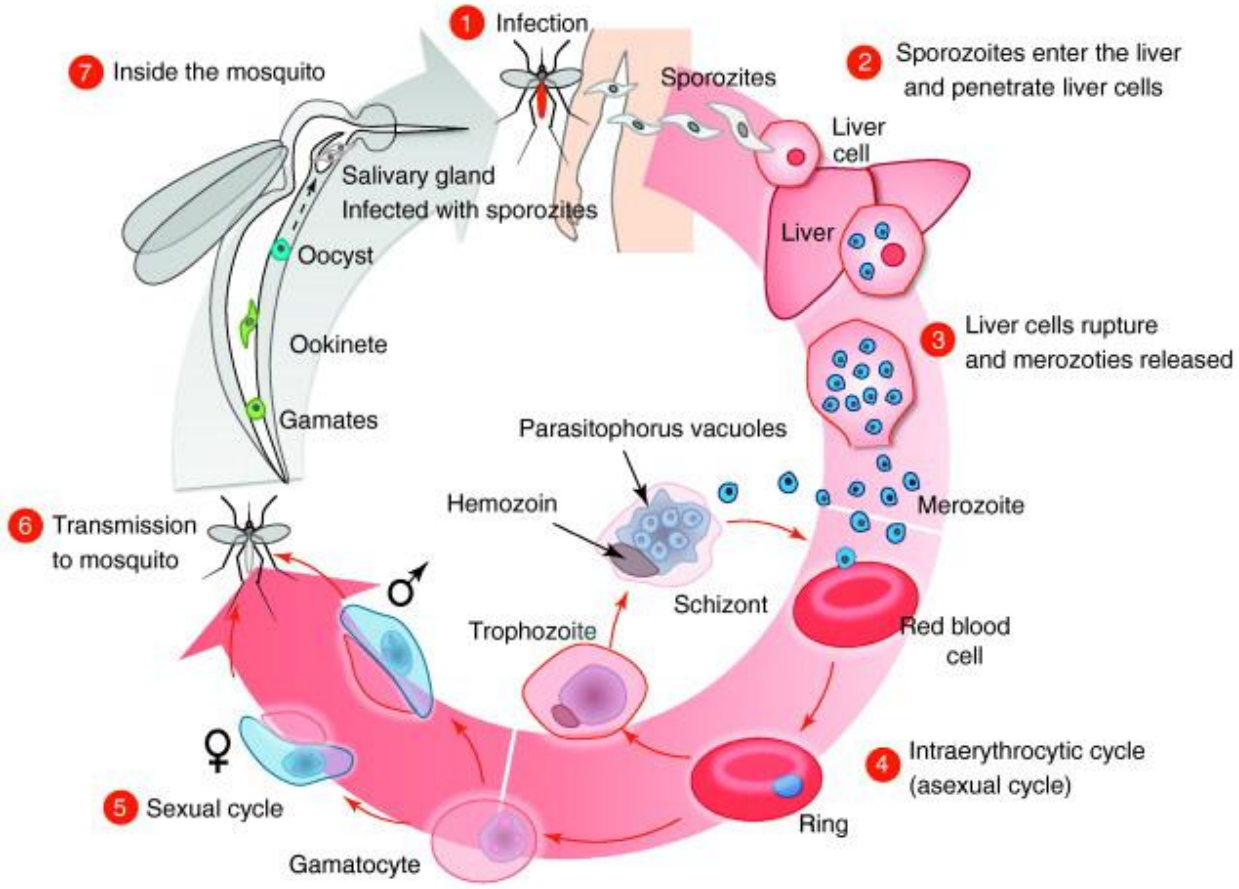


Figure 1.2: The life cycle of human malaria parasite (Cho *et al*., 2012). The life cycle begins when the female *Anopheles* mosquito, infected with malaria parasite, injects sporozoites into the human bloodstream through a bite during a blood feeding (1). Sporozoites then rapidly enter the liver and infect the hepatocytes and multiply asexually until they mature into schizonts (2). During this stage called the incubation stage, no clinical signs or symptoms of malaria are observed. After 7-30 days, the liver schizonts rapture and release thousands of merozoites into the blood (3). In the blood, merozoites infect the red blood cells (RBCs) and undergo additional asexual replication through a set of stages (rings, trophozoite and schizont) (4). Thereafter, mature schizont infected RBCs lyse to release merozoites to infect other RBCs. This causes the initial clinical signs and symptoms of malaria illness. At this stage, some of the merozoites differentiate the sexual stages into gametocytes: macrogametocytes (female) and microgametocytes (male) (5), which are then taken up by the female *Anopheles* mosquito during a blood meal (6). The macrogametocytes and microgametocytes fuse inside the mosquito gut to form ookinetes that develop into oocysts, which matures to produce sporozoites (7). The sporozoites then move to salivary glands of the mosquito, ready to be inoculated into a new human host when the female takes her next blood meal (1) (Cowman *et al*., 2012).

require a blood meal to serve as nourishment for egg development (Sinka *et al.*, 2012). The intensity of malaria transmission varies across continents depending on the type of *Anopheles* species found in each country, their level of interaction with humans, geographical distribution, as well the physiological characteristics of each species (Takken and Knols, 1999; Ndoen *et al*., 2010). The African continent carries the highest malaria burden globally, mainly due to the regions wide spread of the three most efficient *P. falciparum* malaria vectors; *Anopheles arabiensis, An. gambiae* and *An. funestus* (Sinka *et al*., 2012). In South Africa, *An. arabiensis* and *An. funestus* are the most predominant malaria vectors (Brooke *et al*., 2013; Dandalo, 2017).

Anopheles exhibits a complete metamorphosis, cycling between eggs, larvae, pupae and adult stages. Each of these stages can be recognised by its special morphological characteristic (Figure 1.3). The first three stages are aquatic and last about 5-14 days depending on the type of species and environmental temperature (CDC, 2015).

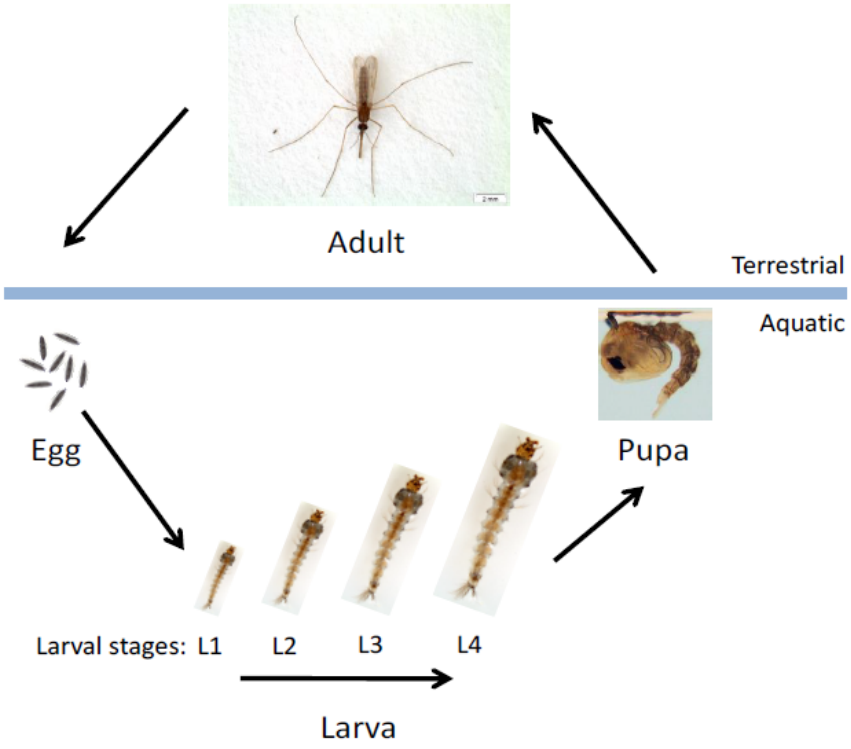


Figure 1.3: The life-cycle of *Anopheles* mosquito (Williams and Pinto, 2012).

Anopheles lay their boat-shaped eggs singly and horizontally on the surface of the water, aided by two lateral air floats that assist in floatation. Larvae moult four times, growing larger after each moult. The larva metamorphosis into a pupa occurs during the fourth instar. During this

developmental stage, the head and thorax are merged into a cephalothorax with the abdomen curving around underneath (Williams and Pinto, 2012). When development stage is complete, the dorsal surface of the cephalothorax splits and the adult mosquito emerges. The adult *Anopheles* mosquito has a slender body with three segments: head, thorax and abdomen. The head consists of the eyes and a pair of long, segmented antennae. The antennae are important for detecting host odours, as well as odours of breeding sites where females lay eggs. Moreover, the head also has an elongate, forward-projecting proboscis used for feeding, and two sensory palps. The thorax has three pairs of legs and a pair of wings used for locomotion. The main function of the abdomen is for food digestion and egg development (Clements, 1992). Both adult males and females feed on nectar and other sources of sugar for energy, but only the mature females feed on blood when egg production occurs. The average life span of *Anopheles* is less than two months; the males live for 10 days while the female lives for about six to eight weeks (Mireji *et al.*, 2010).

1.1.3 Clinical features of malaria

The clinical symptoms associated with malaria vary depending on the species and strain of *Plasmodium* parasite, as well as epidemiology, age and immunity (WHO, 2018). Malaria is classified into two categories: uncomplicated and complicated (severe) malaria (Table 1.2). Uncomplicated malaria is caused by all *Plasmodium* strains and the symptoms associated with this type of malaria are non-specific and do not include any serious organ dysfunction. In contrast, complicated malaria is mainly caused by *P. falciparum*, resulting in severe organ failure, and potential fatality (Bartoloni and Zammarch, 2012; WHO, 2018).

The initial clinical malaria diagnosis is based on the patient's symptoms; however, these are often non-specific and therefore each patient's blood needs to be examined to confirm malaria using parasite-based diagnostic testing, microscopy diagnosis or malaria antigen rapid diagnostic tests before prescribing appropriate antimalarial treatment (Bailey *et al.*, 2013).

1.2 Control and treatment for malaria

The control and treatment for malaria infections are based primarily on two strategies; the control of the *Anopheles* mosquito vectors and control of the malaria parasite through antimalarial chemoprophylaxis and chemotherapy (WHO, 2018).

Table l.2: Clinical and laboratory indicators of uncomplicated and complicated malaria (Blumberg, 2015; Lallo *et al*., 2016; SAMF, 2016; WHO, 2016; Van Zyl, 2016).

Common presenting symptoms	Physical findings	Laboratory findings: uncomplicated malaria	Symptoms of complicated malaria	Laboratory findings: severe malaria
• Headaches • Fatigue • Abdominal discomfort • Chills • Fever • Myalgia • Perspiration • Anorexia • Nausea/vomiting • General malaise	• Elevated temperatures (≥ 37.5°C) • Rigors • Weakness • Splenomegaly • Mild jaundice • Hepatomegaly • Increased respiratory rate	• Microscopic confirmation of malaria/species differentiation • Mild anaemia • Mild thrombocytopenia • Elevated bilirubin • Elevated Aminotransferases	• Fever • Impaired consciousness • Multiple seizures • Prostrations • Respiratory distress/ pulmonary oedema • Jaundice • Spontaneous bleeding	• Parasite density > 4% • Haemoglobin < 5 g/dL • Metabolic acidosis (pH< 7.3; plasma bicarbonate < 15 mmol/L, serum lactate > 5 mmol/L) • Hypoglycaemia (< 2.2 mmol/L) • Renal impairment (oliguria < 0.4 ml/kg bodyweight/hr;serum creatinine > 265 μmol/L) • Thrombocytopenia (< 50,000/μL) • Liver transaminase

1.2.1 Malaria vector control measures

The *Anopheles* vector control relies mainly on two core measures; long-lasting insecticide-treated nets (LLINs) and IRS. The choice of control measure is influenced by vector abundance, behaviour and susceptibility of vectors to the insecticides used in LLINs and IRS (WHO, 2016). LLINs are helpful in reducing contact between mosquitoes and humans by providing physical barrier and an insecticidal effect. IRS involves spraying an effective dose of insecticide with a long residual activity on indoor walls and ceilings where malaria vectors are likely to rest (WHO, 2018). The recommended insecticide classes by WHO for adult malaria vector control with IRS are pyrethroids, organochlorines, carbamates and organophosphates. Pyrethroids are the only class recommended for the treatment of LLINs due to their rapid and effective mechanism of action against malaria vectors, as well as low toxicity to humans (Nauen, 2007).

1.2.2 Chemoprophylaxis

To prevent malaria infections, chemoprophylaxis is recommended for high risks groups and residents in the seasonally endemic areas and travellers to malaria endemic areas (SA-DOH, 2017). Chemoprophylaxis is defined as either the absolute prevention of infection (casual prophylaxis) or the suppression of parasitaemia and its symptoms (suppressive/clinical prophylaxis) (WHO, 2016). The choice of chemoprophylaxis varies depending on the *Plasmodium* species and antimalarial drug resistance prevalent in a country. Chemoprophylactic drugs that are recommended for use in South Africa are mefloquine, doxycycline and a combination of atovaquone and proguanil. If prophylaxis is continued until there are no more parasites in the blood, then a suppressive cure is achieved (SA-DOH, 2017). However, since no chemoprophylaxis is hundred percent effective, patients displaying flu-like symptoms, even whilst taking malaria chemoprophylaxis are advised to seek immediate treatment.

1.2.3 Antimalarial chemotherapy

The recommended antimalarial chemotherapy is based on the type of *Plasmodium* species, severity of the disease, patient characteristics (age, pregnancy and co-morbidity), the availability of medications and resources (SA-DOH, 2018). Antimalarial drugs are broadly classified into five groups based on their similar mechanisms of action and specific activity on various stages of the parasite life cycle (Table 1.3) (Sevene *et al.*, 2010). Most antimalarial drugs act on the pre-erythrocytic life stage and erythrocytic life stages, while a small percentage are active against the sexual stage of the parasite that interrupts transmission (McCarthy and Price, 2015). There are four groups of antimalarial drugs based their activity on various stages of the parasite life cycle (Sevene *et al.*, 2010; McCarthy and Price, 2015):

- **Tissue schizonticides:** act on the primary tissue forms of the *Plasmodium* within the liver, as well as to prevent relapse by acting on the hypnozoites of *P. vivax* and *P. ovale* in the liver.
- **Blood schizonticides:** act on the asexual life cycle stages of the parasite in the erythrocytes, thereby reducing the clinical manifestation of malaria.
- **Gametocytocides:** inhibit the sexual forms of the parasite formed during the erythrocytes stage, thereby preventing transmission of the infection to the mosquito.
- **Sporontocides:** prevent the development of oocysts in the *Anopheles* female mosquitoes.

Table 1.3: Classes of antimalarial drugs based on their mechanism of action and target stages on the parasite life cycle (Balint, 2001; Olliaro, 2001; Biagini *et al*., 2003; Vangapandu *et al*., 2007; Avery *et al*., 2008; Rosenthal, 2009; Kaur *et al*., 2010; Hughes *et al*., 2011; Petersen *et al*., 2011; Vinetz *et al*., 2011).

Antimalarial Drug	Mechanism of action	Activity on parasite life cycle	Examples
Quinoline and derivatives	Inhibit detoxification of haem by the parasite, preventing haemozoin formation	Sporontocide, gametocytocidal, blood and tissue schizontocide	Chloroquine Quinine Mefloquine Primaquine Lumefantrine
Artemisinin and derivatives	Produce free radicals within the parasite which results in alkylation of parasite proteins and lipids	Blood schizontocide and gametocytocidal activity	Artemisinin Artemether Artesunate Dihydroartemisinin
Anti-folates	Inhibits dihydrofolate reductase, interfering with *Plasmodium* pyrimidine synthesis	Blood and tissue schizontocides	Sulphadoxine Pyrimethamine Proguanil Dapsone
Antibiotics	Prevent protein synthesis by inhibits parasite apicoplast organelle function	Blood schizontocides	Doxycycline Clindamycin
Napthoquinones	Inhibit mitochondrial transport chain function of the parasite resulting in the disruption of the regeneration of ubiquinone.	Sporontocide, tissue and blood schizontocide	Atovaquone

In South Africa, the standard treatment for uncomplicated malaria infections (for both *P. falciparum* and non-*P. falciparum* infections) is a fixed dose of artemisinin-based combination therapy (ACTs), artemether-lumefantrine. Oral quinine plus either doxycycline or clindamycin are recommended in cases where artemether-lumefantrine are contraindicated or not available. For mixed infections (*P. falciparum* plus *P. ovale* or *P. vivax*), artemether-lumefantrine is ideally used, followed by primaquine. Patients with complicated malaria are treated with intravenous artesunate or intravenous quinine before completing a full course of oral artemether-lumefantrine (SA-DOH, 2018).

1.2.4 Combination therapy

One of the approaches used to overcome drug resistance is combination therapy, which is based on the synergistic or additive potential of two or more drugs combined to improve therapeutic efficacy and delay the development of resistance to the individual component of the combination (Bloland, 2001). Currently, ACTs are recommended by the WHO for the treatment of *P. falciparum* infections. ACTs have a rapid clinical and parasitological response, reduced malaria transmission, increased cure rates, as well as potential to delay antimalarial drug resistance (WHO, 2018).

While all the prophylactic and treatment regimens are still effective, the increasing number of resistant *Plasmodium* strains has started to limit the efficacy of these regimens. To potentiate this increasing problem, the *Anopheles* vector control measures are also losing their effectiveness due to changing mosquito behaviours, as well as the development of insecticide resistance (Vangapandu *et al*., 2007; Fidock *et al*., 2008; Sinka *et al*., 2012; WHO, 2018).

1.2.5 Insecticide resistance

Insecticide resistance has been reported in all major vector species and all classes of insecticides (Nauren, 2007). Insecticide resistance is the "ability of insects to survive exposure to a standard dose of insecticide, owing to physiological or behavioural adaptation" (WHO, 2016b). The increasing and repeated use of synthetic insecticides over the years has resulted in the development of various insecticide resistance mechanisms in many *Anopheles* species that transmit malaria (IRAC, 2011; WHO, 2015; WHO, 2018). There are four types of resistance mechanisms namely, metabolic, target-site, cuticular and behavioural resistance. These mechanisms can confer resistance to one class of insecticides or cause cross-resistance when resistance to more than one class of insecticide (IRAC, 2011; WHO, 2016).

- **Metabolic resistance:** occurs when detoxifying enzymes in the mosquito metabolises or sequesters insecticide molecules before they elicit a toxic effect. Insect strains that have higher quantities or more efficient forms of these metabolising enzymes, may exhibit phenotypic resistance (Austin *et al*., 2011). Metabolic enzymes associated with resistance in malaria vectors are cytochrome P450-dependent monooxygenases, esterases and glutathione-S-transferases (Nauen, 2007). An increased expression of various monooxygenases is often associated with pyrethroid resistance (Wondji *et al*., 2012; Edi

et al., 2014). Esterase resistance is linked to reduced susceptibility of malaria vectors to both organophosphates and pyrethroids (Brogdon et al., 1999; Vulule et al., 1999) while high expression of glutathione-S-transferase is linked to dichlorodiphenyltrichloroethane (DDT) resistance (Maharaj and Sharp, 2005; Ranson et al., 2001; Riveron et al., 2014).

- **Target-site resistance:** occurs when a genetic mutation has modified the target receptor within the mosquito, thus reducing the toxic effect of the insecticide. The targets of pyrethroids and organochlorines are voltage-gated sodium channels of nerve cell membranes, but the knockdown resistance (*kdr*) mutation reduces channel sensitivity to the binding of these insecticide classes (Bass *et al*., 2007). Similarly, mutations in the gene for acetylcholinesterase called Ace-1R, infers insensitivity to acetylcholinesterase, resulting in resistance to organophosphates and carbamates (Weil *et al*., 2004).
- **Cuticular or reduced penetration resistance:** occurs when the absorption of insecticide into a mosquito is reduced because of changes in the insect's outer cuticle resulting in decreased efficacy of various insecticides (Ahmad *et al*., 2006; Jones *et al*., 2013; WHO, 2016).
- **Behavioural resistance:** is caused by a change in mosquito activity, such as avoidance of insecticide-treated surfaces or changes in feeding or resting patterns in response to the presence of insecticide (Gatton *et al*., 2013).

1.2.6 Parasite resistance to antimalarial drugs

The emergence and spread of resistance parasite strains (especially *P. falciparum*) is one of the greatest challenges for malaria elimination, as it can lead to increased morbidity and mortality in the human host. Antimalarial drug resistance occurs when the resistant-parasite strain survives and multiplies at or above the optimal therapeutic plasma concentration (WHO, 2015). Resistance is more commonly observed in *P. falciparum* and *P. vivax*. *Plasmodium falciparum* has developed resistance to almost all of the recommended standard antimalarial drugs, but the distribution of resistance varies geographically (CDC, 2018). There are reported cases of emergence of *P. falciparum* resistance to artemisinin-based drugs in some parts of Southeast Asia (Blasco *et al*., 2017). Some of the contributing factors to the emergence of drug resistance include:

- **Interaction of drug use patterns:** incorrect dosing, overuse of antimalarial drugs as well as incomplete therapeutic treatment can results in treatment failure in the individuals and can also contribute to the development of drug resistance (Bloland, 2001; Hyde, 2007).
- **Drug profile:** poor pharmacokinetic and pharmacodynamic properties of the drug can result in the parasite being exposed to sub-optimal drug concentrations that increase the probability of resistance development (Petersen *et al*., 2011).
- **Mutations:** Resistance to antimalarial drugs can be due to mutations in the parasite. The mechanism of antimalarial resistance is often due to spontaneous mutation (single point or multiple mutation) that confer reduced affinity of the antimalarial drug to the parasite target site depending on parasite strain or alter drug accumulation within the parasite (Blasco *et al*., 2017). Chloroquine resistance has been associated with an increased level of drug efflux, where the release of drug in chloroquine resistant strains is at a faster rate compared to the chloroquine sensitive parasites. The cause of this resistance is due to an amino acid mutation in *P. falciparum* chloroquine resistance transporter (*Pfcrt*) (Krogstad *et al.*, 1987; Bray *et al*., 1999; Cooper *et al*., 2005). Resistance to antifolate drugs in *P. falciparum* hav been found to be caused by mutations in the genes encoding for dihydrofolate reductase and dihydropteroate synthase (Durand *et al*., 2000; Sibley *et al.*, 2001). The resistance of *Plasmodium* parasites to artemisinin is attributed to early ring stage forms which enter a dormancy state after exposure to artemisinin and then resume growth after the removal of the artemisinin. This is influenced by mutational changes in the gene, *Pfk13* and the poor artemisinin bioavailability (Chinappi *et al*., 2010; Mbengue *et al*., 2015). Resistance to atovaquone occurs due to mutations in the parasite mitochondrial genome which reduces binding affinity of atovaquone at the binding site (Fisher *et al*., 2012).

1.3 Malaria elimination and eradication

The successful eradication of malaria will require continued implementation of measures that will lead to its elimination in countries where there is transmission and prevention of the re-occurrence in countries that are malaria free (WHO, 2018). The WHO (2017) has defined malaria elimination as “the interruption of local transmission (reduction to zero incidence of indigenous cases) of a specified parasite species in a defined geographic area”. While malaria eradication is defined as “the permanent reduction to zero of the world incidence of malaria

infection caused by all species of human malaria parasites". The major barrier to malaria elimination and ultimately its eradication is the increasing levels of resistant *Plasmodium* strains to antimalarials and development of insecticide resistance by *Anopheles* vector. To overcome these challenges, the WHO launched a global technical strategy for malaria in 2016 with the target aim to eliminate malaria by 2030 (WHO, 2018). One of the ways to achieve this set target is through integrated vector management and development of new antimalarial drugs with mechanisms of action targeting various stages of the parasite in both the host and vector.

1.4 Integrated vector management

The main aim of integrated vector management is to improve the efficacy, cost-effectiveness and sustainability of vector control through wide range of complementary non-chemical and chemical interventions (Beier *et al*., 2008; WHO, 2013). The WHO has recommended larval source management as part of integrated vector management used to supplement LLINs and IRS, especially in areas with reported resistance to the current insecticides used. Larval source management targets the immature aquatic stages (Figure 1.3) of the *Anopheles* mosquito vector, thereby decreasing the number of mosquitoes that reach adulthood (Fillinger and Lindsay, 2011; WHO, 2013). This can result in the reduction of malaria transmission, as well as morbidity and mortality. However, it is only effective in areas where the habitats of malaria mosquito larvae are few, fixed and easy to identify, map and eradicate (Beier *et al*., 2008; WHO, 2013). There are four categories of larval source management (Fillinger and Lindsay, 2011; WHO, 2013):

1. **Habitat modification:** permanent alteration to the environment to eliminate larval habitats.
2. **Habitat manipulation:** temporary environment changes to disrupt vector breeding.
3. **Biological control:** the introduction of natural predators to water bodies.
4. **Larviciding:** regular application of chemical and biological insecticides to breeding site.

According to WHO (2013), larval source management can contribute to integrated vector management by targeting outdoor resting and biting mosquitoes as well as malaria hotspots which are helpful to remove residual foci of malaria transmission in elimination programmes. In addition, larval source management can aid in the management of insecticide resistance. The variety in the classes and mechanism of action of different larvicidal agents combined with habit modification and manipulation can minimise overall dependence on the insecticides,

preserve the efficacy of existing insecticides and manage the spread of insecticide resistance once it has emerged (WHO, 2013).

Table 1.4: Types of larvicidal agents used to control aquatic stages of *Anopheles* malaria vector (WHO, 2013).

Larvicidal agent	Mechanism of action
Surface oils and films	Inhibit the ability of larvae to rest and breathe at the surface of the water causing them to drown and die. E.g monomolecular films, biodegradable ethoxylated alcohol surfactants
Bacterial larvicides	Bacteria produces insecticidal crystal proteins which when ingested by the larvae causes pore formation in the larvae midgut thereby interrupting feeding and homeostasis. E.g *Bacillus* species
Synthetic organic chemicals	Inhibit cholinesterase and interferes with the central nervous system of the mosquito vector. E.g organophosphates
Insect growth regulators	Disrupt the metamorphosis of the insect preventing the development into adulthood. E.g methoprene, diflubenzun
Spinosyns	Act as nicotinic acetylcholine receptor allosteric activators

Larval source management was the primary method used for vector control until the introduction of IRS with DDT in the 1950s. Larviciding with Paris green was used to eliminate *An. gambiae* from Brazil by 1940 after its introduction in the late 1920s and from Egypt by 1945. After the introduction of IRS and ITNS, the use of larval source management became less common in Africa. However, there is now renewed interest in larval source management and its practical application as a supplementary intervention to LLINS and IRS especially in areas where there is reported resistance to the current insecticides used (WHO, 2013).

As South Africa shift from malaria control to malaria elimination as well as the WHO target to eliminate malaria by 2030, the current interventions are not enough to eliminate one of Africa's major malaria vector, *An. arabiensis* due to its variable feeding and resting behaviour making it not completely susceptible to current vector control strategies (IRS and ITNS) which target indoor biting and resting (Kitau *et al*., 2012; Dandalo, 2017). In addition to *An. arabiensis* behavioural challenge, insecticide resistance has also been reported (Brooke *et al*., 2015; Messenger *et al*., 2017). Therefore, additional vector control interventions are needed to eliminate this vector and one of the ways identified is targeting the aquatic stages through the use of synthetic larvicides since their breeding sites are locatable (Ghosh *et al*., 2012; WHO,

2013; Muema *et al*., 2017). Only organophosphates are currently approved for larval control due to their efficacy and low persistence in the environment (WHO, 2013). Therefore, new larvicidal agents which are target specific, cost effective and environmentally safe need to be developed as well as new antimalarial drugs.

1.5 Profile for new antimalarial drugs

The ideal new antimalarial drug should meet certain characteristics, namely, rapid efficacy, minimal toxicity against human beings, low cost, be orally bioavailable for easy administration, be suitable for young children and pregnant women. In addition, the drugs must cross the membranes of infected erythrocyte and diffuse into the acidic vacuole of the parasite. (Ridley, 2002; Fidock *et al*., 2004). The new drug needs to be at least additive or synergistic in potency when combined with other antimalarial drugs and have similar pharmacokinetics to reduce the development of resistance (Ashley and Phyo, 2018).

The drug discovery and development of new antimalarial drugs involves screening of compounds *in vitro* using various drug sensitivity assays (such as parasite lactate dehydrogenase (pLDH), fluorescent-based and isotopic assays) to measure their efficacy against parasite growth and development (Noedl *et al*., 2003; Kaddouri *et al*., 2006; Dery *et al*., 2015). The compounds that show potent *in vitro* activity should also exhibit good pharmacokinetic properties as this will contribute to the final clinical success or failure of the compound. Drug properties such as absorption, distribution, metabolism, elimination (ADME) and toxicity are important parameters which can provide preliminary prediction of the *in vivo* properties of the lead compound and its potential to become a drug (Chung *et al*., 2015).

In addition, an ideal antimalarial drug should have a high therapeutic index, in that, the plasma level required to exert a toxic effect would be significantly higher than that required for therapeutic efficacy (Li, 2001). There are several toxicity predictive measures used in various drug discovery and developmental stages such as cytotoxicity assays. Cytotoxicity assays provide a screening method to determine whether the test compounds are harmful to the normal biological processes in human cells compared to the effects they are being tested for in the malaria parasite (Mativandlela, 2008). The ideal antimalarial drug should be target-specific to the parasite and cause no harm to the human host (Mencher and Wang, 2005).

1.6 Specific drug targets in *Plasmodium falciparum*

The main goal of antimalarial drug discovery is to develop drugs that can target specific parasite proteins involved in various metabolic pathways that are different from those of the human host, with reduced risks of potential toxicity (Olliaro, 2001). There are several *Plasmodium* metabolic pathways that have been studied as potential targets for the development of novel antimalarials (Figure 1.4).

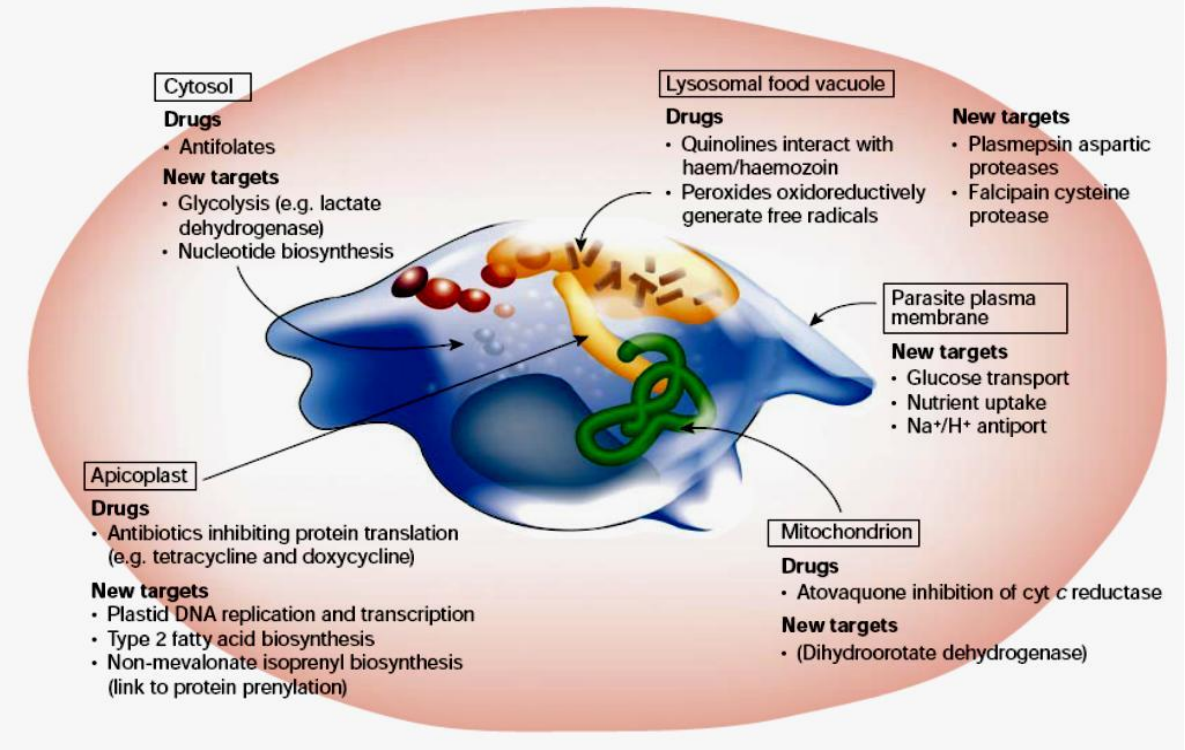


Figure 1.4: Antimalarial drug targets within the *Plasmodium* life cycle (Ridley, 2002).

Some of the investigated targets include:

- **The folate pathway:** which is crucial for parasite growth and survival as it controls the synthesis of DNA and metabolism of certain amino acids. Targeting enzymes such as dihydropteroate synthase that is involved in the biosynthesis of dihydrofolate (which is required for *de novo* folate synthesis), have proven to be effective in inhibiting the growth of sensitive parasites (Nzila *et al*., 2005).
- **The food vacuole:** is the main site for the degradation of host haemoglobin by parasite haemoglobinases, such as aspartic and cysteine proteases. Inhibition of these enzymes can disrupt intra-erythrocytic parasite development by preventing haemoglobin hydrolysis

resulting in parasite starvation and erythrocyte rupture, all culminating in an incomplete erythrocytic life-cycle (Olliaro, 2001; Dahl and Rosenthal, 2005).

- **Glycolysis:** plays a vital role in the energy production during the erythrocytic stages of the malaria parasite; therefore, inhibiting enzymes that are involved in this process can lead to parasite death due to depletion of adenine triphosphate (ATP). One such enzyme is pLDH which is involved in the conversion of lactate to pyruvate and has kinetic differences from the human LDH isoforms. This indicates that pLDH can be selectively targeted for the development of novel antimalarials (Mehta *et al*., 2006; Avery *et al*., 2008).
- **Plasmodial protein kinases:** Inhibition of plasmodial protein kinases can block the intra-erythrocytic development of *P. falciparum* (Olliaro, 2001; Avery *et al.*, 2008).
- **Apicoplast:** Plays a vital role in biosynthesis of lipids and iron metabolism, while the parasite can survive in the absence of apicoplast or chemically damaged apicoplast, they are unable to cause a new infection (Vargas-Parada, 2010).
- **Iron metabolism:** Iron chelators have been proposed as potential agents that can inhibit the development of *Plasmodium* parasites especially *P. falciparum* (Mabeza *et al*., 1999; Choveavx *et al*., 2012). Iron is essential for *P. falciparum* metabolism during the intra-erythrocytic phase. The proposed antimalarial action of iron chelators involves their penetration into the infected parasite erythrocyte, binding iron within the parasite cytosol and from the labile iron pool within the host red blood cell, which is necessary for plasmodial growth (Sekhon and Bimal, 2012). The mechanism by which iron chelators exert their antimalarial effect may either be through isolating iron required for plasmodial replication, or withholding other essential trace elements such as copper and calcium, which are essential to parasite metabolism. Chelators such as desferrioxamine and desferricrocin have been shown to be capable of sequestering iron needed for plasmodial replication *in vitro* (Van Zyl *et al.*, 1992; Mabeza *et al*., 1999). Similarly, 8-hydroxyquinoline and 2,2–bipyridyl binds either ferric or ferrous ions, with the resulting iron complex generating free radicals, which would be effective in inhibiting parasite growth (Mabeza *et al*., 1999).

Exploiting specific targets within the parasite is beneficial not only to decrease the side effect profile of the drug, but also to reduce the risk of drug resistance (Jana and Paliwal, 2007). Several studies have shown that one of the ways to create novel drugs with improved antimalarial properties compared to the existing drugs, is through combination of chemical entities with two or more structural moieties into a single hybrid compound (Raj *et al*., 2015).

1.7 Hybrids compounds

Hybridisation is a concept that involves covalent linking of two molecules, each with their own mechanism of action to produce a single hybrid compound with dual activity (Meunier, 2008; Walsh and Bell, 2009). Hybrids compounds are absorbed, distributed, metabolised and eliminated at one single rate, thereby reducing the risk of drug interactions and this offers an advantageous effect compared to combination therapy that can result in pharmacological interactions between the drugs (Muregi and Ishih, 2010). Various hybrid compounds such as quinoline-artemisinin hybrids have been shown to possess potent *in vitro* antimalarial activity with minimum toxicity (Capela *et al.*, 2011; Wang *et al*., 2014; Lödige and Hiersch, 2015).

In this study, novel quinoxaline-phenyl and quinoxaline-quinoline hybrids with either a chalcone or pyrazoline linker were synthesized and tested for their *in vitro* antimalarial and insecticidal activity as part of identifying new antimalarial and larvicide agents (Figure 1.5). The toxicological profile of hybrids compounds in the literature were mostly tested on human cancer cells (Capela *et al.*, 2011). Similarly, these novel hybrids were evaluated using several methodology as well as comparing their effect on normal human cells and human cancer cells to provide further information of unknown spectrum of activity of these novel hybrids. Individually, these compounds have been found to possess various biological activities as described below.

1.7.1 Quinoxaline

A quinoxaline is a nitrogen containing heterocyclic compound with a benzene ring and pyrazine ring (Figure 1.5). Quinoxaline and its derivatives have been identified as important heterocyclic compounds due to diverse pharmacological properties including anti-oxidant, antibacterial, antifungal, anticancer and antimalarial activity (Raju *et al*., 2015; Kaushal *et al*., 2019). The first publication on the antimalarial activity of quinoxaline-based compounds was by Haworth and Robinson (1948) when it was reported that the presence of the chlorine atom of 2,3-dichloquinoxaline and 1,4-dichlorophthazine was crucial for the inhibitory activity against *P. gallinaceum*. Thereafter, Crowther *et al*. (1949) synthesized a new series of dialkylaminoquinoxaline and found similar results. Since then, there has been growing evidence that compounds with a quinoxaline nucleus have potential *in vitro* antimalarial effects against *Plasmodium* strains including, *P. falciparum* (Ortega *et al*., 2002; Aguirre *et al*., 2004;

Estevez *et al*., 2011; Montana *et al*., 2014). In addition, quinoxaline and its derivatives have wide application in agriculture as insecticides, fungicides and herbicides.

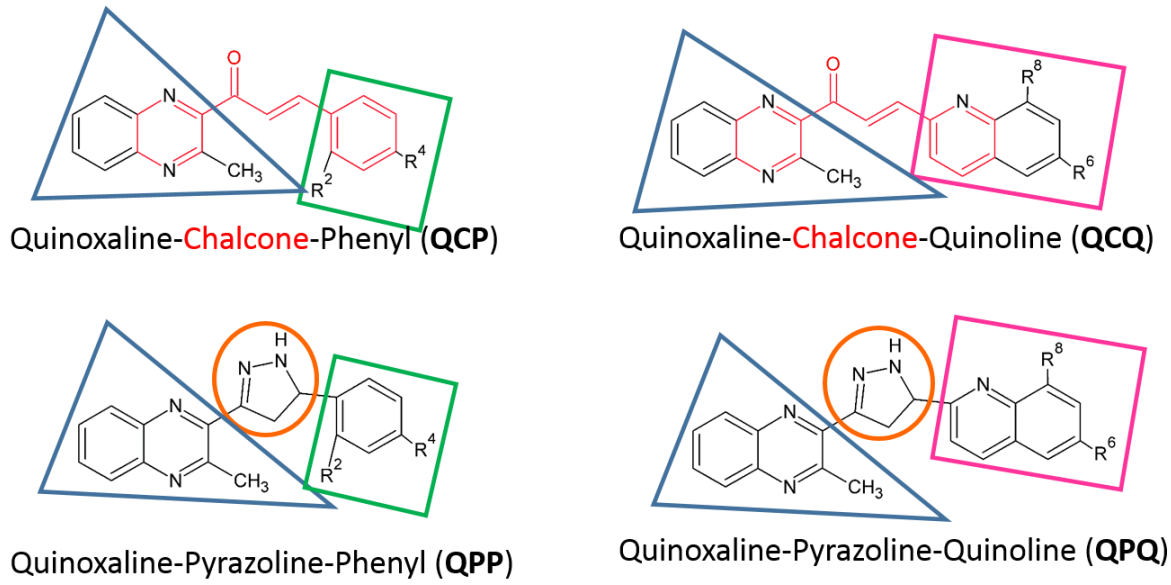


Figure 1.5: The structural components of the test compounds. These included a quinoxaline (blue triangle) with either a phenyl (green square) or quinoline (pink square); with either a chalcone (red text) or pyrazoline (orange circle) linker.

1.7.2 Chalcones

Chalcones (1,3-diphenyl-2-propen-1-ones) (Figure 1.5) are open-chain precursors for biosynthesis of flavonoids and isoflavonoids and occur mainly as polyphenolic compounds abundant in fruits, spices and vegetables (Go, 2003; Ren *et al*., 2003; Nowakowska, 2007). Chalcones exist as either *trans* (E, **1**) or *cis* (Z, **2**) isomers having two aromatic rings that are joined by a three carbon α-, β-unsaturated carbonyl system. The *E*-isomer is more stable, which makes it the predominant configuration among the chalcones. The configuration of the *Z*-isomer is unstable, due to the strong steric effects between the carbonyl group and A-ring (Go, 2003; Aksöz *et al*., 2011).

Chalcones possess a variety of biological activities including anti-inflammatory, anti-oxidant, anticancer, antiprotozoal and antibacterial activity (Nowakowska, 2007; Mishra *et al*., 2008). The activities have been largely attributed to the presence of α,β-unsaturated carbonyl ketone moiety in chalcones (Won *et al*., 2005). The antimalarial activity of chalcones was first reported in oxygenated chalcone, licochalcone A, which was isolated from Chinese liquorice.

Licochalcone A was found to have similar activity when tested against chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) *P. falciparum* strains with 50% parasite inhibition at 0.6 µg/ml (Chen *et al*., 1994; Go *et al*., 2004).

1.7.3 Quinoline

Quinolines (1-aza-napthalene or benzo[*b*]pyridine) are nitrogen containing heterocyclic aromatic compounds (Figure 1.5). The quinoline structure and its derivatives are pharmacologically active substances displaying a broad range of biological activity; including analgesic, anti-inflammatory, anticancer, antiviral and cardiovascular activity (Wozniacka *et al*., 2002; Solomon and Lee, 2011).

The isolation of quinine, cinchonine and quinoline in the 18th century from the bark of the *Cinchona* tree constituted the first successful malaria medication (Wozniacka *et al*., 2002; Solomon and Lee, 2009). Chloroquine is quinoline derivative that has been used for decades as the primary antimalarial drug due to its favourable pharmacokinetic profile, low cost and clinical efficacy (Fidock *et al*., 2004; Sharma, 2005). However, there is now widespread resistance by *P. falciparum,* thus making chloroquine less effective as an antimalarial drug (Fidock *et al*., 2004).

1.7.4 Pyrazoline

Pyrazoline and its derivatives are members of nitrogen-containing heterocycles with a C=N bond (Figure 1.5) and are found in natural products in the form of vitamins, alkaloids and pigments (Yusuf and Jain 2014). Pyrazoline and its derivatives have varied pharmacological activities such anticancer, anti-oxidant, antimicrobial and antidepressant effects (Amin *et al*., 2013; Kumar *et al*., 2013; Patel *et al*., 2014; Rao and Kalaichelvan, 2015). However, there is limited literature describing the antimalarial efficacy of pyrazolines (Insuasty *et al*., 2015; Mishra *et al*., 2017).

The rationale of synthesising novel quinoxaline-based derivatives was to determine if hybridisation of quinoxaline with a quinoline or phenyl scaffold, linked by biologically active chalcone or pyrazoline moiety could produce compounds that acted in a complementary manner to inhibit parasite growth and cause lethality in *Anopheles* eggs and larvae more effectively than the exciting antimalarial and insecticides agents; whilst causing minimal toxicity to the host cell and non-target organisms.

1.8 AIM AND OBJECTIVES

The aim of this study was to determine the effect of thirty-five novel quinoxaline-based hybrid compounds on the asexual blood stages of *P. falciparum* and the aquatic life-cycle stages of the malaria vector, *An. arabiensis*; as well as determine their preliminary toxicological profile.

To achieve this aim, objectives of this study were to:

- Assess the *in vitro* antimalarial activity of the test compounds against the chloroquine-sensitive NF54 strain of *P. falciparum.*
- Determine the pharmacological interaction between a standard antimalarial drug, quinine and two of the active compounds on antimalarial activity.
- Assess the ovicidal and larvicidal activity of the compounds against malaria vector, *An. arabiensis* (KWAG strain) compared to positive control, DDT.
- Evaluate the preliminary toxicological profile of the compounds against human red blood cells, human epithelial kidney cells, human chronic myelogenous leukaemia and *Artemia franciscana*.
- Determine the stage-specific morphological effects of the two active compounds in comparison to quinine.
- Assess the iron chelating and free radical scavenging properties of the test compounds to further elucidate a potential antimalarial mechanism of action.
- Predict drug-like and pharmacokinetic properties of the active compounds in comparison to standard antimalarial drug, quinine.

CHAPTER TWO - METHODS AND MATERIALS

2.1 Synthesis and chemistry of the test compounds

A total of thirty-five novel quinoxaline-based hybrid derivatives were synthesized by Prof N Koorbanally and Dr S Sewpersad from the Department of Chemistry at the University Of KwaZulu-Natal, South Africa. The compound structure was verified by NMR and IR and the purity of the compounds were determined to be more than 98%. The derivatives consisted of two different sets of hybrids; eighteen quinoxaline-phenyl hybrids with either chalcone (**QCP** compounds) or pyrazoline linker (**QPP** compounds) (Table 2.1) and seventeen quinoxaline-quinoline hybrids with either chalcone (**QCQ** compounds) or pyrazoline linker (**QPQ** compounds) (Table 2.2). Compound **QCP4**, **QPP4**, **QCQ4**, **QPQ4** and **QPQ9** were not received and therefore were excluded from the study.

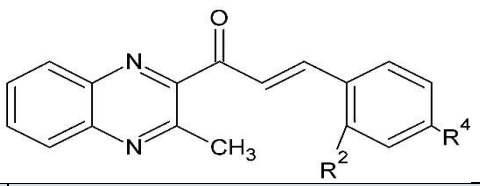
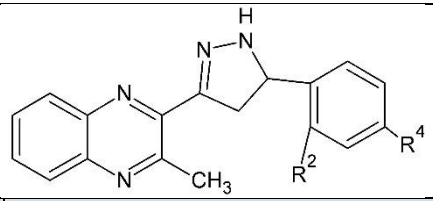
All the chemicals and reagents used in this study were of the highest quality and were purchased from reputable companies. The test compounds and controls were dissolved in dimethyl sulfoxide (DMSO; Merck) to prepare 10 mM stock solutions and stored in 1 ml aliquots at -70˚C until needed for experiments. The final concentration of DMSO in all experiments was ≤ 1%, where in preliminary experiments was verified not to inhibit viability of cells or inhibit activity in all assays. For all experiments, negative background controls were included in the assays to be used in the calculation to determine final compound effect and as such were not reported for each assay.

2.2 *In vitro* parasite culturing

2.2.1 *In vitro* parasite culture maintenance

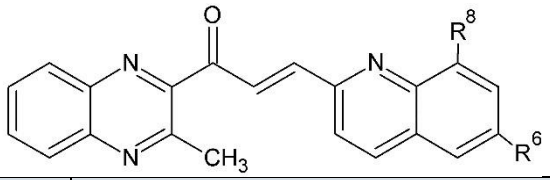
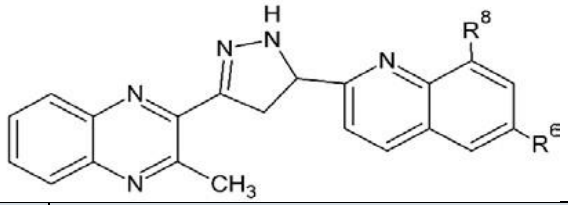
The asexual blood stages of chloroquine-sensitive NF54 strain of *P. falciparum* were cultured *in vitro* according to the method of Trager and Jensen (1976) and Van Zyl *et al.* (2006) with slight modifications. The initial parasite culture was obtained from the Department of Molecular Medicine and Haematology at the University of the Witwatersrand, South Africa and were maintained in 75 cm^2 tissue culture flask (Sigma-Aldrich®). The parasitaemia was maintained between 5-10% with a 5% haematocrit and the complete culture media was replaced daily (Section 2.2.4). The parasite culture was incubated at 37ºC in a 5% carbon dioxide, 3% oxygen and 92% nitrogen environment and was synchronised when the parasites were predominately in early ring stage (Section 2.2.5). A Giemsa-stained thin blood smear was

Table 2.1: The tested quinoxaline-phenyl hybrids with either; **A:** chalcone (**QCP** compounds) or **B**: pyrazoline linker (**QPP** compounds).

A 				**B** 			
Code	**R-group**	**Name**	**MW (g/mol)**	**Code**	**R-group**	**Name**	**MW (g/mol)**
QCP1	2-F	(E)-3-(2-fluorophenyl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	292.31	**QPP1**	2-F	2-(5-(2-fluorophenyl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	306.34
QCP2	2-Cl	(E)-3-(2-chlorophenyl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	308.76	**QPP2**	2-Cl	2-(5-(2-chlorophenyl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	322.79
QCP3	2-Br	(E)-3-(2-bromophenyl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	353.21	**QPP3**	2-Br	2-(5-(2-bromophenyl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	367.24
QCP5	2-CH_3	(E)-3-(2-methylphenyl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	288.34	**QPP5**	2-CH_3	2-(5-(2-methylphenyl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	302.37
QCP6	4-F	(E)-3-(4-fluorophenyl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	292.31	**QPP6**	4-F	2-(5-(4-fluorophenyl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	306.34
QCP7	4-Cl	(E)-3-(4-chlorophenyl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	308.76	**QPP7**	4-Cl	2-(5-(4-chlorophenyl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	322.79
QCP8	4-Br	(E)-3-(4-bromophenyl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	353.21	**QPP8**	4-Br	2-(5-(4-bromophenyl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	367.24
QCP9	4-OCH_3	(E)-3-(4-methoxyphenyl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	304.34	**QPP9**	4-OCH_3	2-(5-(4-methoxyphenyl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	318.37
QCP10	4-CH_3	(E)-3-(4-methylphenyl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	288.34	**QPP10**	4-CH_3	2-(5-(4-methylphenyl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	302.37

MW: Molecular Weight

Table 2.2: The tested quinoxaline-quinoline hybrids with either; **A:** chalcone (**QCP** compounds) or **B**: pyrazoline linker (**QPP** compounds).

A 				B 			
Code	**R-group**	**Name**	**MW (g/mol)**	**Code**	**R-group**	**Name**	**MW (g/mol))**
QCQ1	8-F	(E)-3-(8-fluoroquinolin-2-yl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	343.35	**QPQ1**	8-F	2-(5-(8-fluoroquinolin-2-yl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	357.38
QCQ2	8-Cl	(E)-3-(8-chloroquinolin-2-yl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	359.81	**QPQ2**	8-Cl	2-(5-(8-chloroquinolin-2-yl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	373.84
QCQ3	8-Br	(E)-3-(8-bromoquinolin-2-yl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	404.26	**QPQ3**	8-Br	2-(5-(8-bromoquinolin-2-yl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	418.29
QCQ5	8-CH_3	(E)-3-(8-methylquinolin-2-yl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	339.39	**QPQ5**	8-CH_3	2-(5-(8-methylquinolin-2-yl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	353.42
QCQ6	6-F	(E)-3-(6-fluoroquinolin-2-yl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	343.35	**QPQ6**	6-F	2-(5-(6-fluoroquinolin-2-yl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	357.38
QCQ7	6-Cl	(E)-3-(6-chloroquinolin-2-yl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	359.81	**QPQ7**	6-Cl	2-(5-(6-chloroquinolin-2-yl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	373.84
QCQ8	6-Br	(E)-3-(6-bromoquinolin-2-yl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	404.26	**QPQ8**	6-Br	2-(5-(6-bromoquinolin-2-yl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	418.29
QCQ9	6-OCH_3	(E)-3-(6-methoxyquinolin-2-yl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	355.39	**QPQ10**	6-CH_3	2-(5-(6-methylquinolin-2-yl)-4,5-dihydro-1Hpyrazol-3-yl)-3-methylquinoxaline	353.42
QCQ10	6-CH_3	(E)-3-(6-methylquinolin-2-yl)-1-(3methylquinoxalin-2-yl)prop-2-en-1-one	339.39				

MW: Molecular Weight

prepared daily to determine percentage parasitaemia and observe any morphological changes (Section 2.2.5).

A human ethics waiver was obtained from the University of the Witwatersrand Human Research Ethics Committee (Medical) for the use of human blood for *in vitro* malaria parasite culturing and experimental studies (waiver number: W-CBP-180420-2; Appendix A). A Biosafety clearance certificate was obtained from the Witwatersrand Institutional Biosafety Committee (protocol number: 20180102; Appendix B) for the *in vitro* laboratory studies.

2.2.2 Preparation of incomplete culture and experimental media

The incomplete culture media was prepared by dissolving 10.4 g/L Roswell Park Memorial Institution (RPMI)-1640 medium (Gibco®), 20 mM D-glucose monohydrate (Merck), 30 mM N-2-hydroxyelthlypiperazine-N'-2-ethane-sulfonic acid (HEPES) buffer (Boehringer Mannheim®), 0.1 mM gentamicin (Sigma-Aldrich®) and 0.3 mM hypoxanthine (Sigma-Aldrich®) in autoclaved Milli-Q® water. The solution was then stirred for one hour and sterilized through a Sterivex™-GS 0.22 µm filter and stored at 4ºC until required. For incomplete experimental media, the same procedure was followed, with the exclusion of gentamicin.

2.2.3 Preparation of lipid rich bovine serum AlbuMAX™

A stock solution of 5% (w/v) lipid rich bovine serum AlbuMAX™ (Gibco®) was prepared in incomplete culture medium (Section 2.2.2). Once dissolved at 37°C for at least 2 hours, the solution was sterilised through a Sterivex™-GS 0.22 µm filter and stored at -20˚C until needed.

2.2.4 Preparation of complete culture and experimental media

The complete culture and experimental media were prepared by supplementing the incomplete culture and experimental media (Section 2.2.2) with sterile 5% (w/v) sodium bicarbonate (Sigma-Aldrich®) and 10% (v/v) lipid enriched bovine serum AlbuMAX™ (Section 2.2.3).

2.2.5 Preparation of Giemsa stained thin blood smear

The percentage parasitaemia and parasite blood stages were determined daily by preparing a thin blood smear on a clean microscope slide from a settled parasite culture. The smear was air dried before being stained using the Rapi-diff stain kit (Clinical Sciences Diagnostics) that consisted of three solutions. Solution 1 (thiazine) was used to fix the slide for 5 seconds,

solution 2 (eosin) was used to stain the RBCs for 20 seconds and then washed under gentle flowing tap water. Solution 3 (methylene blue) stained the parasites purple after 60 seconds of staining and the slide was washed with tap water. The slide was then air dried before being viewed microscopically under oil immersion (1000x) using a Nikon Optiphot phase contrast 2 light microscope (CFW10x). Ten fields were counted to determine the percentage parasitaemia using Equation 2.1 and the percentage of each parasite stage determined; as well as the morphological features of the parasites to ensure they were growing under optimal conditions.

$$\% \text{ Parasitemia} = \frac{\text{Total number parasitized RBCs}}{\text{Total number of RBCs (uninfected and parasitized)}} \times 100$$

Equation 2.1: The formula used to calculate percentage parasitaemia from the Giemsa stained thin blood smear.

2.2.6 Sorbitol synchronisation of the parasite culture

To obtain a synchronous ring staged culture for experimentation and optimal parasite growth, the D-sorbitol method was used (Lambros and Vanderberg, 1979). The malaria parasites grow in synchrony *in vivo* and in human host however, when grown *in vitro* they tend to lose the synchronicity in their life cycle. Therefore, to maintain *in vitro* culture synchrony, the parasite culture was suspended in 5% (w/v) D-sorbitol that lysed the RBCs infected with late mature stages of the parasite (trophozoites and early schizonts) by osmosis, while the ring-staged and uninfected RBCs remained unaffected (Lambros and Vanderberg, 1979).

Briefly, the percentage rings in the asexual blood stages of parasite were determined using the Giemsa-stained thin blood smear (Section 2.2.5) and when predominantly in the ring stage, the culture was transferred into a 50 mL tube and centrifuged at 400 *g* for 5 minutes. The supernatant was replaced with 10 ml of 5% (w/v) D-sorbitol (Sigma-Aldrich®) and incubated for 12 minutes at 37ºC. Thereafter, the parasite suspension was vortexed for 15 seconds before being centrifuged at 400 *g* for 5 minutes. The pellet was washed twice in sterile phosphate buffered saline (PBS, pH 7.40). The synchronised parasite culture was then re-suspended in complete culture medium (Section 2.2.4) and incubated at 37ºC in a 5% carbon dioxide, 3% oxygen and 92% nitrogen atmosphere.

PBS (pH 7.40) was prepared by dissolving 2 mM of potassium phosphate monobasic (Fluka®), 4 mM potassium chloride (Sigma-Aldrich®), 4 mM sodium phosphate dibasic dehydrate

(Sigma-Aldrich®) and 4 mM sodium chloride (Sigma-Aldrich®) in Milli-Q® water. The pH was then adjusted to 7.40 with 10 mM sodium hydroxide (NaOH, Sigma-Aldrich®) before the solution was autoclaved (121ºC at 1.2 kg/cm^2 for 20 minutes) and stored at 4ºC until required.

2.2.7 Preparation of uninfected red blood cells

The whole human blood sample was obtained from healthy volunteers within the University of the Witwatersrand Faculty of Health Sciences, Johannesburg (waiver number: W-CBP-180420-2, Appendix A). The blood was collected and stored in Vacuette® blood collection tubes containing Acid Citrate Dextrose Solution B (ACD). The blood was washed twice in PBS (pH 7.40) at 617 *g for* 5 minutes and the supernatant was discarded. The remaining packed RBCs was re-suspended in an equal volume of incomplete experimental culture medium (Section 2.2.2) to store as a 50% haematocrit suspension at 4ºC for a week.

2.3 *In vitro* antimalarial activity

2.3.1 Parasite lactate dehydrogenase antimalarial assay

The pLDH assay was used to determine the sensitivity of the asexual blood stages of the chloroquine-sensitive *P. falciparum* NF54 strain, to the test compounds according to Makler and Hinrichs (1993) with minor modifications. The basis of this assay involves the ability of *Plasmodium* to synthesise lactate dehydrogenase (LDH) as a terminal enzyme in the glycolytic pathway during its blood stage cycle. The parasite LDH differs from the human LDH by its ability to use 3-acetylpyridine adenine dinucleotide (APAD) as a coenzyme to convert lactate to form pyruvate, while reducing APAD to APADH (Makler and Hinrichs, 1993). In the pLDH assay, pyruvate is formed when viable pLDH enzyme from the parasite infected blood lysate is mixed with buffered solution of APAD and L-lactate (known as the Malstat™ reagent) and the addition of nitroblue tetrazolium (NBT) and phenazine ethosulfate (PES). The yellow NBT is reduced by APADH to a blue formazan salt that can be measured spectrophotometrically (Makler *el al.*, 1998) (Figure 2.1).

2.3.2 Preparation of parasites for pLDH assay

Using a synchronised parasite culture predominantly composed of rings, a Giemsa-stained thin blood smear was prepared to determine the percentage parasitaemia using Equation 2.1 (Section 2.2.5). Based on this, the parasitaemia was adjusted to 2% and a 2% haematocrit

suspension was prepared using washed uninfected RBCs (Section 2.2.7) (older than three days) and complete experimental medium (Section 2.2.4).

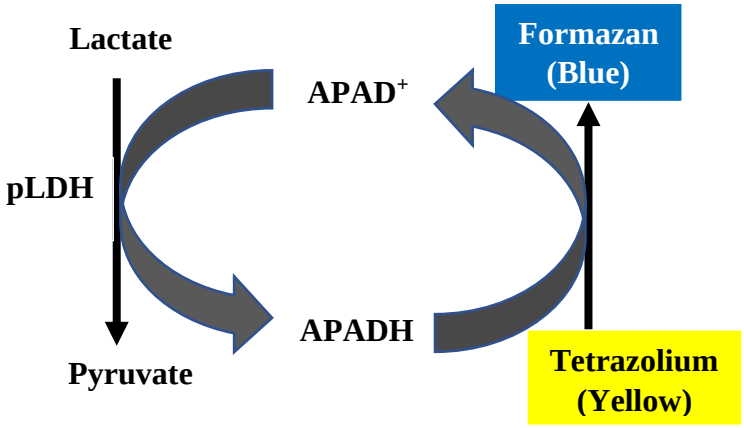


Figure 2.1: The Malstat™ reaction for the detection of pLDH in viable *P. falciparum* (adapted from Markwalter *et al.*, 2015).

2.3.3 Preparation of test compounds and control drugs

The stock solutions (10 mM) of the test compounds and two antimalarial reference drugs; quinine hydrochloride dihydrate (Sigma-Aldrich®) and dihydroartemisinin (DHA; Sigma-Aldrich®) were diluted using incomplete experimental media (Section 2.2.2) to the screening concentration of 50 µM taking the nine times dilution factor into account.

2.3.4 Preparation of pLDH assay reagents

2.3.4.1 Phenazine ethosulfate solution

To make the PES solution, 0.1 mg/mL PES (Sigma-Aldrich®) was dissolved in autoclaved Milli-Q® water and sterilised through a Sterivex™-GS 0.22 µm filter and stored for maximum of six months at 4ºC covered with foil to prevent light exposure.

2.3.4.2 Nitroblue tetrazolium solution

The NBT powder (Sigma-Aldrich®) (2 mg/mL) was dissolved in autoclaved Milli-Q® water and sterilised through a Sterivex™-GS 0.22 µm filter and was stored for no more than a week at 4ºC covered in foil to prevent light exposure.

2.3.4.3 Malstat™ reagent

Malstat™ reagent was prepared by dissolving 40 µL Triton X-100™ (Sigma-Aldrich®), 0.18 mM calcium-L-lactate (Sigma-Aldrich®) and 0.5 mM APAD (Sigma-Aldrich®) in trisaminomethane (TRIS) buffer (27.5 mM TRIS base powder (Prolabo®) dissolved in autoclaved Milli-Q® water). The pH was adjusted to 9.10 with NaOH, before the prepared reagent was sterilised through Sterivex™-GS 0.22 µm filter and stored at 4ºC, covered in foil to prevent light exposure, for no more than a week.

2.3.5 Experimental protocol for pLDH assay

The 2% parasitaemia and 2% haematocrit parasite ring suspension (100 µL) was plated into all the wells of a sterile 96-well microtiter plate, except for two that were used as the uninfected RBCs control. To the latter two wells, 100 µL of a 2% haematocrit of washed uninfected RBCs (Section 2.2.7) was plated. The plate was then incubated in an air-tight humidified glass desiccator at 37°C for 24 hours. Thereafter, 12.5 µL diluted test compounds and reference drugs were added to duplicate wells, except for the drug-free parasitised RBCs control wells and the uninfected RBC control wells, to which 12.5 µL complete media was added. The plate was incubated for further 48 hours under the same conditions and thereafter frozen at -70°C for one hour. The plates were thawed at 37°C for 45 minutes, before shaken at 1200 rpm for 2 minutes on an orbital plate shaker (MRC Thermo-Shaker™ MB100-4P). Thereafter, 25 µL of the blood lysate was transferred from each well to a new sterile 96-well microtiter plate. To which 100 µL Malstat™ reagent was added together with 20 µL of a 1:1 ratio of NBT and PES mixture. The plate was then incubated at 37°C for 40 minutes and 50 µL of 5% (v/v) acetic acid was added to each well to stop the reaction.

2.3.6 Data analysis

The plate was read at 620 nm using a UV-Vis plate spectrophotometer (Labsystems iEMS Reader MF) with Ascent™ software version 2.6 and the percentage parasite growth was calculated using Equation 2.2. For those compounds that exhibited more than 60% inhibitory activity after the initial screening, six 1:2 serial dilutions were prepared to determine the half maximal inhibitory concentrations (IC_{50}) from a log sigmoidal dose response curves using GraphPad Prism software version 5 (GraphPad Software, Inc, USA).

$$\%\ \text{Parasite growth} = \frac{\text{Absorbance}_{\text{compound}} - \text{Absorbance}_{\text{blank}}}{\text{Absorbance}_{\text{parasite control}} - \text{Absorbance}_{\text{blank}}} \times 100$$

Equation 2.2: Calculation of percentage parasite growth.

Based on the IC_{50} values, the *in vitro* antimalarial activity of the active compounds was classified according to modified criteria described by Batista *et al*. (2009) and Moseley (2012) as follows: IC_{50} < 5 µM, excellent/potent activity; IC_{50} of 5-10 µM, good/promising activity; IC_{50} values of 10-20 µM, moderate activity; IC_{50} of 20-50 µM, low activity and IC_{50} > 50 µM as inactive.

2.4 Combination studies

According to the WHO recommendation, the standard malaria treatment should consist of two or more drugs in order to reduce the development of drug resistance and to increase the therapeutic effect (WHO, 2015). When drugs are combined, there are three possible pharmacological interactions, namely additive, synergistic or antagonistic, with the former two the preferred interactions (Bell, 2005).

2.4.1 Experimental protocol for combination studies

Based on the individual *in vitro* antimalarial IC_{50} values (Section 2.3.5), two active test compounds with low toxicity against RBCs (Section 2.6.1) and the human epithelial kidney cells (Section 2.6.2) were combined with the standard antimalarial drug, quinine to determine their pharmacological interaction. The pLDH assay (Section 2.3.5) was used for the combination of the compounds with quinine (Van Zyl *et al.*, 2010). The working solutions of the test compounds and quinine were prepared at concentrations 18 times higher than the final concentration required in the experiment to account for the nine times dilution factor of the pLDH assay (Section 2.3.5) and the two times dilution factor generated by combining equal volumes of the test compound and quinine. The test compounds and quinine were combined in different concentration ratios such that as the concentration of the one increased, the concentration of the other decreased (Table 2.5). Serial dilutions were prepared from each set of concentration ratios, from which log sigmoidal dose response curves were constructed using GraphPad Prism software version 5 (GraphPad Software, Inc, USA) to determine IC_{50} values for the drug alone and the combinations.

Table 2.3: Concentration ratios used for the combination between quinine and two active test compounds.

Concentration (µM)	A	B	C	D	E	F	G	H
Quinine	0	0.001	0.01	0.1	1	2.5	5	25
Test compound	200	150	100	50	10	2.5	1	0

2.4.2 Data analysis

The IC_{50} values for each combination were used to calculate the relative ratios according to Equation 2.3. An isobologram was constructed using the relative ratios to determine whether the pharmacological interaction was synergistic, additive or antagonistic.

$$(X{:}Y) = \frac{IC_{50\text{ of compound X in combination}}}{IC_{50\text{ of compound X alone}}} : \frac{IC_{50\text{ of compound Y in combination}}}{IC_{50\text{ of compound Y alone}}}$$

Equation 2.3: Calculation of relative ratios between the test compounds and quinine.

To verify the strength of the interaction interpreted from the isobologram, the sum of the fractional inhibitory concentration (∑FIC) of two test compounds was calculated for each combination (Equation 2.4). The calculated ∑FIC was interpreted as follows: if ∑FIC >1.25, as antagonistic, 0.75>∑FIC<1.25 as additive and ∑FIC <0.75 as synergistic (Van Zyl *et al.*, 2010).

$$\sum \text{FIC} = \frac{IC_{50\text{ of compound X in combination}}}{IC_{50\text{ of compound X alone}}} + \frac{IC_{50\text{ of compound Y in combination}}}{IC_{50\text{ of compound Y alone}}}$$

Equation 2.4: ∑FIC calculation from the IC_{50} values of compound Y and X (Van Zyl *et al.*, 2010).

2.4.3 Morphological and stage specific effects of the compounds on *P. falciparum*

The morphological and stage specific effects of the two active compounds used in combination studies (Section 2.4.1) and quinine was determined on the asexual blood stage of the chloroquine-sensitive NF54 strain of *P. falciparum* (Moseley, 2012; with slight modifications). The working solution of the test compounds and quinine was prepared to their respective IC_{50} values in incomplete experimental culture media (Section 2.2.2) to determine the concentration effects on the malaria parasite. The synchronised ring staged parasites (5.4 mL; Section 2.2.5)

prepared at a 2% parasitaemia and 2% haematocrit in complete culture media (Section 2.2.4) were incubated with 600 µL compounds/quinine in 25 cm^3 tissue culture flasks (Sigma Aldrich®). The untreated control was also prepared at 2% haematocrit and 2% parasitaemia in the absence of any compound/quinine. The cultures were incubated at 37˚C in a 3% oxygen, 4% carbon dioxide and 93% nitrogen environment. Every 8 hours, the flasks were gently agitated to re-suspend the culture and 200 µL suspension was removed from each flask to make Giemsa stained thin blood smears (Section 2.2.6). The blood smears were analysed using an Olympus®, CKX41 microscope at 1000x magnification to determine total percentage parasitaemia, percentage parasitaemia at each stage, as well as observe any morphological changes in the parasite compared to the untreated and quinine controls.

2.5 Mammalian *in vitro* toxicity studies

2.5.1 Haemolysis assay

To confirm if the test compounds had a direct inhibitory effect on the intra-erythrocytic malaria parasite and not on the human RBC membrane, the *in vitro* haemolytic activity of the test compounds and controls on healthy uninfected RBCs was determined (Hayat *et al.*, 2011; with minor modifications).

2.5.1.1 Preparation of RBC suspension

Freshly drawn human blood (not older than three days) was washed twice in PBS (pH 7.40) (Section 2.2.7) and adjusted to a 2% haematocrit suspension in complete experimental medium (Section 2.2.4).

2.5.1.2 Preparation of test compounds and controls

A 10 mM stock solution for each compound and reference drugs, quinine (Sigma-Aldrich®) and DHA (Sigma-Aldrich®) was prepared and from this stock, a 50 µM screening concertation was prepared taking the dilution factor of ten into account.

2.5.1.3 Experimental protocol for haemolysis assay

A 2% haematocrit (180 µL) suspension was plated in all the wells of a sterile 96-well microtiter plate, except for row H that served as a row for controls. For the 0% haemolysis negative controls, 20 µL of incomplete media (Section 2.2.4) was plated in triplicate wells and for the 100% haemolysis positive control, 20 µL of 0.2% (v/v) Triton X-100™ (Sigma-Aldrich®) was plated in the remaining wells of row H. The test and control compounds (20 µL; 50 µM) were

added to the remaining wells before being incubated at 37˚C for 48 hours in an airtight humidified glass desiccator. After which, the RBC pellets were gently disrupted in an orbital plate shaker (MRC Thermo-Shaker™ MB100-4P) at 1200 rpm for 2 minutes, before being centrifuged at 400 g for 5 minutes on the Thermo Scientific Heraeus Megafuge 40 Centrifuge. The supernatant (50 µL) was then pipetted into a new non-sterile 96-well microtiter plate with 150 µL sterile Milli-Q® water before the absorbance was read.

2.5.1.4 Data analysis

The plate was read at 540 nm on the UV-Vis plate spectrophotometer (Labsystems iEMS Reader MF) with Ascent™ software version 2.6. The test compounds that exhibited more than 60% haemolytic activity at 50 µM, were further diluted in order to determine IC_{50} values from log sigmoidal dose response curves generated by using GraphPad Prism software version 5 (GraphPad Software, Inc, USA). The percentage haemolysis was calculated using Equation 2.5.

$$\%\ \text{Haemolysis} = \frac{\text{Absorbance}_{\text{test compound}} - \text{Absorbance}_{0\%\ \text{haemolysis control}}}{\text{Absorbance}_{100\%\ \text{haemolysis}} - \text{Absorbance}_{0\%\ \text{haemolysis control}}} \times 100$$

Equation 2.5: Equation used to calculate percentage haemolysis of the compounds and controls.

2.5.2 Cell viability assay

The cell viability assay was used to determine whether the test compounds possess any cytotoxic effects to mammalian cell lines using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as a substrate (Mosmann, 1983; Van Zyl *et al.*, 2006). The MTT viability assay is based on the reduction of the yellow water-soluble tetrazolium MTT to a purple insoluble formazan by active mitochondrial dehydrogenase enzymes in metabolically active cells (Mosmann, 1983; Dreiem *et al.*, 2005) (Figure 2.2).

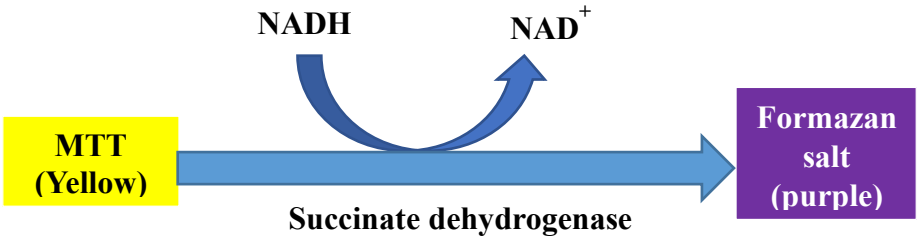


Figure 2.2: The reduction of MTT to formazan by metabolically active cells (adapted from van Meerloo *et al*., 2011). The enzyme succinate dehydrogenase catalyses the reduction of MTT to formazan salt using NADH and NADPH as co-factors.

2.5.2.1 Maintenance of cell culture

The test compounds were evaluated for their cytotoxicity profile against two human cell lines; the human epithelial kidney (HEK-293; Graham) and chronic myelogenous leukaemia (K562) cell lines. The cells were continuously maintained in Dulbecco's Modified Eagle's Medium (DMEM) at 37°C in 5% carbon dioxide incubator (Forma Scientific Water-Jacketed Incubator). The human epithelial kidney cell line was trypsinized at least once a week when 90% cell confluency was reached, and media was changed at least twice a week for both cell lines. Ethics waiver (reference number: W-CJ-170410-1) was obtained from the University of the Witwatersrand Human Research Ethics Committee (Medical) allowing the use of cell lines for *in vitro* laboratory studies (Appendix C).

2.5.2.2 Preparation of incomplete cell culture media

DMEM culture media: This media was prepared by dissolving 13.5 g/L DMEM powder (Sigma-Aldrich®) and 44 mM $NaHCO_3$ (Sigma-Aldrich®) in autoclaved Milli-Q® water and the solution was sterilised through a 0.22 µm Sterivex™-GS filter before being stored at 4ºC until required.

Ham's nutrient mixture F-12: This media was made by dissolving 10.6 g/L Ham's nutrient mixture powder (Sigma-Aldrich®) and 44 mM $NaHCO_3$ (Sigma-Aldrich®) in autoclaved Milli-Q® water and the solution was sterilised through a 0.22 µm Sterivex™-GS filter before being stored at 4ºC until required.

2.5.2.3 Preparation of complete cell culture media

The complete cell culture media was made by mixing 1:1 ratio of DMEM culture media and Hams F12 culture media (Section 2.6.2.2) together with 10% (v/v) fetal bovine serum.

2.5.2.4 Preparation of MTT solution

The MTT solution was prepared by dissolving MTT powder (Sigma-Aldrich®) (5 mg/mL) in PBS (pH 7.40) and sterilised through a 0.22 µm Sterivex™-GS filter unit, before being stored covered in foil at 4 ºC until required.

2.5.2.5 Preparation of cell suspension for MTT assay

The cell density (number of cells per mL) in the cell suspension was determined by staining the cells in a 1:1 ratio with a 0.2% (w/v) trypan blue solution (Sigma-Aldrich®) in PBS (pH 7.40). Thereafter, 20 mL of cell suspension was placed on the haemocytometer to count the number of cells using a Nikon Optiphot phase contrast 2 light microscope (CFW10x) at 100x magnification and the number of cells were adjusted using complete experimental cell media (Section 2.5.2.3) to 10,000 cells per well for the MTT viability assay.

2.5.2.6 Preparation of test compounds and control drugs for MTT assay

The stock solutions (10 mM) of the test compounds and reference drugs; camptothecin (Sigma-Aldrich®), quinine (Sigma-Aldrich®) and DHA (Sigma-Aldrich®) were diluted in DMSO to the screening concentration of 50 µM, taking the ten times dilution factor into account.

2.5.2.7 Experimental protocol for the MTT viability assay

The cell suspension (180 µL) was plated to each well of a sterile 96-well microtiter plate except for the cell-free control wells (row H1-3), in which 200 µL of complete cell experimental media (Section 2.6.2.3) was plated. Thereafter, 20 µL test compound was added to triplicate wells and 20 µL complete cell culture media was added in the 100% cell viability positive control wells (row H4-12). After a 44 hour incubation period, 40 µL MTT solution was added to all the wells and incubated for further 4 hours at 37°C in 5% carbon dioxide under humidified conditions. Thereafter, the plates were centrifuged at 400 *g* for 5 minutes and 150 µL supernatant from each well was replaced with 200 µL DMSO to stop the reaction and dissolve the purple formazan crystals. The plate was shaken with an orbital plate shaker (MRC Thermo-Shaker™ MB100-4P) at 1200 rpm for 5 minutes before reading the absorbance.

2.5.2.9 Data analysis

The absorbance of the plate was read at a test wavelength of 540 nm (purple formazan colour) and reference wavelength of 690 nm (unreacted MTT colour) using the UV-Vis plate spectrophotometer (Labsystems iEMS Reader MF) with Ascent™ software version 2.6. The percentage cell viability of the test compounds and reference drugs were calculated according

to Equation 2.6. The IC_{50} values for test compounds that had less than 60% cell viability at the 50 µM screening concentration, were calculated from log sigmoidal dose response curves generated using Graphpad Prism Software version 5.0

$$\%\ \text{Cell viability} = \frac{\text{Absorbance}_{\text{drug(540nm)}} - \text{Absorbance}_{\text{drug(690nm)}}}{\text{Absorbance}_{\text{drug free control(540nm)}} - \text{Absorbance}_{\text{drug free control(690nm)}}} \times 100$$

Equation 2.6: Percentage cell viability calculation.

2.5.3 Selectivity index

To determine whether the test compounds were more selective towards the malaria parasite or the human kidney epithelial cells, the IC_{50} values from the antimalarial (Section 2.3.5) and cytotoxicity studies (Section 2.6.2) were used to calculate the safety indices (S.I) for *P. falciparum* ($S.I_{pf}$) using Equation 2.7. From the literature, $S.I_{pf}$ of less than 10 is considered toxic towards the human kidney epithelial cells, while $S.I_{pf}$ greater than 10 is considered non-toxic and is favourable to the malaria parasite (Pink *et al*., 2005; Badisa *et al*., 2009). The higher the $S.I_{pf}$ value, the more selective the compound is towards the malaria parasite.

$$\text{Safety Index } (S.I_{pf}) = \frac{\text{Cytotoxicity } (IC_{50})}{\text{Antimalarial activity } (IC_{50})}$$

Equation 2.7: Safety index calculation for *P. falciparum* compared to the human kidney epithelial cells (Badisa *et al*., 2009).

The degree of selectivity of the test compounds against human kidney epithelial cells (HEK-293) compared to chronic myelogenous leukaemia (K562) cells was determined by calculating the selectivity index for normal: cancer ($S.I_{nc}$) values according to equation 2.8. The $S.I_{nc}$ of greater than 10 was considered more selective towards the chronic myelogenous leukaemia cells (Badisa *et al*., 2009).

$$\text{Selectivity Index } (S.I_{nc}) = \frac{\text{Cytotoxicity of HEK} - 293\ (IC_{50})}{\text{Cytotoxicity of K562 cells } (IC_{50})}$$

Equation 2.8: Selectivity index calculation for human kidney epithelial cells compared to chronic myelogenous leukaemia cells (Badisa *et al*., 2009).

2.6 Anti-oxidant activity

2.6.1 DPPH• free radical scavenging assay

The free radical scavenging properties of the test compounds was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free-radical scavenging assay according to Gülçin *et al*. (2004) and Hayat *et al*. (2011) with slight modifications. This assay measures the ability of an anti-oxidant to scavenge the stable free-radical of DPPH• converting it to non-radical stable form, 2, 2'-diphenyl-1-picrylhydrazine (DPPH-H). The scavenging activity can then be expressed as the percentage decrease in absorbance which corresponds to the colour change from purple (DPPH•) to yellow (DPPH-H) (Gülçin *et al*., 2004).

2.6.1.1 Preparation of the solutions

A 100 µM DPPH• solution (Sigma-Aldrich®) was prepared in HPLC grade methanol (Rochelle Chemicals) and stored in the dark at 4°C for 24 hours before use. The stock solution (10 mM) of test compounds and positive controls, ascorbic acid (Riedel de Haen) and Trolox (Sigma-Aldrich®), were diluted in DMSO (Merck) to the screening concentration of 50 µM taking the five times dilution factor of the experiment into account.

2.6.1.2 Experimental protocol

The test compounds and positive controls (50 µL) were plated into a 96-well microtiter plate along with 200 µL DPPH•. The blank control consisted of 50 µL DMSO and 200 µL HPLC grade methanol and the drug-free control consisted of 50 µL DMSO and 200 µL DPPH•. The plates were then incubated in the dark at room temperature for 30 minutes; after which the absorbance was read at 540 nm using a UV-Vis plate spectrophotometer (Labsystems iEMS Reader MF) with Ascent™ software version 2.6. The free-radical scavenging activities of the test compounds were expressed as the percentage decrease in the absorbance in comparison to the drug-free DPPH• control using Equation 2.9.

$$\%\ \text{Scavenging activity} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{blank}}) - (\text{Absorbance}_{\text{compound}} - \text{Absorbance}_{\text{blank}})}{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{blank}}} \times 100$$

Equation 2.9: Calculation of percentage free radical scavenging activity.

2.6.1.3 Data analysis

The IC_{50} values were calculated for test and control compounds that exhibited greater than 60% $DPPH^{\bullet}$ free-radical scavenging activity at the 50 µM screening concentration from log sigmoidal dose response curved generated using Graphpad Prism Software version 5.0.

2.6.2 Iron chelating activity

The iron chelating properties of the test compounds was determined using the ferrozine-iron chelating assay according to Dinis *et al*. (1994) with slight modifications. This assay was based on the ability of ferrozine to form a purple coloured complex with ferrous ion (Fe^{2+}). The compounds with chelating properties prevented the formation of the ferrozine-Fe^{2+} complex by chelating ferrous ion before ferrozine. This resulted in a decrease in the purple colour of the ferrozine-Fe^{2+} complex corresponding to a decreased in absorbance at 540 nm (Gülçin *et al*., 2004).

2.6.2.1 Preparation of the test compounds and controls

The stock solution (10 mM) of test compounds and positive control, ethylenediaminetetraacetic acid (EDTA; Rochelle Chemicals), were diluted using DMSO (Merck) to the screening concentration of 50 µM taking the 7.5 times dilution factor of the experiment into account.

A 1.2 mM ferrozine (Fluka) solution was prepared in Milli-Q® water and sterilised through a 0.22 µm Sterivex™-GS filter unit before being stored at 4˚C until needed. A 0.48 mM ferrous chloride solution (Fluka) was freshly prepared in 5% (w/v) ammonium acetate (Fluka) before each experiment.

2.6.2.2 Experimental protocol

Test compounds and EDTA were plated out (20 µL) in triplicate well of a non-sterile 96-well microtiter plate, along with 30 µL $FeCl_2$. The blank control consisted of 100 µL MilliQ™ water and 30 µL $FeCl_2$, while the drug-free control consisted of 100 µL DMSO and 30 µL $FeCl_2$. The plate was incubated in the dark at room temperature for 5 minutes before adding 100 µL ferrozine to all the wells, except for the blank control well. Thereafter, the plate was incubated again in the dark at room temperature for 30 minutes and the absorbance was read at 540 nm using an UV-Vis plate spectrophotometer (Labsystems iEMS Reader MF) with Ascent™

software version 2.6. The percentage iron chelating activity of the test compounds and EDTA were calculated using Equation 2.10.

$$\%\ \text{Scavenging activity} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{blank}}) - (\text{Absorbance}_{\text{compound}} - \text{Absorbance}_{\text{blank)}}}{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{blank}}} \times 100$$

Equation 2.10: Percentage iron chelating activity calculation.

2.6.2.3 Data analysis

The IC_{50} values were calculated for EDTA and test compounds which exhibited more than 60% iron chelation activity at the 50 µM screening concentration from log sigmoidal dose response curved generated using Graphpad Prism Software version 5.0.

2.7 Prediction of drug-likeness and pharmacokinetic properties

2.7.1 Measurement of drug-likeness

The molecular properties of the active compounds against *P. falciparum* (Section 2.3.5) were predicted *in silico* for drug-likeness properties based on Lipinski rule of five (RO5) using web-based software Chemicalize (www.chemicalize.com) in comparison to quinine. The RO5 is a guide to evaluate whether a chemical compound with a certain pharmacological or biological activity has properties that would make it a likely drug to be orally bioactive in humans (Lipinski *et al*., 1997). The RO5 states that compounds are likely to have good permeability or absorption if there is no more than one violation to the rule, where molecular weight <500, hydrogen bond acceptors (HBA) <10, hydrogen bond donor (HBD) <5 and logP <5 are considered (Lipinski *et al*., 2001). The test compounds that violated more than one rule were considered as being non drug-like or having poor pharmacokinetic properties for oral bioavailability (Bhal *et al.*, 2007). The partition coefficient (logP) was used as predictor of the lipid solubility of the test compounds. The compound structures were constructed using ACD/ChemSketch software (free version).

2.7.2 Ionisation prediction as a function of pH

The pKa values of the active compounds were predicted *in silico* using web-based software, chemicalize (www.chemicalize.com) to determine the degree of ionisation of the compounds at RBC cytosol, pH 7.4 and parasite digestive food vacuole, pH 5 using Henderson-Hasselbasch equation (Equation 2.11).

$$\mathrm{pKa} = \mathrm{pH} + \log \frac{\text{acid}}{\text{base}}$$

Equation 2.11: Henderson-Hasselbalch equation (Henderson, 1908; Hasselbasch, 1917).

The Henderson-Hasselbalch equation (Equation 2.11) relates the ratio of ionised to unionised forms of weak acids or bases to the drug's pK_a and the pH of the medium (Aulton, 2002; Rosenthal, 2007). The relative concentrations of the ionic and molecular forms of the drug depend on the pK_a of the drug and the pH of the medium (Knittel and Zavod, 2008). Since most drugs are absorbed in unionised form via passive diffusion (Rang *et al.*, 2012b), the degree of ionisation of a drug at a certain pH is of great importance. The pK_a of a drug influences solubility, protein binding and permeability, which in turn directly affects pharmacokinetic characteristics of the drug (Manallack, 2007). There is a direct link between the degree of ionisation of a drug and its solubility in aqueous medium (Figure 2.3).

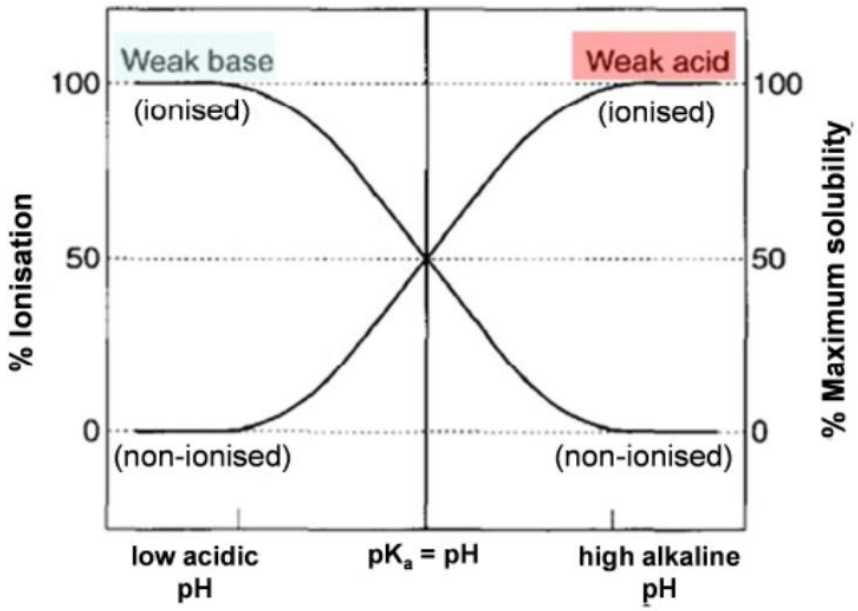


Figure 2.3: Changes in ionisation and relative solubility of weakly acidic and weakly basic drugs as a function of pH (Aultun, 2000; Motau, 2015).

2.7.3 Metabolism and *in vivo* toxicity predictions

Metabolism and *in vivo* toxicity for the active compounds was predicted using web-based software, Toxtree v3.1 (http://toxtree.sourceforge.net/). During drug metabolism, lipophilic

drugs are transformed into more water-soluble molecules so they can be more easily excreted from the body (Li, 2001; Tingle and Helsby, 2006). Enzymes involved in metabolism include; phase I drug metabolizing enzymes which introduce a polar functional group to xenobiotics and phase II drug metabolizing enzymes which are responsible for conjugating a xenobiotic or its metabolite (Iyer, and Sinz 1999; Asha and Vindyavathi, 2010). It has been stated that the majority of drugs in clinical trials fail due to unexpected adverse drug properties. Therefore, predicting potential toxicity early on drug discovery can aid in identifying compounds with high toxicity potential (Li, 2001).

2.8 Vector studies

The test compounds were screened against *An. arabienses* eggs and larvae over a 72 hour period to determine whether they possessed ovicidal and larvicidal activities. The *Artemia* lethality assay was conducted to evaluate whether the inhibitory effect of the test compounds was specific to *An. arabiensis* eggs and larvae or non-specific towards non-target aquatic organism (Colombo *et al*., 2001; Ruebhart *et al*., 2009).

2.8.1 Mosquitoes

The test compounds were screened against eggs and larvae of *An. arabiensis* (KwaZulu-Natal species G; KWAG) mosquito strain obtained from WITS Research Institute for Malaria (WRIM) Maureen Coetzee insectary at the University of the Witwatersrand, Johannesburg. The mosquitoes were reared as per the methods of Hunt *et al*. (2005). A clearance waiver (reference: R van Zyl waiver (*Anopheles*) 07-11-2017-0) was obtained from the Animal Research Ethics Committee at University of the Witwatersrand permitting the use of *Anopheles* mosquitoes' eggs and larvae for research purposes (Appendix D.1); with subsequent approval for Ms Malimabe under this umbrella waiver (Appendix D.2).

2.8.2 Ovicidal activity

2.8.2.1 Preparation of the test compounds and controls

The stock solution (10 mM) of test compounds and positive controls, formalin and dichlorodiphenyltrichloroethane (DDT; Sigma-Aldrich®), were diluted using DMSO (Merck) to the screening concentration of 0.05 µM taking the 100 times dilution factor of the experiment into account.

2.8.2.2 Experimental protocol

The ovicidal assay was carried out according to Su and Mulla (1998) with slight modifications. Briefly, fresh *An. arabiensis* eggs (0 – 6 hours old) were placed into a large plastic cup containing 200 ml sterile distilled water. A suspension of 50 µl was then pipetted out into a clean filter paper and 20 eggs were counted using Donegan Da-2 OptiVISOR (1.5x magnification) before being plated into the wells of a 48-well microtiter plate by rinsing the filter paper with 490 µl sterile distilled water. The test compounds and controls (10 µl) were then added and the hatch rate was assessed after 24, 48 and 72 hour incubation at 27°C to determine percentage egg hatchability (Equation 2.12).

$$\%\ \text{Egg hatchability} = \frac{\text{Number of eggs hatched}}{\text{Total number of eggs plated per well}} \times 100$$

Equation 2.12: Percentage egg hatchability calculation.

2.8.3 Larvicidal activity

2.8.3.1 Preparation of the test compounds and controls

The stock solution (10 mM) of test compound and positive control, DDT (Sigma-Aldrich®), were diluted using DMSO (Merck) to the screening concentration of 0.05 µM taking the 100 dilution factor of the experiment into account.

2.8.3.2 Experimental protocol

The larvicidal activity of the test compounds was evaluated according to the standard guidelines of the WHO (2005) and Kathrada (2017) with slight modifications. The larvae were fed standard larval food twice daily during the duration of experiment (Hunt *et al*., 2005). Briefly, a batch of twenty 3rd instar larvae was placed into plastic cups containing 24.75 ml of distilled water along with 250 µL of test compound/controls. The number of moribund and dead larvae were counted after 24, 48 and 72 hour post-treatment incubation period at 25°C to determine percentage larvae mortality (Equation 2.13).

$$\%\ \text{Mortality} = \frac{\text{Number of dead larvae}}{\text{Total larvae per cup}} \times 100$$

Equation 2.13: Percentage larvae mortality calculation.

Larvae was recorded as dead if it remained immobile when prodded. Moribund were larvae that did not rise to the surface or show characteristic diving reaction when the water was disturbed (WHO, 2005). The compounds that demonstrated more than 60% mortality, were further investigated with 1:2 serial dilutions to determine the lethal concentration required to kill 50% of larvae (LC_{50}) (values using Probit analysis (IBM SPSS statistical version 23 software Package, IBM Corp.). The morphological alterations were observed and photographically recorded at 24, 48 and 72 hour incubation period compared to the controls using BestScope compound microscope (WF10X/22) with TCapture version 3.9.0.602.

2.8.4 *Artemia* lethality assay

2.8.4.1 Hatching of eggs

To hatch, *Ar. franciscana* eggs (Ocean Nutrition™) were placed in salt water (32 g/L) (Aquarium Systems®). The hatching apparatus consisted of a rotary pump placed inside the plastic container to allow bubbling of water for dispersion of eggs and aeration. The container was then incubated at 27°C (Figure 2.4). After 24 hours, the newly hatched nauplii were placed into a sorting tray containing 200 mL salt water.

2.8.4.2 Preparation of the test compounds and controls

The stock solution (10 mM) of the test compounds and positive controls, potassium dichromate (Sigma-Aldrich®) and DDT (Sigma-Aldrich®), were diluted using DMSO (Merck) to the screening concentration of 50 µM taking the 100 times dilution factor of the experiment into account.

2.8.4.3 Experimental protocol

The assay was carried out according to the method of Ruebhart *et al.* (2009) and Kathrada (2017) with slight modifications. A clearance waiver (reference: R van Zyl-(*Artemia*) waiver 07-11-2017-0) was obtained from the Animal Research Ethics Committee at University of the Witwatersrand permitting the use of *Artemia* eggs and nauplii for research purposes (Appendix E); with subsequent approval for Ms Malimabe under this umbrella waiver (Appendix D.2). A nauplii suspension containing between 20-40 nauplii (50 µL) was added to 197.5 µL of salt water in each well of a 48 well microtiter plate and then 2.5 µL test compound/controls was added.

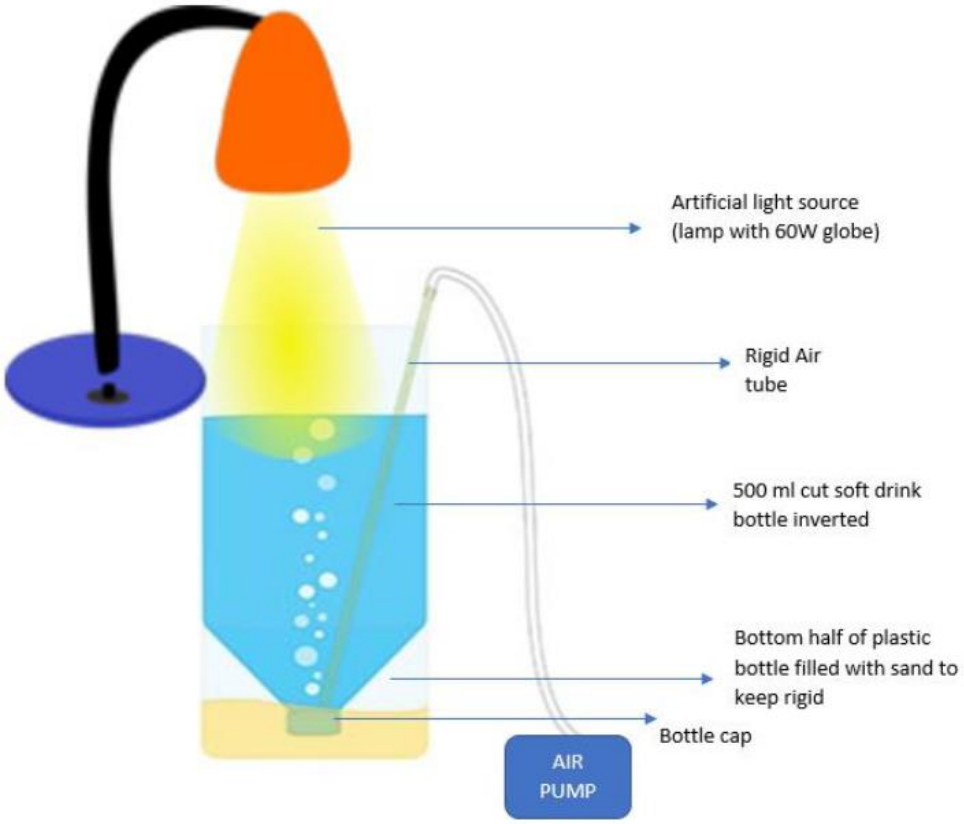


Figure 2.4: The design of the *Artemia* hatchery setup (Kathrada, 2017).

The plates were incubated at 27°C and the dead nauplii were counted after 24, 48 and 72 hour incubation period and after the 72 hour time point, all the remaining live nauplii were killed with 50% (v/v) acetic acid (Sigma-Aldrich®), to allow for the total number of nauplii to be counted. The negative control consisted of only *Ar. franciscana* nauplii and saltwater.

2.8.4.4 Data analysis

The percentage nauplii mortality was calculated according to Equation 2.13 and the LC_{50} values were determined for test compounds exhibiting more than 60% mortality. The morphological alterations were observed and photographically recorded 24, 48 and 72 hour incubation period and compared to the controls using Olympus (CKX53) microscope (40x).

$$\%\ \text{Nauplii mortality} = \frac{\text{Number of dead nauplii}}{\text{Total number of nauplii}} \times 100$$

Equation 2.14: Percentage nauplii mortality calculation.

2.9 Statistical Analysis

To determine if there were statistical differences/similarities between the test compounds and the positive controls, Mann-Whitney U-test was performed, where a p-value < 0.05 was considered significant. The results were reported as mean ± standard deviation (s.d.) of three independent replicates, with exception of larvicidal assay where the experiment was repeated five times.

CHAPTER THREE – RESULTS

3.1 Antimalarial activity

3.1.1 Quinoxaline-phenyl hybrids

The two sets of novel quinoxaline-based hybrid compounds possessed varying *in vitro* antimalarial activity at 50 µM against the intra-erythrocytic parasite. In the quinoxaline-phenyl hybrids set, the compounds with a chalcone link (**QCP** compounds) inhibited a high percentage of the parasite growth (99.9%), except for compound **QCP10**, 38.37 ± 4.97% (Table 3.1). The compounds with pyrazoline link (**QPP** compounds) had a percentage parasite growth ranging between 21.11 ± 0.90 and 77.98 ± 3.35% at 50 µM, in comparison to the positive controls, DHA and quinine with 0.10 ± 2.21% and 5.20 ± 0.98%, respectively (Table 3.1).

Table 3.1: The *in vitro* antimalarial activity of quinoxaline-phenyl hybrids with either chalcone (**QCP** compounds) or pyrazoline linker (**QPP** compounds) against the asexual blood stages of *P. falciparum* NF54 strain at 50 µM, in comparison to positive controls, DHA and quinine (n = 3, mean ± s.d). Refer to table 2.1 for compound codes.

QCP compound code	Parasite growth (%) ± s.d.	QPP compound code	Parasite growth (%) ± s.d.
QCP1	0.10 ± 1.82	**QPP1**	66.42 ± 2.03
QCP2	0.10 ± 3.33	**QPP2**	17.23 ± 0.19
QCP3	0.10 ± 2.25	**QPP3**	56.12 ± 1.38
QCP5	0.10 ± 3.45	**QPP5**	22.21 ± 0.66
QCP6	0.10 ± 0.41	**QPP6**	21.11 ± 0.90
QCP7	0.10 ± 5.84	**QPP7**	66.78 ± 1.31
QCP8	0.10 ± 5.26	**QPP8**	67.49 ± 5.54
QCP9	0.10 ± 0.56	**QPP9**	77.98 ± 3.35
QCP10	61.63 ± 4.97	**QPP10**	76.85 ± 5.16
DHA	0.10 ± 2.21	**Quinine**	5.20 ± 0.98

Out of eighteen quinoxaline-phenyl hybrids screened for *in vitro* antimalarial activity, twelve compounds inhibited the growth of intra-erythrocytic parasite, *P. falciparum* and warranted further investigation. Overall, compound **QCP3** was the most potent antimalarial in comparison to quinine (Figure 3.1).

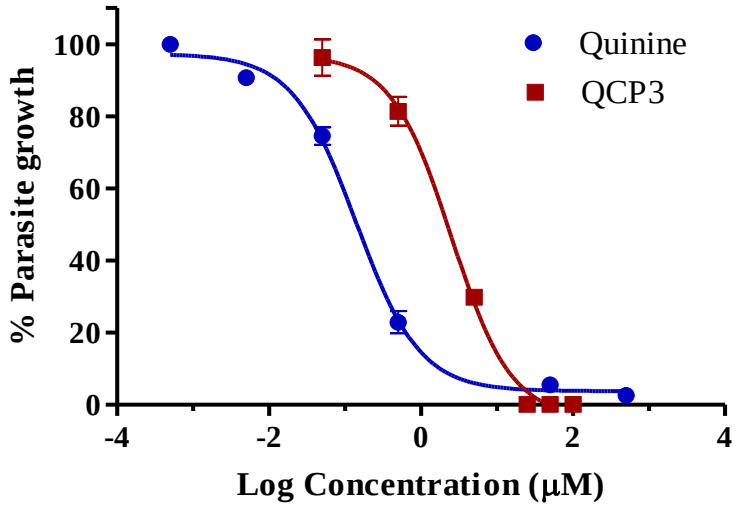


Figure 3.1: Dose dependent antimalarial inhibitory effect of the most active compound, **QCP3** against the *in vitro* asexual blood stages of *P. falciparum* in comparison to standard antimalarial drug, quinine.

The active quinoxaline-phenyl hybrids with a chalcone link (**QCP** compounds) displayed potent to low antimalarial activity as IC_{50} values ranged between 4.19 ± 0.52 and 30.00 ± 2.79 µM; while the compounds with pyrazoline link (**QPP** compounds) had moderate to low activity with IC_{50} values ranging between 14.83 ± 1.99 and 24.03 ± 3.45 µM (Figure 3.2). Compounds, **QCP3** (IC_{50} value: 4.19 ± 0.52 µM), **QCP8** (IC_{50} value: 6.06 ± 0.57 µM) and **QCP7** (IC_{50} value: 9.71 ± 0.42 µM) were the most active; while, compound **QCP5** exhibited the lowest activity (IC_{50} value: 30.00 ± 2.79 µM) in the **QCP** set. The most potent compound, **QCP3** was however, 38 times less active than quinine (IC_{50} value: 0.11 ± 0.01 µM) (Figure 3.2). The antimalarial activity of the active quinoxaline-phenyl compounds was significantly different to the control quinine with *p*-values < 0.05.

3.1.2 Quinoxaline-quinoline hybrids

In the quinoxaline-quinoline hybrid set, the compounds with a chalcone link (**QCQ** compounds) had a percentage parasite growth of 0.10%, except for compound **QCQ8** with 17.61% at 50 µM. While, the compounds with a pyrazoline link (**QPQ** compounds) had a percentage parasite growth ranging between 9.34 and 52.87% at 50 µM. In comparison, positive controls, DHA and quinine resulted in 0.10 ± 2.21% and 5.20 ± 0.98% parasite growth, respectively (Table 3.2).

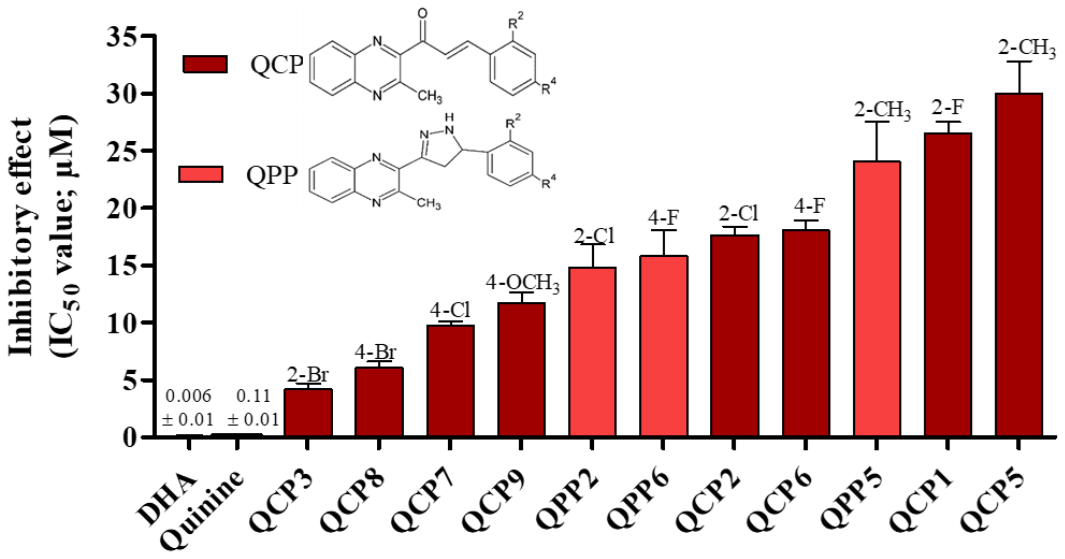


Figure 3.2: The antimalarial inhibitory effect (IC_{50} values) of the active quinoxaline-phenyl hybrid with chalcone (**QCP** compounds) or pyrazoline (**QPP** compounds) against *P. falciparum* NF54 strain in comparison to antimalarial drug controls, DHA and quinine.

Table 3.2: The *in vitro* antimalarial activity of quinoxaline-quinoline hybrids with either chalcone (**QCQ** compounds) or pyrazoline linker (**QPQ** compounds) against the asexual blood stages of *P. falciparum* NF54 in comparison to positive controls, DHA and quinine at 50 µM(n = 3, mean ± s.d). Refer to table 2.2 for compound codes.

QCQ compound code	Parasite growth (%) ± s.d.	QPQ compound code	Parasite growth (%) ± s.d.
QCQ1	0.10 ± 0.01	**QPQ1**	47.43 ± 0.62
QCQ2	0.10 ± 3.12	**QPQ2**	48.14 ± 4.72
QCQ3	0.10 ± 2.06	**QPQ3**	32.62 ± 1.31
QCQ5	0.10 ± 1.91	**QPQ5**	36.72 ± 3.78
QCQ6	0.10 ± 2.31	**QPQ6**	9.34 ± 1.32
QCQ7	0.10 ± 1.51	**QPQ7**	47.20 ± 3.05
QCQ8	17.61 ± 1.43	**QPQ8**	4.48 ± 0.97
QCQ9	0.10 ± 4.58	**QPQ10**	52.87 ± 3.90
QCQ10	0.10 ± 3.50		
DHA	0.10 ± 2.21	**Quinine**	5.20 ± 0.98

Thirteen out of seventeen quinoxaline-quinoline hybrids inhibited more than 60% of parasite growth which warranted further investigation (Table 3.2). The quinoxaline-quinoline hybrids with a chalcone link (**QCQ** compounds) displayed promising to moderate activity with IC_{50} values ranging between 6.45 ± 0.37 to 14.37 ± 2.79 µM; while compounds with pyrazoline link (**QPQ** compounds) had moderate to low activity, with IC_{50} values between 14.03 ± 2.66 and 22.60 ± 3.15 µM (Figure 3.3). In comparison, the IC_{50} values of DHA and quinine were 0.006 ± 0.01 µM and 0.11 ± 0.01 µM, respectively. Overall, five compounds with a chalcone link exhibited the highest activity in the quinoxaline-quinoline set with IC_{50} values less than 10 µM; namely, **QCQ9** (IC_{50} value: 6.45 ± 0.37 µM), **QCQ1** (IC_{50} value: 6.70 ± 0.94 µM), **QCQ3** (IC_{50} value: 7.41 ± 1.25), **QCQ5** (IC_{50} value: 8.27 ± 1.06 µM) and **QCQ2** (IC_{50} value: 9.05 ± 1.06 µM). Of the seventeen compounds, compound **QPQ5** had the lowest antimalarial activity with IC_{50} value of 22.60 ± 3.15 µM. The antimalarial activity of the active quinoxaline-quinoline compounds was significantly different to the control quinine with *p*-values < 0.05.

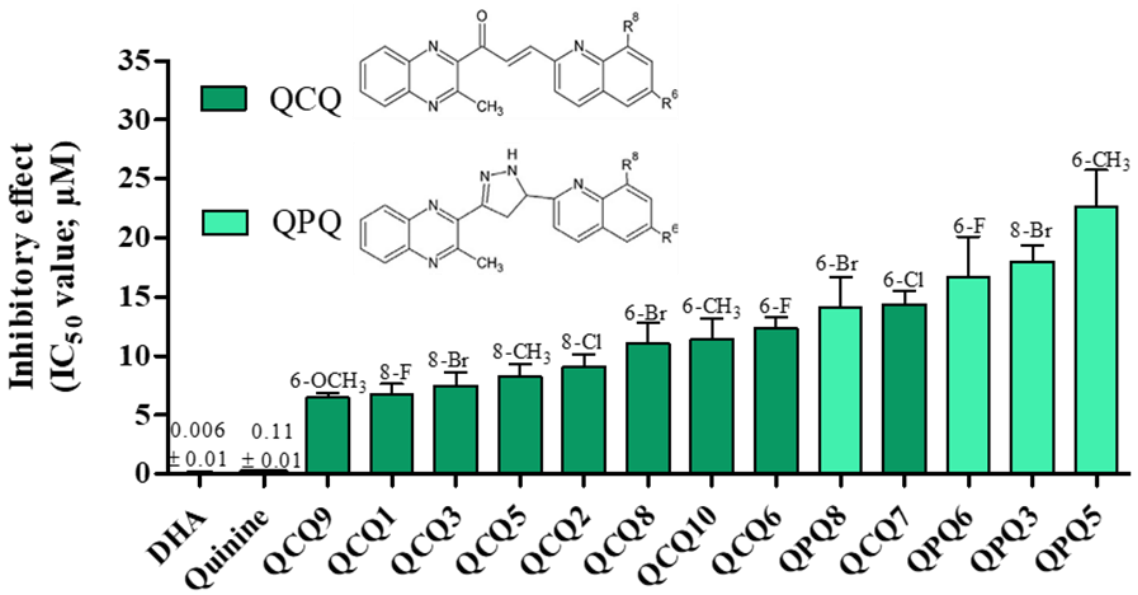


Figure 3.3: The antimalarial inhibitory effect (IC_{50} values) of the active quinoxaline-quinoline hybrids (**QCQ** and **QPQ**) compounds against *P. falciparum* NF54 strain in comparison to antimalarial drug controls, DH A and quinine.

3.1.3 Stage specific and morphological effects

Both compound **QCP7** and **QCP8** displayed similar stage specific and morphological effects on *P. falciparum* at their IC_{50} concentrations in comparison to the untreated and quinine parasite control over 72 hours (Figure 3.4). There were no notable changes observed in parasite

morphology after 16 hours post-treatment for both compounds and controls. After 24 hours, both **QCP7** and **QCP8** showed a lag in progression from rings to trophozoite stage (Figure 3.5A), while the untreated parasite control, progressed to the trophozoite stage (Figure 3.5B). After 32 hours, the untreated parasite progressed to late trophozoite stage and only 30% of the treated parasite with **QCP7** and 40% with **QCP8**, appeared to be in early trophozoite stage with reduction in size. The haemozoin formation was visible, but the crystals were not as compact as in the untreated control.

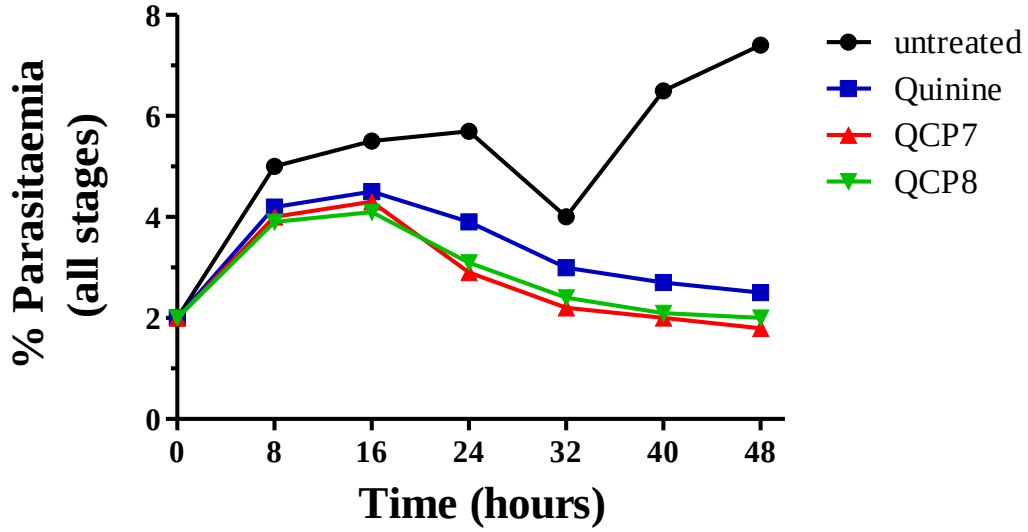


Figure 3.4: Total percentage parasitaemia of the treated parasites (**QCP7**, **QCP8** and quinine) compared to the untreated control over a single 48 hour cycle at their respective IC_{50} values.

When the untreated trophozoite progressed into a schizont, both the treated compounds parasites remained in trophozoite stage with significant reduction in total parasitaemia (Figure 3.4). Quinine showed a lag in progression from trophozoite to schizonts (Figure 3.5C). Both compound **QCP7** and **QCP8** inhibited cycle completion and progression into the new cycle compared to the untreated parasites. Both test compounds and quinine showed low parasitaemia over time in comparison to the untreated (Figure 3.4).

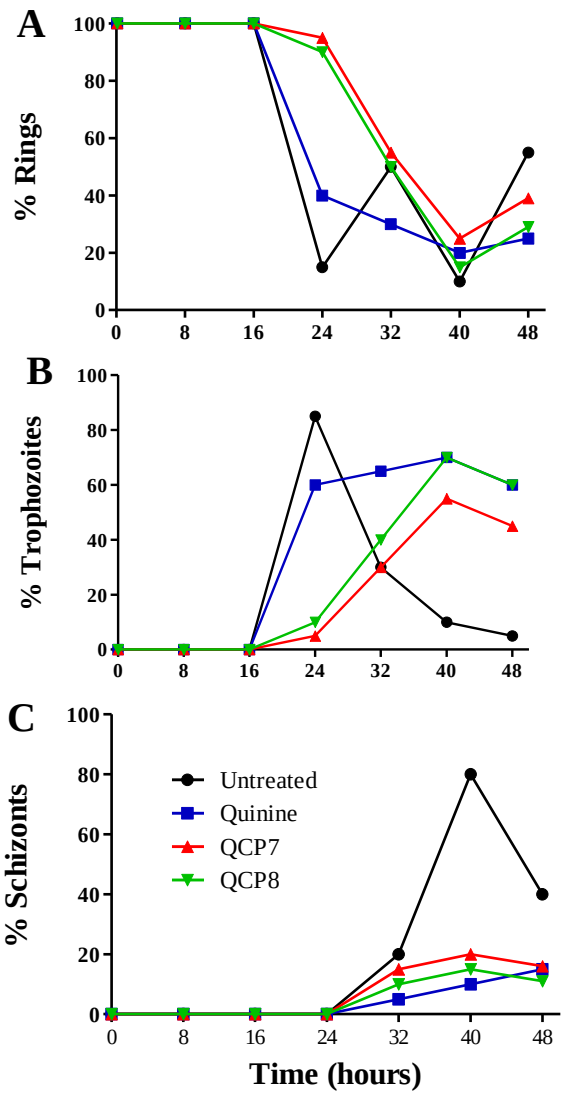


Figure 3.5: Stage-dependant effect of **QCP7** and **QCP8** at their IC_{50} values; 6.06 ± 0.57 µM and 9.71 ± 0.42 µM, respectively in comparison to quinine (IC_{50}: 0.11 ± 0.01 µM) and untreated parasites. The % ring (A), trophozoite (B) and schizont (C) progression over 48 hours illustrated the inhibitory effect of the two **QCP** compounds on the various stages.

3.2 Preliminary mammalian toxicological studies

3.2.1 Haemolytic activity

Both sets of novel quinoxaline-based hybrid compounds induced negligible haemolysis (0.1 ± 0.01%) when compared to the positive control, 0.2% (v/v) TritonX-100™ that resulted in 100 ± 0.01% haemolysis at 50 µM. The haemolytic activity induced by the test compounds was comparable to that of antimalarial drug control, quinine (0.1 ± 0.01%) at 50 µM.

3.2.2 MTT cytotoxic activity

3.2.2.1 Quinoxaline-phenyl hybrids

Both the quinoxaline-phenyl compounds with a chalcone (**QCP** compounds) and a pyrazoline (**QPP** compounds) link exhibited low cytotoxicity against the human kidney epithelial cells, with a percentage cell viability ranging between 53.91% and 100.00% at 50 µM (Table 3.3). Four compounds with a chalcone link did not follow this trend by displaying high cytotoxic effects with percentage cell viability of less than 40% at 50 µM; namely, **QCP1** (IC_{50} value: 18.12 ± 2.11 µM), **QCP2** (IC_{50} value: 36.77 ± 0.89 µM), **QCP3 (**IC_{50} value: 21.16 ± 1.86 µM) and **QCP5** (IC_{50} value: 20.39 ± 2.59 µM). Their cytotoxicity was, however, significantly different (p value < 0.05) to the positive control, camptothecin (IC_{50} value: 0.15 ± 0.01 µM) (Figure 3.6).

Table 3.3: The cytotoxicity profile of the quinoxaline-phenyl hybrids with a chalcone (**QCP)** and pyrazoline linker (**QPP**) at 50 µM against the human kidney epithelial (HEK-293) and chronic myelogenous leukaemia (K562) cells. None of the compounds exhibited haemolytic activity at 50 µM in comparison of positive control, 0.2% TritonX-100™ with 100 ± 0.01% haemolysis (n = 3, mean ± s.d). Refer to table 2.1 for compound codes.

Cytotoxic profile					
QCP compound code	**HEK-293 (%) ± s.d.**	**K562 (% cell growth) ± s.d.**	**QPP compound code**	**HEK-293 (%) ± s.d.**	**K562 (% cell growth) ± s.d.**
QCP1	28.29 ± 4.74	3.68 ± 0.34	**QPP1**	96.53 ± 2.87	81.26 ± 7.02
QCP2	35.90 ± 4.37	3.54 ± 0.64	**QPP2**	89.65 ± 4.99	63.36 ± 2.60
QCP3	32.27 ± 6.94	1.53 ± 0.21	**QPP3**	51.46 ± 5.06	73.92 ± 1.62
QCP5	25.94 ± 4.48	4.06 ± 0.66	**QPP5**	86.95 ± 8.67	53.91 ± 2.36
QCP6	61.45 ± 7.23	10.67 ± 1.25	**QPP6**	83.52 ± 4.54	88.91 ± 9.55
QCP7	63.51 ± 9.09	26.13 ± 2.28	**QPP7**	65.67 ± 6.47	97.65 ± 6.43
QCP8	57.88 ± 3.07	24.59 ± 1.89	**QPP8**	75.64 ± 9.30	86.50 ± 4.68
QCP9	68.82 ± 8.85	39.97 ± 2.71	**QPP9**	100.00 ± 7.69	90.63 ± 9.10
QCP10	85.39 ± 0.83	15.29 ± 1.03	**QPP10**	78.27 ± 7.03	81.12 ± 2.87
Campto-thecin	30.00 ± 2.36	27.08 ± 2.91	**Quinine**	83.35 ± 2.62	98.99 ± 0.78
DHA	89.31 ± 4.59	15.71 ± 2.91			

In contrast, only the compounds with a chalcone link (**QCP** compounds) displayed antileukemic activity against the chronic myelogenous leukaemia cells in a dose-dependent manner with cell viability of less than 40% at 50 µM (Table 3.3). The IC_{50} values ranged between 6.85 ±0 .79 and 36.42 ± 1.44 µM (Figure 3.6). Of these compounds, five exhibited the high activity with IC_{50} values less than 10 µM, **QCP2** (6.85 ± 0.79 µM), **QCP3** (9.18 ± 1.33 µM), **QCP1** (9.80 ± 1.35 µM) and **QCP9** (9.94 ± 1.16 µM). **QCP7** (IC_{50} value: 36.42 ± 1.44 µM) had the least activity against the K562 chronic myelogenous leukaemia cells. The most active compound of this set, **QCP2** (6.85 ± 0.79 µM) was 343-fold less active compared to the positive control, camptothecin (0.02 ± 0.01 µM) and the *p* value was < 0.05.

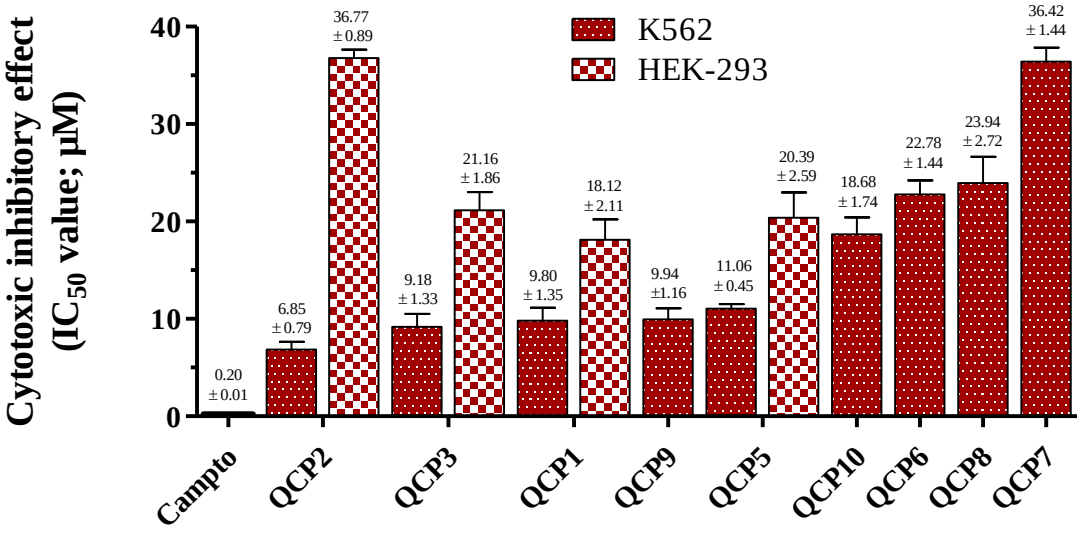


Figure 3.6: The cytotoxic inhibitory effect of quinoxaline-phenyl hybrids with either a chalcone (**QCP** compounds) or pyrazoline linker (**QPP** compounds) against human kidney epithelial (HEK-293) and chronic myelogenous leukaemia (K562) cells compared to positive control, camptothecin.

3.2.2.2 Quinoxaline-quinoline hybrids

In the quinoxaline-quinoline hybrid set, all nine compounds with a chalcone link (**QCQ** compounds) exhibited high cytotoxicity against both the human kidney epithelial and chronic myelogenous leukaemia cells at 50 µM, with the percentage cell viability less than 40% at 50 µM (Table 3.4). The IC_{50} values ranged between 13.66 ± 0 46 to 35.72 ± 5.64 µM when compared to camptothecin (0.15 ± 0.01 µM) (Figure 3.7). In contrast, none of the compounds with the pyrazoline link (**QPQ** compounds) displayed cytotoxicity against both the human

kidney epithelial and chronic myelogenous leukaemia cells at 50 µM (52.00 ± 9.44% and 100.00 ± 5.99%) (Table 3.4).

Table 3.4: The cytotoxicity profile of the quinoxaline-quinoline hybrids with a chalcone (**QCQ)** and pyrazoline linker (**QPQ**) at 50 µM in comparison to controls. None of the compounds exhibited haemolytic activity at 50 µM in comparison of positive control, 0.2% TritonX-100™ with 100 ± 0.01% haemolysis (*n* = 3, mean ± s.d). Refer to table 2.2 for compound codes.

Cytotoxic profile					
QPP compound code	**K562 (% Cell growth) ± s.d.**	**K562 (% Cell growth) ± s.d.**	**QPP compound code**	**Human kidney epithelial cell (%) ± s.d.**	**Chronic myelogenous leukaemia cells**
QCQ1	7.99 ± 0.93	2.62 ± 0.38	**QPQ1**	87.33 ± 4.86	56.24 ± 5.58
QCQ2	23.23 ± 3.54	1.59 ± 0.14	**QPQ2**	78.65 ± 4.47	69.60 ± 7.72
QCQ3	21.80 ± 2.36	2.62 ± 0.19	**QPQ3**	100.00 ± 5.99	61.86 ± 0.77
QCQ5	28.34 ± 4.64	2.44 ± 0.09	**QPQ5**	56.03 ± 2.50	52.00 ± 4.83
QCQ6	20.05 ± 1.20	2.54 ± 0.45	**QPQ6**	90.15 ± 1.34	52.71 ± 9.44
QCQ7	28.10± 3.00	4.81 ± 0.94	**QPQ7**	96.67 ± 5.12	55.62 ± 3.19
QCQ8	32.57 ± 5.05	15.38 ± 0.24	**QPQ8**	57.31 ± 1.08	75.92 ± 9.13
QCQ9	13.29 ± 1.71	3.01 ± 0.62	**QPQ10**	77.98 ± 3.22	56.55 ± 8.43
QCQ10	22.45 ± 2.82	4.39 ± 0.40	**Quinine**	83.35 ± 2.62	98.99 ± 0.78
Camptothecin	30.00 ± 2.36	27.08 ± 2.91	**DHA**	89.31 ± 4.59	15.71 ± 2.91

3.2.3 Selectivity index

The selectivity index $S.I_{pf}$ and $S.I_{nc}$ values were calculated for thirteen compounds, four quinoxaline-phenyl hybrids with a chalcone link (**QCP1, QCP2, QCP3, QCP5**) and all nine quinoxaline-quinoline hybrids with chalcone link (**QCQ** compounds) in comparison to the controls (Table 3.5). The cytotoxicity IC_{50} values and hence the $S.I_{pf}$ and $S.I_{nc}$ values were not determined for the rest of the compounds since the cell viability of human kidney epithelial cells in the presence of these compounds was greater than 50% at 50 µM (Section 3.3). Overall, the compounds had low $S.I_{pf}$ and $S.I_{nc}$ values ranging between 0.68 and 5.49 indicating non-selective cytotoxicity rather than selectivity towards the parasite compared to the $S.I_{pf}$ of

1278.91 and 25232.42 for quinine and DHA, respectively which showed selectivity to the malaria parasite rather than human cells.

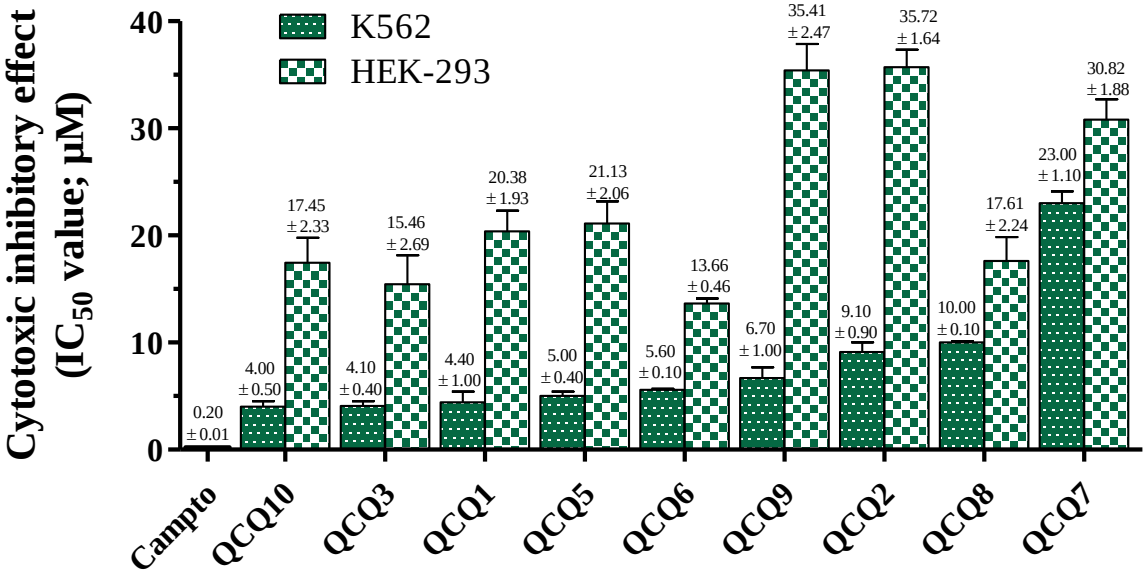


Figure 3.7: The cytotoxic inhibitory effect of quinoxaline-quinoline hybrids with either chalcone (**QCQ** compounds) or pyrazoline linker (**QPQ** compounds) against human kidney epithelial and chronic myelogenous leukaemia cells compared to positive control, camptothecin.

Table 3.5: The selectivity index (or safety profile) of quinoxaline-quinoline hybrids (**QCQ** compounds) and four quinoxaline-phenyl hybrids (**QPQ** compounds).

Compound code	$S.I_{pf}$	$S.I_{nc}$	Compound code	$S.I_{pf}$	$S.I_{nc}$
QCQ1	3.04	4.63	**QCP1**	0.68	2.65
QCQ2	3.95	3.93	**QCP2**	2.09	3.75
QCQ3	2.09	3.77	**QCP3**	5.05	2.31
QCQ5	2.56	4.23	**QCP5**	0.68	1.84
QCQ6	1.11	2.44			
QCQ7	2.14	1.34			
QCQ8	1.59	1.76	**Camptothecin**	n.d	1.33
QCQ9	5.49	5.29	**Quinine**	1278.91	0.5
QCQ10	1.54	4.36	**DHA**	25232.42	39.14

n.d.: not determined

3.3 Combination studies

The two active compounds (Section 3.2) with low toxicity profile (section 2.4.1) and more favourable safety index (Section 3.2.3) towards the malaria parasite were combined with quinine to determine whether the pharmacological interaction was synergistic, additive and antagonistic (Section 2.2). As such, the two quinoxaline-phenyl hybrids with a chalcone linker, **QCP7** (9.71 ± 0.42 µM) and **QCP8** (6.06 ± 0.57 µM) were selected for combination studies with quinine.

When combined with quinine, both compound **QCP7** and **QCP8** displayed an overall synergistic interaction (Figure 3.8). The ∑FIC values for compounds, **QCP7** and **QCP8** compounds were 0.385 and 0.251, respectively, confirming the synergistic interaction as determined from the isobolograms.

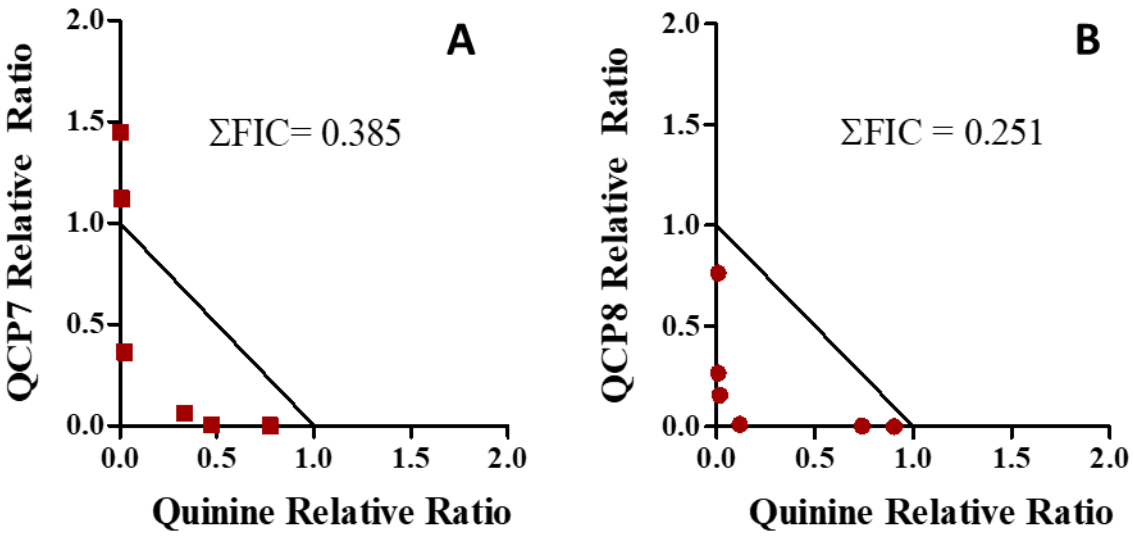


Figure 3.8: Isobologram showing the synergistic pharmacological interactions of **A: QCP7** and **B: QCP8** compounds when combined with quinine.

3.4 Anti-oxidant activity

3.4.1 Iron chelating properties

Both sets of quinoxaline-based hybrid compounds had low binding affinity for Fe (II) as overall percentage iron chelation ranged between 0.1 ± 1.85 and 36 ± 3.71%, in comparison to the positive control, EDTA (96.64 ± 1.52%) (Table 3.6). Of the quinoxaline-phenyl hybrids, three compounds with a pyrazoline link (**QPP6, QPP9, and QPP3)** displayed the highest iron

chelating activity with 33.18 ± 1.38%, 24.84 ± 2.48% and 21.77 ± 2.37%, respectively. The quinoxaline-quinoline hybrids, **QPQ8** with a pyrazoline link displayed the most activity with 36.13 ± 3.74%, followed by two compounds with a chalcone link, **QCQ3** (22.67 ± 2.86%) and **QPQ1** (22.00 ± 0.72%) (Table 3.6).

Table 3.6: Iron chelation and free radical scavenging properties of quinoxaline-phenyl hybrids with a chalcone (**QCP**) and pyrazoline (**QPP**) in comparison to positive controls at 50 µM *(n* = 3, mean ± s.d).

QCP compound code	Iron chelation (%) ± s.d.	Free radical scavenging activity (%) ± s.d.	QPP compound code	Iron chelation (%) ± s.d.	Free radical scavenging activity (%) ± s.d.
QCP1	17.34 ± 1.73	5.59 ± 0.36	**QPP1**	18.75 ± 1.71	2.67 ± 0.10
QCP2	18.89 ± 0.25	7.94 ± 1.23	**QPP2**	17.83 ± 0.26	1.75 ± 0.11
QCP3	18.11 ± 1.01	0.10 ± 2.23	**QPP3**	21.77 ± 2.37	2.57 0.26
QCP5	16.71 ± 0.92	0.10 ± 1.8	**QPP5**	0.10 ± 2.23	1.55 ± 0.19
QCP6	4.98 ± 0.55	0.10 ± 1.56	**QPP6**	33.18 ± 1.38	5.37 ± 0.85
QCP7	0.55 ± 0.02	0.10 ± 1.43	**QPP7**	20.27 ± 0.80	3.22 ± 0.16
QCP8	12.99 ± 0.35	0.10 ± 0.49	**QPP8**	18.75 ± 0.88	9.51 ± 0.58
QCP9	15.35 ± 0.86	0.10 ± 0.19	**QPP9**	24.84 ± 2.48	6.90 ± 0.58
QCP10	2.30 ± 0.23	15.10 ± 0.50	**QPP10**	2.70 ± 0.21	3.39 ± 0.13
EDTA	96.64 ± 1.52	n.d	**Ascorbic acid**	n.d	67.52 ± 1.76
Trolox	n.d	72.29 ± 3.16			

n.d.: not determined

3.4.2 Free radical scavenging activity

None of the compounds (50 µM) from both sets had the ability of scavenging the free radical of $DPPH^{\bullet}$ as efficiently as the two positive controls, Trolox (72.29 ± 4.37%) and ascorbic acid (67.52 ± 1.76%). **QCP10** with a chalcone link displayed the highest free radical scavenging activity of 15.10 ± 0.50% in quinoxaline-phenyl hybrid set (Table 3.3); while **QPQ2** with a pyrazoline link in quinoxaline-quinoline hybrid set scavenged 19.23 ± 0.73% of the free radicals (Table 3.7).

Table 3.7: Iron chelation and free radical scavenging properties of quinoxaline- quinoline hybrids with a chalcone (**QCQ**) and pyrazoline (**QPQ**) in comparison to positive controls at 50 µM (n = 3, mean ± s.d).

Compound	% Iron chelation (%) ± s.d.	Free radical scavenging activity (%) ± s.d.	Compound	% Iron chelation (%) ± s.d.	Free radical scavenging activity (%) ± s.d.
QCQ1	7.47 ± 0.86	1.54 ± 0.27	**QPQ1**	22.00 ± 0.72	5.61 ± 0.24
QCQ2	3.07 ± 0.17	0.10 ± 0.38	**QPQ2**	14.59 ± 1.14	19.23 ± 0.73
QCQ3	22.67 ± 2.86	0.62 ± 0.12	**QPQ3**	0.10 ± 1.85	3.30 ± 0.03
QCQ5	9.73 ± 0.64	3.47 ± 0.05	**QPQ5**	0.10 ± 3.49	0.10 ± 1.08
QCQ6	4.65 ± 0.08	7.98 ± 0.45	**QPQ6**	13.33 ± 1.00	2.64 ± 0.07
QCQ7	8.20 ± 0.80	0.74 ± 0.05	**QPQ7**	18.62 ± 0.78	4.19 ± 0.55
QCQ8	4.58 ± 0.57	1.70 ± 0.13	**QPQ8**	36.13 ± 3.74	0.62 ± 0.04
QCQ9	8.08 ± 0.57	4.54 ± 0.29	**QPQ10**	3.65 ± 0.13	7.03 ± 0.41
QCQ10	5.94 ± 0.92	7.94 ± 0.76	**Ascorbic acid**	n.d	67.52 ± 1.76
EDTA	96.64 ± 1.52	n.d	**Trolox**	n.d	72.29 ± 3.16

n.d.: not determined

3.5 Prediction of drug-likeness and pharmacokinetic properties

3.5.1 Drug-likeness predictions

The molecular properties of the active compounds against *P. falciparum* were predicted *in silico* for drug-likeness properties based on Lipinski rule of five (Table 3.8). All the active compounds possessed good drug-likeness properties based on Lipinski's rule and none violated the RO5. Ten out of twenty-four active compounds had a logP value equivalent to that of quinine with logP value of 3.44, whilst compounds **QPP2**, **QPP5** and **QPP6** had the same HBA and HBD as quinine (Table 3.8).

3.5.2 Ionisation prediction as a function of pH

The predicted pKa values were used to determine how much of the ionised compound can penetrate through the parasite membrane and accumulate its digestive vacuole (Table 3.9).

Table 3.8: The predicted molecular properties of the active compounds against *P. falciparum* based on Lipinski rule (Lipinski *et al*., 1997).

Hybrid set	Compound code	MW (g/mol) (<500)	Log p (<5)	HBA (<10)	HBD (<5)	# of RO5 violation (<1)
Quinoxaline-chalcone-phenyl (QCP)	**QCP1**	292.31	3.88	3	0	0
	QCP2	308.76	4.34	3	0	0
	QCP3	353.21	4.50	3	0	0
	QCP5	288.34	4.25	3	0	0
	QCP6	292.31	3.88	3	0	0
	QCP7	308.76	4.34	3	0	0
	QCP8	353.21	4.50	3	0	0
	QCP9	304.34	3.58	4	0	0
Quinoxaline-pyrazoline-phenyl (QPP)	**QPP2**	322.79	3.64	4	1	0
	QPP5	302.37	3.55	4	1	0
	QPP6	306.34	3.18	4	1	0
Quinoxaline-chalcone-quinoline (QCQ)	**QCQ1**	343.35	4.27	4	0	0
	QCQ2	359.81	4.73	4	0	0
	QCQ3	404.26	4.89	4	0	0
	QCQ5	339.39	4.64	4	0	0
	QCQ6	343.35	4.27	4	0	0
	QCQ7	359.81	4.73	4	0	0
	QCQ8	404.26	4.89	4	0	0
	QCQ9	355.39	3.97	5	0	0
	QCQ10	339.39	4.64	4	0	0
Quinoxaline-pyrazoline-quinoline (QPQ)	**QPQ3**	418.29	4.20	5	1	0
	QPQ5	353.42	3.94	5	1	0
	QPQ6	357.38	3.57	5	1	0
	QPQ8	418.29	3.20	5	1	0
Reference drug	**Quinine**	324.42	3.44	4	1	0

3.5.3 Metabolism and *in vivo* toxicity

The *in silico* predictions for the metabolism of the active compounds indicated that the active compounds with chalcone linker in quinoxaline-phenyl hybrid (**QCP** compounds) and in quinoxaline-quinoline (**QCQ** compounds) hybrid set were predicted to be metabolised through aromatic hydroxylation by CYP3A4 enzymes. In contrast, the compounds with pyrazoline linker in both sets (**QPP** and **QPQ** compounds) were predicted to be metabolised through N-

dealkylation. From the toxicity predictions, all twenty-four active compounds displayed high class toxicity potential.

Table 3.9: Predicted percentage ionisation and absorption of the selected compound with *in vitro* antimalarial activity in comparison to quinine.

Hybrid set	Compound code	pKa	% ionisation		% Absorption
			pH 7.4	pH 5	
Quinoxaline-chalcone-phenyl (QCP)	**QCP1**	1.29	0.00	0.02	100.00
	QCP2	1.29	0.00	0.02	100.00
	QCP3	1.29	0.00	0.02	100.00
	QCP5	1.29	0.00	0.02	100.00
	QCP6	1.29	0.00	0.02	100.00
	QCP7	1.29	0.00	0.02	100.00
	QCP8	1.29	0.00	0.02	100.00
	QCP9	1.29	0.00	0.02	100.00
Quinoxaline-pyrazoline-phenyl (QPP)	**QPP2**	0.18	0.00	0.00	100.00
	QPP5	0.18	0.00	0.00	100.00
	QPP6	0.18	0.00	0.00	100.00
Quinoxaline-chalcone-quinoline (QCQ)	**QCQ1**	1.58	0.00	0.04	100.00
	QCQ2	2.33	0.00	0.21	100.00
	QCQ3	2.58	0.00	0.38	100.00
	QCQ5	3.89	0.03	7.20	99.97
	QCQ6	2.99	0.00	0.97	100.00
	QCQ7	2.94	0.00	0.86	100.00
	QCQ8	2.96	0.00	0.90	100.00
	QCQ9	3.95	0.04	8.18	99.96
	QCQ10	4.00	0.04	9.09	99.96
Quinoxaline-pyrazoline-quinoline (QPQ)	**QPQ3**	2.09	0.00	0.12	100.00
	QPQ5	3.47	0.01	2.87	99.99
	QPQ6	2.53	0.00	0.34	100.00
	QPQ8	2.47	0.00	0.29	100.00
Reference drug	**Quinine**	13.89, 9.05	2.19	99.99	97.81

3.6 Vector studies

3.6.1 Ovicidal activity

Out of the thirty-five compounds screened for ovicidal activity, twenty-eight possessed no ovicidal activity on the *An. arabiensis* (KWAG strain) eggs after 72 hours, with an average of 90% of the eggs hatching after 48 hours. Only seven compounds displayed minimal ovicidal activity, namely, two quinoxaline-phenyl hybrids with a chalcone link, **QCP6** (14%), **QCP5** (8%) and one with a pyrazoline link, **QPP7** (1%). Three quinoxaline-quinoline with a chalcone link, **QCQ1** (5%), **QCQ9** (5%), **QCQ7** (1%) and one with a pyrazoline link, **QPQ6** (2%). In the untreated control, 100% of the eggs hatched after 48 hours; whilst the positive control, formalin resulted in 100% ovicidal activity over 48 and 72 hours. The LC_{50} value of formalin was determined to be 0.005 ± 0.01 µM. The eggs treated with DDT resulted in 56% of the egg hatching after 48 hours and 72% after 72 hours.

3.6.2 Larvicidal activity

Test compounds possessed low larvicidal activity in a time-dependent manner. Seven of the **QPP** compounds resulted in 5-13% mortality, three **QCP** compounds between 18-23%, and two **QPQ** compounds resulted in 6-11% mortality (Figure 3.9). The rest of the compounds, including all nine **QCQ** compounds exhibited 0% larvicidal activity over 72 hours of exposure at 0.05 µM. No larvicidal activity (0% mortality) was observed in the untreated control over the 72 hour period. Whilst the positive control, DDT exhibited 86% larvae mortality after 24 hours and 100% larval mortality after 48 hours at 0.05 µM.

The LC_{50} values of DDT were determined to be 0.159 ± 0.02 µM after 24 hours, 0.035 ± 0.12 µM after 48 hours and 0.004 ± 0.01 µM after 72 hours. After 72 hours, pupae formation was observed with three of the compounds, namely QCP**9** (8% pupation), **QPP5** (6%) and **QCP6 (**12%), as well as in the untreated control (8%). There was no pupae formation in the DDT treated larvae.

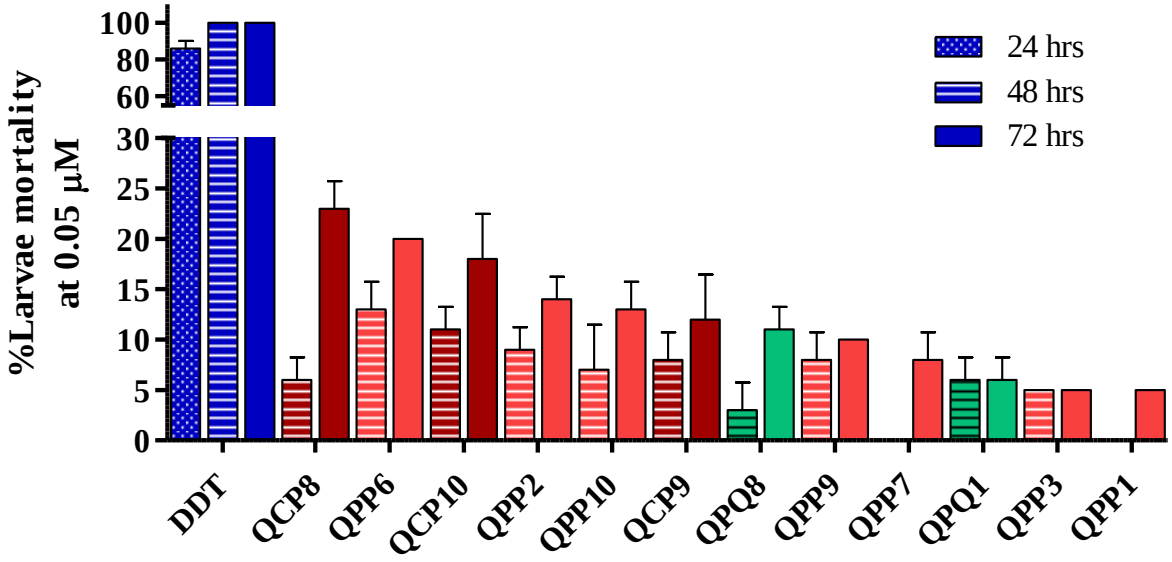


Figure 3.9: Larvicidal activity of the quinoxaline-based hybrid compounds against 3rd instar *An. arabiensis* (KWAG strain) larvae in comparison to the positive control, DDT over 72 hour time interval. Out of the thirty-five compounds, only twelve caused larvae mortality over the 72 hours in a time-dependent manner at 0.05 µM, but larvicidal activity was low when compared to DDT. Figure key: 24 hours indicated by bars with dots; 48 hours indicated by bars with horizontal lines and 72 hours indicated by empty bars.

3.6.3 Morphological effects of the compounds on the *An. arabiensis* larvae

The morphological changes were only observed for the larvae treated with compounds **QCP8**, **QPP10**, **QPP2** and **QPP6** (Table 3.10**)**. The untreated larvae (negative control) displayed normal morphological features which contrasted to those altered features of larvae treated with the positive control, DDT. Compound **QCP10** caused enlargement of the head and thorax, while compound **QPP2** resulted in the loss of palmate hairs. Compound **QCP8** and **QPP6** compromised the proper development of the pupae.

3.6.4 *Artemia* lethality assay

Thirty-two compounds out of thirty-five compound screened at 50 µM against *Ar. franciscana* nauplii showed negligible cytotoxicity with the overall percentage nauplii mortality less than 20% in comparison to the 100% by positive control, potassium dichromate over 72 hour exposure (Table F.1 and F.2). Only three **QPQ** compounds exhibited time- and dose-dependent cytotoxicity against *Ar. franciscana*. Compound **QPQ2** exhibited the highest cytotoxicity with a similar inhibitory activity to that of potassium dichromate. Both, compound **QPQ2** and

potassium dichromate resulted in 70% nauplii mortality after 48 hours and 100% nauplii mortality after 72 hours with no mortality observed after 24 hours (Figure 3.10). After 72 hours, the LC_{50} value for **QPQ2** was determined to be 1.05 µM, whilst for potassium dichromate and DDT were 16.61 and 6.69 µM, respectively. Compound **QPQ5** had the second highest percentage nauplii mortality of 30.49% after 48 hours and 48.48% after 72 hours, whilst compound **QPQ3** caused 11.85% mortality after 48 hours and 24.01% after 72 hours.

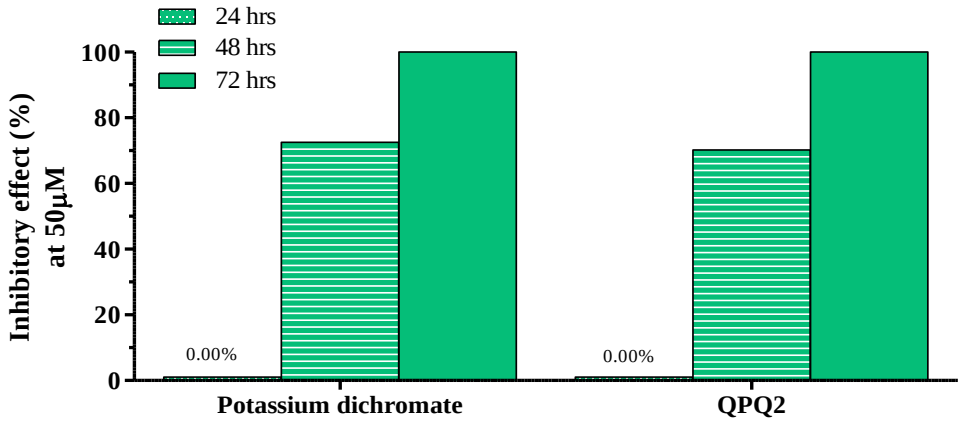


Figure 3.10: Inhibitory effect of quinoxaline-quinoline hybrid with a pyrazoline link, compound **QPQ2** against *Ar. franciscana* nauplii in comparison to the positive control, potassium dichromate over at 72 hours post-treatment at 50 µM.

3.6.5 Morphological alterations of the compounds on the *Artemia* nauplii

Morphological alterations in the nauplii were only observed when treated with compounds **QPQ2** and **QPQ5,** as well as potassium dichromate (Table 3.11). The rest of the compounds did not exhibit any visible morphological changes, however the mobility of the nauplii treated with compounds **QPQ3**, **QPQ6**, **QPQ8** and **QPQ10** was reduced when compared to the untreated control.

Table 3.10: The morphological effect of compound **QCP8**, **QCP10, QPP2** and **QPP6** against 3rd instar *An. arabiensis* larvae over a 72 hour period to observe any developmental alteration, compared to the positive and negative controls.

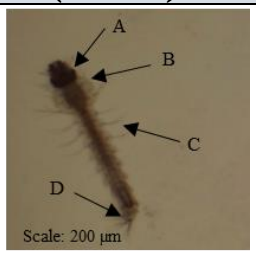
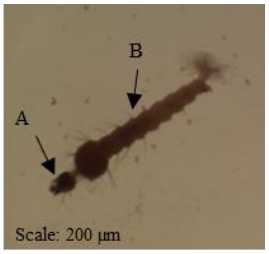
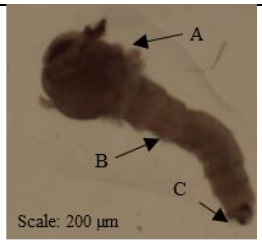
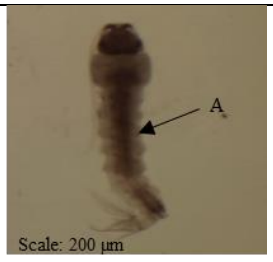
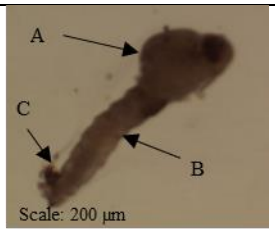
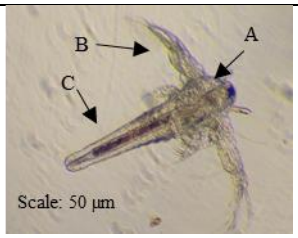
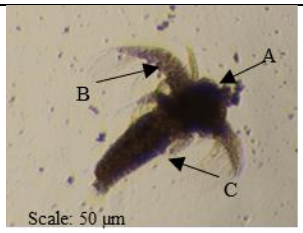
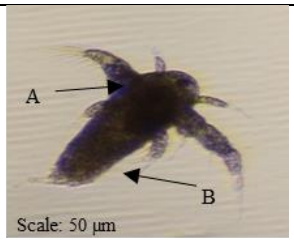
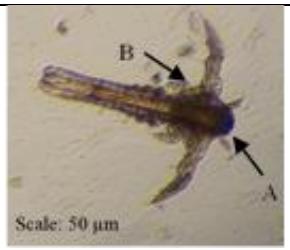
Compound	*An. arabiensis* (KWAG) larvae	Morphological changes
Negative control (untreated)		Normal head (A) and thorax (B) Presence of palmate hairs (C) Presence of tracheal gills (D)
Positive control (DDT)		Head reduced (A) Reduced palmate hairs (B)
QCP8		Distorted head (A) No palmate hairs (B) No tracechea gills (C) Altered pupa development
QCP10		Enlarged head and thorax (A) No palmate hairs (B)
QPP2		No palmate hairs (A)
QPP6		Distorted head and thorax (A) No palmate hairs (B) Tracheal gills and ventral brush under developed (C)

Table 3.11: The morphological effects of compounds **QPQ2** and **QPQ5** against *A. franciscana* nauplii 72 hours post treatment in comparison to the positive and negative controls.

Compound	***A. franciscana* nauplii**	**Morphological alterations**
Negative control (untreated)		Eye visible (A) Antennae and swimming appendages fully developed (B) Clear colour (C)
Positive control (Potassium dichromate)		Eye not visible (A) Antennae and swimming appendages not well developed (B) Colour darkened (C) Reduced size
QPQ2		Swimming appendages not well developed (A) Colour darkened (B) Reduced size
QPQ5		Eye not visible (A) Colour darkened (B)

CHAPTER FOUR – DISCUSSION

The rapid emergence of resistant strains of malaria parasites, especially *P. falciparum* has necessitated the continuous search and screening of alternative novel compounds in order to identify pharmacologically active agents with improved efficacy and minimal toxicity to the host cell (Smilkstein *et al*., 2004; Baniecki *et al*., 2007). As such, many studies seem to support the concept of molecular hybridization, in which two or more active pharmacophores are covalently linked into a single chemical entity, as the next generation of antimalarial drugs (Muregi and Ishih, 2010; Raj *et al*., 2015). The prime force driving the concept of hybrids is their potentially higher efficacy than that of either of the parent compounds administered as monotherapy or as a fixed-dose combination. With the added advantage of potentially lower toxicity against human host cells (Murugan *et al*., 2015; Raj *et al*., 2015; De Oliveria *et al*., 2017). In many cases, the hybrid compounds have displayed increased *in vitro* potency towards both drug resistant and non-resistant strains of *Plasmodium* (Bellot *et al*., 2010; Singh *et al*., 2017; Wang *et al*., 2014; Reddy *et al*., 2017).

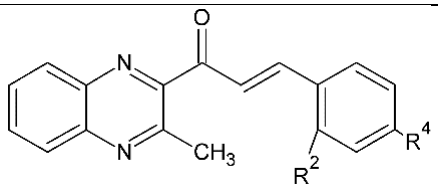
In the same manner, this present study was conducted to examine the *in vitro* antimalarial, larvicidal, as well as ovicidal activity of thirty-five quinoxaline-based hybrids (**QCP**, **QPP**, **QCQ** and **QPQ** compounds) and their preliminary toxicological profile. The test compounds exhibited variable inhibitory properties including antimalarial, anti-leukemic as well as cytotoxicity proprieties depending on the set of hybrids compounds, the type of linker (chalcone or pyrazoline) used and well as the type of R-group and its position as discussed below (Table 4.1)

4.1. Antimalarial activity

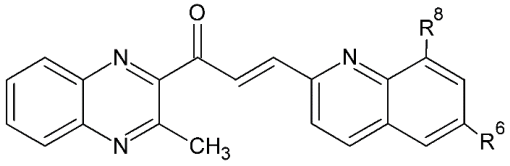
4.1.1 Influence of structure on antimalarial activity

The asexual blood stages of *P. falciparum* provide a good initial screening of compounds to identify those with potential to act as blood schizonticides to reduce the clinical symptoms of malaria (Table 1.2) (Bhattacharya *et al*., 2009). All the test compounds in this study consisted of methylquinoxalin-2-yl as the core structure (Figure 1.5). Many studies have reported on the antimalarial activity of the quinoxaline and its derivatives. Zarranz *et al*. (2005) observed improved antimalarial activity in compounds with non-substituted 3-arylquinoxaline 1,4-dioxide in positions 6 and 7 of the aromatic ring and with a hydrogen or chloro substituent in

Table 4.1: Collated summary of the inhibitory properties of the quinoxaline-phenyl hybrids with chalcone linker (**QCP** compounds) and the quinoxaline-quinoline hybrids with chalcone linker (**QCQ** compounds) based on the position of the R-group on the phenyl ring.



R-group at C_2	*P. falciparum**	Anti-cancer K562*	Cyto-toxicity HEK-293*	R-group at C^4	*P. falciparum**	Anti-cancer K562*	Cyto-toxicity HEK-293*
QCP1 2-F	26.46 ± 1.04	6.85 ± 1.35	18.12 ± 2.11	**QCP6** 4-F	18.03 ± 0.91	22.78 ± 1.44	>50**
QCP2 2-Cl	17.62 ± 0.78	6.85 ± 0.79	36.77 ± 0.89	**QCP7** 4-Cl	9.71 ± 0.42	36.42 ± 1.44	>50**
QCP3 2-Br	4.19 ± 0.54	9.18 ± 1.33	21.16 ± 1.86	**QCP8** 4-Br	6.06 ± 0.57	23.94 ± 2.72	>50**
QCP5 2-CH_3	30.00 ± 2.79	11.05 ± 0.45	20.39 ± 2.59	**QCP10** 4-CH_3	>50**	18.68 ± 1.78	>50**
***** All units in µM ± s.d. ******IC_{50} value not determined as percentage parasite growth/cell viability was >60% at 50 µM.				**QCP9** 4-OCH_3	11.69 ± 0.95	9.94 ± 1.16	>50**



R-group at C_8	*P. falciparum**	Anti-cancer K562*	Cyto-toxicity HEK-293*	R-group at C^6	*P. falciparum**	Anti-cancer K562*	Cyto-toxicity HEK-293*
QCQ1 8-F	6.70 ± 0.94	4.40 ± 1.00	20.38 ± 1.93	**QCQ6** 6-F	12.34 ± 0.97	5.60 ± 0.10	13.66 ± 0.46
QCQ2 8-Cl	9.0 ± 1.06	9.10 ± 0.90	35.72 ± 1.64	**QCQ7** 6-Cl	14.37 ± 1.12	23.00 ± 1.10	30.82 ± 1.18
QCQ3 8-Br	7.41 ± 1.25	4.10 ± 0.40	15.46 ± 2.69	**QCQ8** 6-Br	11.07 ± 1.69	10.00 ± 0.10	17.61 ± 2.24
QCQ5 8-CH_3	8 ± 1.06	5.00 ± 0.40	21.13 ± 2.06	**QCQ10** 6-CH_3	11.36 ± 1.80	4.00 ± 0.50	17.45 ± 2.33
***** All units in µM ± s.d.				**QCQ9** 6-OCH_3	6.45 ± 0.37	6.70 ± 1.00	35.41 ± 2.47

the *para* position of the phenyl ring. It was concluded that the di-N-oxide moiety was necessary for antimalarial activity as the reduced quinoxaline was found to be inactive. To further improve the antimalarial efficacy of quinoxalines , it was found that 3-methyl-2-[3-(3,4,5-trimethoxyphenyl-prop-2-enoyl)] quinoxaline 1,4-di-N-oxide (1a; IC_{50} = 6.2 µM) and 3-methyl-2-[3-(naphth-2-yl-prop-2-enoyl)] quinoxaline 1,4-di-N-oxide (2a; IC_{50} = 5.8 µM) were the more active but still not comparable to chloroquine (IC_{50} = 0.173 µM). The structural-activity relationship revealed the importance of chalcone moiety linked to the quinoxaline for enhanced antimalarial activity (Gil *et al*., 2014).

In this present study, two of the sets of hybrids consisting of quinoxaline linked to either phenyl (**QCP** compounds) or quinoline (**QCQ** compounds) by chalcone moiety displayed an overall improved activity compared to those with a pyrazoline linker (**QPP** and **QPQ** compounds); which further indicated that chalcones can increase antimalarial activity of quinoxaline. Chalcones have been found to have selectively inhibited various strains of *P. falciparum* by acting on all asexual stages of the parasite (Chen *et al*., 1994; Bhattacharya *et al*., 2009). The *in vitro* antimalarial activity of chalcones and its derivatives is mainly determined by the properties of ring B with the size and hydrophobicity of the substituents identified as critical parameters (Liu *et al*., 2001; Go *et al.*, 2004). Previously reported chalcones derivatives including the alkoxylated chalcones were more active (IC_{50} values: < 6.5 µM) than the hydroxylated chalcones (4-chloro-2',4'-dihydroxychalcone; IC_{50} value: 12.3 µM) against chloroquine-resistant K1 strain of *P. falciparum* (Liu *et al*., 2001).

In comparison, 1-(4-benzimidazol-1-yl-phenyl)-3-(2,4-dimethoxy-phenyl)-propen-1-one was found to be the more potent than previously reported antimalarials with an IC_{50} value of 1.1 µg/ml, as compared to licochalcone (IC_{50} value: 1.43 µg/ml) (Yadav *et al*., 2012). The improved activity was contributed to the presence of two methoxy groups at position 2 and 4, which were found to be essential for optimum antimalarial activity, where substitutions with 3,4-dimethoxy and 2,5-dimethoxy resulted in decreased activity.

The similar influence of the R-group on the antimalarial activity was observed with quinoxaline hybrids with chalcone linker (**QCP** and **QCQ** compounds) (Table 4.1). The structure-activity relationship on the **QCP** compounds demonstrated that compounds with a fluoro and chloro substitution at the C_4 position of the phenyl ring (**QCP6, QCP7**), yielded an improved antimalarial activity with a decrease in IC_{50} values, in comparison to those with substitution at the C_2 position (**QCP1**, **QCP2**). The position of the chloro group seemed to have significant

influence on the activity, where the IC_{50} value of **QCP2** (17.62 ± 0.78 µM) with the chloro group substitution at C_2 was almost double that of **QCP7** (9.71 ± 0.42 µM) substituted at the C_4 position. Whilst compound **QCP6** with a fluoro group on the C_4 position was 1.5 times more active than when substituted at the C_2 position **QCP1**. The exception to the observed trend was with compound **QCP3**, which exhibited an overall potent activity (IC_{50} value: 4.19 ± 0.54 µM) (Figure 3.1) with a bromo group at the C_2 position of the phenyl ring, and in comparison to compound **QCP8** where the bromo group was at the C_4 position, resulted in decreased antimalarial activity (IC_{50} value: 6.06 ± 0.57 µM) (Figure 3.2). The substitution of the methyl group at C_2 (**QCP5)** of the phenyl ring resulted in low activity (IC_{50} value: 30 ± 2.79 µM) and when the same group was placed at C_4, the antimalarial activity diminished as percentage parasite growth was 61.63 ± 4.97% at 50 µM (Table 3.1). Compound **QCP9** was the only compound with substitution of methoxy group at C_4 position and displayed moderate activity (IC_{50} value: 11.69 ± 0.95 µM).

It was observed that when the phenyl ring was replaced with a quinoline ring, as seen in the **QCQ** compounds, the antimalarial activity improved with an overall IC_{50} values of less than 15 µM (Figure 3.3). It was noted that the compounds with R-group substitutions at position C_8 of the quinoline ring (**QCQ1**, **QCQ2, QCQ3** and **QCQ5**) displayed promising antimalarial activity as their IC_{50} values were < 10 µM. However, when the substitution was at C_6 (**QCQ6, QCQ7, QCQ8** and **QCQ10**), the IC_{50} values increased by at least two units resulting in an overall moderate antimalarial activity with IC_{50} values ranging between 11:07 ± 1.69 to 14.37 ± 1.12 µM. Compound **QCQ9** was the exception with the highest antimalarial activity (IC_{50}: 6.45 ± 0.37 µM) in the set, with a methoxy-group substitution at position C_6 of the quinoline ring. The fluoro substitution at position C_8 had a significant influence on the antimalarial activity of **QCQ1** (IC_{50} value: 6.70 ± 0.94 µM); however, when positioned at C_6 (**QCQ6**), the IC_{50} value doubled (12.34 ± 0.97 µM), thus decreasing the overall antimalarial activity (Figure 3.3).

Domínquez *et al.* (2001) demonstrated that a single bromo- or chloro-substitution on C_4 of the phenyl ring resulted in the elimination of any antimalarial activity in both chloroquine-sensitive and –resistant parasites, with resultant IC_{50} values greater than 200 µM. However, di-chloro substitutions at the C_2, C_4 and C_2, C_5 positions produced compounds with IC_{50} values of 19 µM and 48.6 µM, respectively; whilst di-substitution at C_3 and C_4 reduced the antimalarial activity. However, the study by Liu *et al*. (2001) reported contrasting results where structural analysis

indicated that 3-quinolinyl derivatives were more active than 4-quinolinyl derivatives, regardless of ring substitutions. It was also noted that electron-acceptor groups, such as difluoro- or dichloro- at C_2, C_3 or C_2, C_4 positions on ring A were not necessarily contributors to antimalarial activity. The study identified 1-(2',3',4'-trimethoxyphenyl)-3-(3-quinolinyl)-2-prop-1-one as a promising inhibitor of parasite growth against chloroquine-resistant K1 strain of *P. falciparum*, with an IC_{50} value of 2 μM (Liu *et al*., 2001). Sharma *et al*. (2009) also demonstrated that the activity of 4-oxo and 4-amino linked quinoline-chalcones displayed no activity against the NF-54 chloroquine-sensitive strain of *P. falciparum,* when the R group was a pyridine ring, or a chloro-, bromo-, nitro-, methyl- or methoxy- substituted phenyl ring. The same study found that none of the compounds with halogen substitutions on the phenyl ring exhibited sub-micromolar IC_{50} values and indicted that the 7-chloroquinoline moiety possibly contributed to the compound's activity, by inhibiting haemozoin formation (Guantai *et al*., 2010). However, there was no observed correlation between the β-haematin inhibitory and antimalarial activity of the tested compounds (Guantai *et al*., 2010). Ferrer *et al*. (2009) also found that a chloro-substitution on C_4 of the phenyl ring in a series of chloroquinoline-chalcones conferred β-haematin inhibitory activity, whereas a chloro-substitution at C_3 abolished β-haematin inhibitory activity.

The selection of linkers in this study clearly influenced the efficacy of the compound in inhibiting the growth of the intra-erythrocytic parasite (Figure 3.2 and 3.3). This is reflected in previous reports (Mott *et al*., 2012; Raj *et al*., 2014; Araujo *et al*., 2009). In a study to evaluate the influence of different linkers to covalently bond quinolines and artemisinin, it was found that cyclic linkers should be avoided as they contribute to decreased antimalarial activity. This was observed with the decrease in activity of the **QPP** and **QPQ** compounds where the cyclic nature of pyrazoline could have contributed to this outcome (Table 3.1 and Table 3.2). In addition, the length of the linker should be shortened to two or three carbon atoms as hybrids with a linker chain length of greater than three were found to be less potent than chloroquine (Lombard *et al*., 2013).

4.1.2 Possible mechanism of action of quinoxaline-hybrids

4.1.2.1 Inhibition of *P. falciparum* cysteine protease

The latter structure-activity relationships aid in a better understanding of how the chalcones and quinolines exert their antimalarial activity. The exact mechanism of action for both

chalcones and quinoline is not clearly understood, however various mechanisms have been proposed. The chalcones have been proposed to act on different metabolic pathways of the parasite depending on their side chain substitution (Go, 2003; Kumar *et al*., 2007). The predominantly proposed antimalarial mechanism of action of chalcones is the inhibition of *P. falciparum* cysteine protease, which is one of the main enzymes involved in the degradation of haemoglobin within the acidic food vacuole of the intra-erythrocytic parasite (Shenai *et al*., 2000).

Li *et al*. (1996) proposed the trophozoite cysteine protease (falcipain-2) as the most likely target enzyme for the chalcone derivatives (Mishra *et al*., 2008). The results from the stage specific and morphological effects of **QCP7** and **QCP8** on *P. falciparum* revealed that these two compounds acted mainly at the trophozoite stage and resulted in reduced haemozoin formation (Figure 3.5).

Mahesh *et al.* (2014) outlined that an aromatic moiety, hydrogen bond donors between 0 to 2 and hydrogen bond acceptors between 2 to 6, as well as a basic nature of the molecule are necessary for falcipain inhibitory activity. Both compound **QCP7** and **QCP8** had all the necessary structural features, with hydrogen bond donors (0) and acceptors (3) within the range (Table 3.8) and are both weak bases. Therefore, it can be proposed that these two compounds inhibited the activity of cysteine protease at trophozoite stage.

Molecular modelling studies have revealed that chalcones are stable in the presence of cysteine protease enzymes and assumes a linear or planar conformation to fit into the long cleft of the active site of the malarial cysteine protease enzyme to inhibit its function (Mishra *et al*., 2008; Li *et al*., 1995). The *in vitro* activity against *P. falciparum* (3D7) of a triazolyl-substituted chalcone was proposed to be due to protonation of the nitro- groups in the triazolyl moiety in the acidic food vacuole, that resulted in an enhanced interaction of the compound with the histidine residue of the cysteine protease enzyme (Figure 4.1) (Mishra *et al*., 2008).

4.1.2.2 Inhibition of haemozoin formation

Chalcones have also been shown to inhibit the polymerisation of haem into haemozoin (Orij, 2001) (Figure 4.2). The metabolic processes of the parasite peaks during the trophozoite-stage, with ingestion of ~80% of the haemoglobin in the host RBCs being one of main processes (Bozdech *et al*., 2003; Hanssen *et al*., 2010; Tilley *et al*., 2011). After ingestion, haemoglobin-

containing vesicles bud off from the cytostome and fuse to the digestive food vacuole (Sullivan, 2002; Lazarus *et al*., 2008; Tilley *et al.*, 2011). The food vacuole is highly acidic, containing

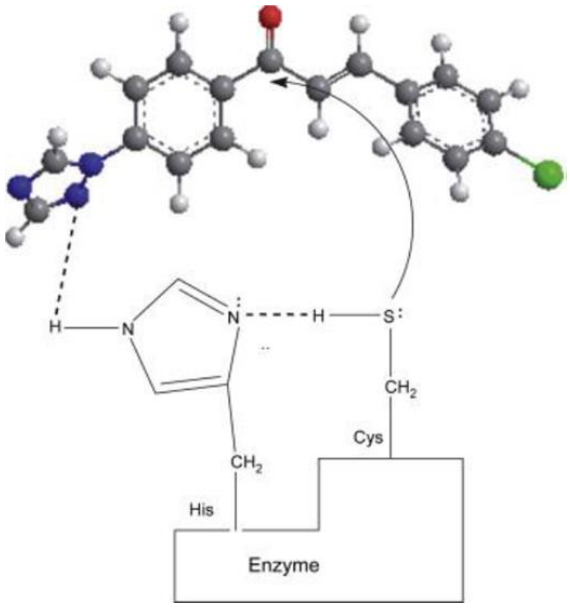


Figure 4.1: The proposed interaction of a chalcone with the histidine residue at the active site of cysteine protease enzyme of the malaria parasite (Mishra *et al.*, 2008).

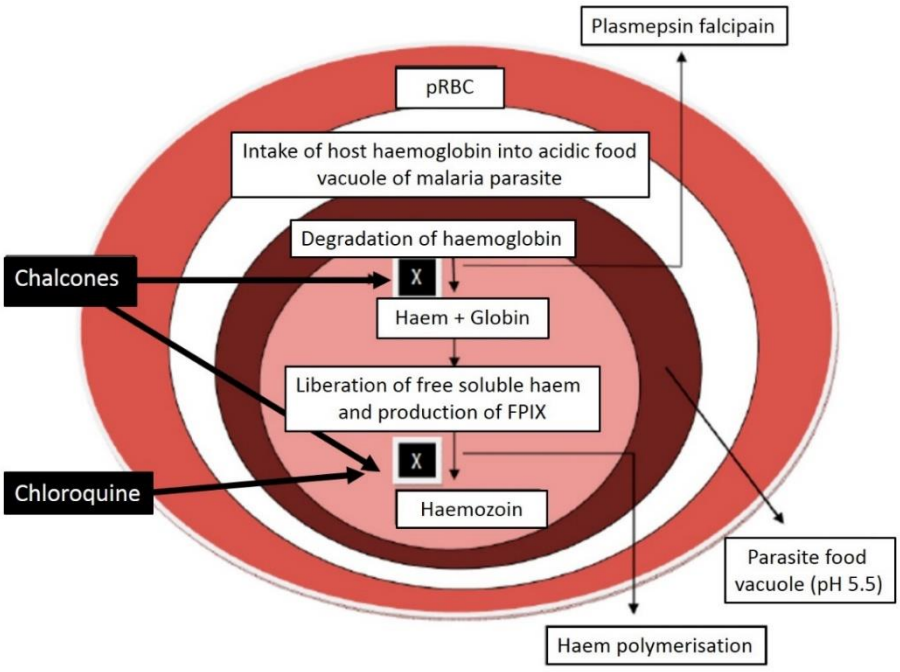


Figure 4.2: Mechanism of action of chalcones and chloroquine in malarial parasite (adapted from Sinha *et al.*, 2013).

various parasitic proteases responsible for haemoglobin degradation into peptides and haem units, Fe (II)-protoporphyrin-IX (Banerjee *et al*., 2002; Egan, 2008; Skinner-Adams *et al*., 2009). The released haem is highly toxic to the parasite and may move from the food vacuole into the parasite cytoplasm where it undergoes redox reactions to form reactive oxygen species (ROS). The iron present in haem is also capable of reacting with oxygen to form ROS via the Fenton reaction. Intracellular ROS attack parasitic macromolecules such as DNA and proteins, and causes lipid peroxidation (Müller, 2004; Zhan *et al*., 2006). Therefore, haem is detoxified by the parasite to form the inert polymer, haemozoin, or be degraded by glutathione (GSH) (Müller, 2004; Garavito *et al.*, 2007).

The results from combination studies showed that both compound **QCP7** and **QCP8** had an overall synergistic interaction in combination with antimalarial drug, quinine (Figure 3.4). This was a favourable interaction as the synergistic combination can aid in the delay of resistant parasites developing, as well as minimising the cost and dose of the treatment regimen (Bell, 2005). The observations from the stage specific and morphological studies, indicates that the synergic interaction could have been due to the **QCP** compounds along with quinine inhibiting different steps in the haemozoin formation pathway (Nowakowska, 2017). Similar results from a combination of chalcone derivatives with artemisinin resulted in a synergistic interaction by decreasing haemozoin formation against *P. falciparum* 3D7 strain (Bhattacharya *et al.*, 2009).

The presence of the quinoline ring in the quinoxaline-quinoline hybrids, as well as the chalcone linker (**QCQ** compounds) seemed to have enhanced their antimalarial activity (Table 4.1). The pKa values of the **QCQ** compounds doubled that of the **QCP** compounds when the quinoline replaced the phenyl group (Table 3.9), along with the IC_{50} values of the **QCQ** compounds, on average being half that of the **QCP** compounds. In contrast, the replacement of the phenyl group with quinoline on the quinoxaline-pyrazoline hybrids, increased the pKa ~15 fold; which could account for the improved accumulation of the quinoxaline-quinoline hybrid into the malaria parasite and possibly the acidic food vacuole causing parasite death. (Table 3.9).

Most structure-activity relationships, as well mechanistic studies, have shown that compounds possessing the quinoline structure are capable of inhibiting haemozoin formation, except primaquine (Egan *et al.*, 2000; Olliaro, 2001; Biagini *et al*., 2003; Vangapandu *et al*., 2007). Primaquine exhibits its antimalarial activity by acting as a pro-oxidant generating reactive oxygen species within the RBC that induces lipid peroxidation and inhibits parasite growth (Basso *et al*., 2011). Other mechanisms by which these test compounds could have inhibited

parasite growth were by inhibiting protein synthesis, intercalation into parasite DNA, inhibiting DNA replication, the inhibition of food vacuole phospholipases, and the inhibition of aspartic proteinases. However, these may be exhibited at higher concentration (Olliaro, 2001).

Several studies have shown that chalcones with the α, β-unsaturated carbonyl group can form a conjugate with GSH, one of the main anti-oxidants in the parasite. This results in preventing the degradation of Fe (III) protoporphyrin-IX in the parasite (Nadelmann *et al.*, 1997; Go, 2003; Frölich *et al*., 2005). Therefore, the antimalarial activity of the quinoxaline-quinoline hybrids with a chalcone linker (**QCQ** compounds) may have been due the dual action of the quinoline and chalcone. The quinoline could have inhibited the haemozoin formation, while the α, β-unsaturated carbonyl group on the chalcone inhibited the GSH-mediated degradation of Fe (III) protoporphyrin-IX causing oxidative stress that resulted in parasite death. The latter is a similar mechanism of action to that of chloroquine (Figure 4.3). Chloroquine, like other quinoline-based antimalarial drugs that act through inhibition of haemozoin formation, have also been proposed to exert their antimalarial activity by inhibiting of GSH-mediated haem degradation (Müller, 2004; Kumar *et al*., 2007).

The activity of the quinoline derivatives was observed to be reduced when the chalcone linker was replaced with a pyrazoline linker, as seen in the **QPQ** compounds where only four compounds exhibited antimalarial activity with IC_{50} values ranging between 14.03 ± 2.66 and 22.60 ± 3.15 μM (Figure 3.3). A similar trend was observed when the quinoline ring was replaced with a phenyl ring in the **QPP** compounds and the chalcone linker replaced with a pyrazoline linker. Only three compounds displayed antimalarial activity with IC_{50} values ranging between 14.83 ± 1.99 and 24.03 ± 3.45 μM (Figure 3.2).

Even though pyrazolines and quinolines share the same mechanism of action by inhibiting haemozoin formation (Acharya *et al.*, 2010), the **QPQ** and **QPP** compounds did not exhibit a high antimalarial activity as was expected (Figure 3.2 and 3.3). A series of pyrazole-pyrazoline compounds substituted with benzenesulfonamide were reported to exhibit antimalarial activity with IC_{50} values less than 2 μM on both chloroquine-sensitive (3D7) and -resistant (RKL-9) strains of *P. falciparum*. A mechanistic study revealed that these compounds inhibited β-haematin (Kumar *et al.*, 2018). Similarly, a series of 1, 3, 5-trisubstituted pyrazolines were also found to possess antimalarial activity by inhibiting haemozoin formation *in vitro.* A correlation was observed between the antimalarial and β-haematin inhibitory activity (Acharya *et al*., 2010).

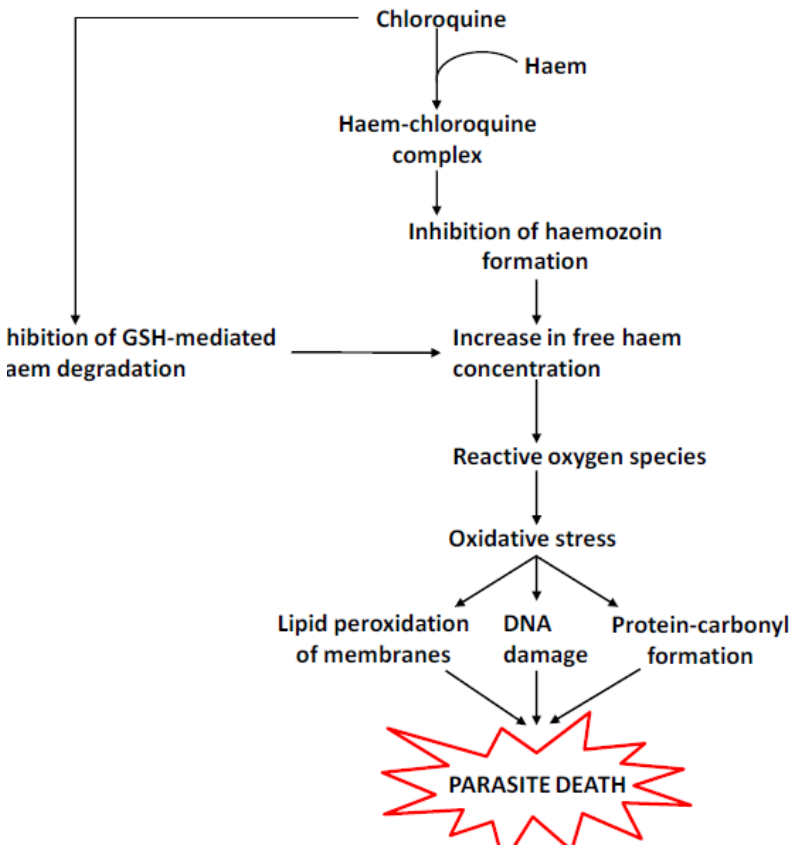


Figure 4.3: Proposed mechanism of chloroquine (Kumar *et al.*, 2007; Moseley, 2012).

4.1.2.3 Induction of apoptosis

As with many of the antimalarial drugs, it would appear that these quinoxaline hybrids have more than one mechanism by which they kill the malaria parasite (Egan *et al.*, 2000). A series of stilbene-chalcone hybrids were found to be specific for the ring and trophozoite stage of the parasite and did not affect the schizont stage, egress of merozoites, or the process of merozoite invasion. The stilbene-chalcone hybrids were found to block the progression of the parasite life cycle at the ring or the trophozoite stages. A potential target as previously identified is falcipain-2, found in both rings and trophozoites to ensure an amino acid supply for the developing parasite. However, these stilbene-chalcone hybrids were shown to have no inhibitory effect on the enzyme. Dominguez *et al.* (2001) also reported that the hybrid quinolinylchalcones failed to inhibit the falcipain-2 activity (IC_{50}: >100 μM), even though they inhibited the growth of the malaria parasite with an IC_{50} value of ~1 μM.

As such an alternative mechanism was sought. Apoptosis, a well-known programmed cell death mechanism in multicellular organisms, is not well studied in unicellular organisms. These chalcone derivatives induced cell shrinkage, chromatin condensation, DNA fragmentation, and

loss of mitochondrial membrane potential in the parasite, thereby suggesting their ability to cause apoptosis in malaria parasite (Sharma *et al*., 2012).

A similar trend was observed for compounds **QCP7** and **QCP8**, where the rings development lagged behind that of the rings in quinine and the untreated control (Figure 3.5A); but a small percentage of the parasites progressed into the trophozoite and schizont stages (Figure 3.5 B, C). Even though cell shrinkage was noted, further investigations are required to verify an apoptotic or nonapoptotic death. The quinoline hybrids could have also induced apoptosis, as it has been shown that chloroquine-induced oligonucleosomal DNA fragmentation suggesting programmed cell death in non-synchronized erythrocytic stages of chloroquine-sensitive *P. falciparum* strain (Picot *et al.*, 1997). Subsequent studies have shown that chloroquine also induces the hallmark features of apoptosis including nuclear chromatin condensation, loss of mitochondrial membrane potential, and changes in plasma membrane permeability.

4.1.3 Haemolysis

Haemolysis and subsequent anaemia in malaria-infected patients is often a concern as the infection rate increases (Roux *et al*., 2010). As such, the therapeutic drugs should not contribute to further lysis to compromise for the safety of the patient. This lesson was learnt with primaquine, which is a quinoline derivative that is known to cause haemolytic anaemia, especially in patients with glocuse-6-phosphate dehydrogenase (G6PD) deficiency and this has limited its use (Ashley *et al*., 2014).

In this study, all thirty-five compounds did not compromise the human RBC membrane integrity by inducing haemolysis (0.10 ± 0.01% lysis) in comparison to the 100% lysis control, TritonX-100™ (Section 3.2.1). This indicated that the *in vitro* antimalarial action of the test compounds was due to a direct inhibitory effect on intra-erythrocytic malaria parasite (Hayat *et al*., 2011). This was a favourable property for the development of these test compounds as antimalarial drugs.

Chalcones have also been shown to inhibit the parasite-induced new permeation pathways formed in the erythrocytic membrane at the end of the ring-stage (Go *et al*., 2004; Bhattacharya *et al*., 2009). Go *et al*. (2004) found that only dimethoxy- and methoxy-chalcones were capable of inhibiting sorbitol-induced haemolysis. Bhattacharya *et al*. (2009) reported that out of the three tested chalcone derivatives with IC_{50} values below 10 μM against *P. falciparum*, only a

triazole-substituted chalcone and not a pyrrole or benzotriazole-substituted chalcone, inhibited sorbitol-induced haemolysis. However, inducing haemolysis of uninfected red blood cells is not a potential risk of these compounds tested in this study.

4.2 Cytotoxicity

The first concern with novel compounds is the selectivity or safety of the compound, where the compound should selectively target the malaria parasite without causing cellular damage or death in the human host. Of the compounds tested, only four **QCP** compounds with substitutions at C_2 of the phenyl ring (**QCP1, QCP2, QCP3** and **QCP5)** displayed cytotoxicity against the human kidney epithelial cells. Low $S.I_{pf}$ values ranging between 0.68 and 5.05 (Table 3.5) were determined, indicating non-selectivity for the malaria parasite or the human host cells.

The high cytotoxicity towards human epithelial cells and hence the very low safety indices of **QCQ** compounds is of concern (Figure 3.6 and 3.7; Table 3.5). General high cytotoxicity of **QCQ** could have been due to the interaction of α, β-unsaturated carbonyl group on chalcone and the nitrogen on the quinoline ring. Chalcones with α, β-unsaturated carbonyl group have also shown to alkylate proteins and when this alkylation is not specific to the parasite, it can be highly toxic to the host cell (O' Neill and Posner 2004). Therefore, it is possible that the **QCQ** compounds exerted their antimalarial and cytotoxicity action through non-specific chalcone-mediated protein alkylation due to the presence of α, β-unsaturated carbonyl that acted as an electrophile, while the nitrogen on the quinoline ring (Figure 1.5) donated the electrons by acting as nucleophile which posed increased toxicity potential.

In comparison, the $S.I_{pf}$ values of quinine and DHA were 1278.91 and 25232.42, respectively indicating a preferred site of action within the malaria parasite and the potential for reduced toxicity and side effects in the human host (Figure 3.2; Table 3.5). This high S.I also indicates a preferred safety profile, especially since the rest of the **QCP** compounds displayed favourable *in vitro* cytotoxicity against the human kidney epithelial cells where the percentage cell viability was greater than 50%. This is a favourable outcome considering 100% of the compound was predicted to be absorbed into the human host, as was quinine (Table 3.9). However, the side effect profile of quinine is known to require monitoring due to its narrow therapeutic range resulting in an increased risk of cinchonism side effects and hypoglycaemia.

Based on the predicted metabolism and toxicity profile to be encountered in the human host, the low $S.I_{nc}$ obtained *in vitro*, matches that of the *in silico* predictions for a high class toxicity potential of the 24 active compounds (Section 3.6.3). Among the quinoxaline class of compounds, quinoxaline-1,4-dioxides have been reported to possess liver and adrenaline toxicities (Ihsan *et al.*, 2010). Toxicity is a major concern and should be addressed carefully. In addition, potential drug interactions need to be considered, since these **QCP** and **QCQ** hybrids are predicted to be metabolised by hepatic cytochrome P450 CYP3A4 enzymes, along with many other therapeutic medications. The metabolism of quinoxaline-8-carboxylic acid in humans revealed that a major detoxification pathway was catalysed by cytochrome P450 1A2, as well as xanthine oxidase through oxidative metabolism (Kaushal *et al*., 2019). An overall favourable safety profile following oral ingestion along with selectivity towards malaria parasite, *P. falciparum*, is preferred for any novel compound (Table 3.5).

4.3 Anticancer activity

An ideal anticancer drug should exhibit high potency and specificity in killing cancer cells without causing toxicity to normal cells. As such, the hybrids were tested on normal cells and for comparative activity against human cancerous cells, chronic myelogenous leukaemia cells (section 2.5.2). It has been previously reported that quinoxaline hybrids possess anticancer properties (Kumar *et al.*, 2003; Solomon and Lee, 2011; Wang *et al.*, 2017).

The quinoxaline-hybrids with pyrazoline linker (**QPQ** and **QPP**) were relatively ineffective against the chronic myelogenous leukaemia (K562) (Table 3.3 and 3.4). Through a series of 3, 5-disubstituted pyrazolines, Lu *et al.* (2017) showed that the introduction of alkoxy, and fluorine functionalities exhibited higher activity than unsubstituted pyrazolines when tested against human non-small-cell lung cancer cell line (A549). As such the inactivity of the **QPQ** and **QPP** hybrids could be due to the unsubstituted pyrazolines. Pyrazoline derivatives have been found to possess *in vitro* anticancer activity against MCF-7 and MDA-MB-231 breast adenocarcinoma cancer cell lines effect with IC_{50} value of 1.86 and 2.45 μM, respectively (Dinesha *et al.*, 2015). Similarly, pyrazolines have been found to be effective against hepatocarcinoma HepG-2 cells by inducing apoptosis and causing cell cycle arrest at G2/M phase (Wang *et al.*, 2017). Apoptosis was also the mechanism by which a series of benzochalcones bearing a pyrazoline nucleus inhibited colorectal cancer (IC_{50} value: 2.40 μM)

with the cell cycle being inhibited through the dysregulation of cell cycle regulatory cyclin B1 (Shin *et al*., 2013).

In contrast, the quinoxaline- hybrids with chalcone linker (**QCP** and **QCQ**) were found to be more active against the K562 leukaemia cell line (Table 4.1). The quinoxaline group has been linked to many functional groups to improve upon its anticancer properties (Figure 4.4**)**. Quinoxaline-1, 4-dioxides (QdNOs) acted as potent hypoxia selective cytotoxins modulating hypoxia inducible factor-1alpha (HIF-1a) expression in lung and mammary adenocarcinoma (Weng *et al.*, 2008). Whilst the methyl and benzyl derivatives of 3-aylquinoxalines were potent inhibitors of vascular endothelial growth factor (VEGF) that stimulates angiogenesis (Whatmore *et al*., 2002). In contrast, isoindolo [2,1-a] quinoxaline derivatives have been shown to possess a dual mechanism of action by inhibiting tubulin polymerization, as well as topoisomerase I.

Along with the antileukaemic properties in this study (Table 4.1), chalcones have been shown to possess anticancer activity and inhibit the growth of various tumour cells including breast cancer cell lines (MCF7, MB468) prostate cancer cells, leukaemia cells (Chen *et al*., 1994; Kumar *et al*., 2003; Li *et al*., 2012). The anticancer properties of chalcones have been found to be mainly influenced by the substitutions on the two aryl rings of chalcone molecule and their substitution patterns. For an improved anticancer activity, literature suggests the implementation of three structural adaptations, namely, structural manipulation of both aryl rings, replacement of aryl rings with heteroaryl scaffolds and molecular hybridization through conjugation with other pharmacologically active scaffolds (Karthikeyan *et al*., 2014).

Chalcones exhibit anticancer properties through multiple mechanism such as cell cycle disruption and angiogenesis inhibition. The antiproliferative activity of chalcones is due to their capacity to inhibit tubulin assembly and stop uncontrolled proliferation of cancer cells. The induction of apoptosis by chalcones often comprises the mitochondrial pathways, down-regulation of anti-apoptotic proteins, and/or death receptor pathways (Pan *et al*., 2005; Nishimura *et al*., 2007; Sakai *et al*., 2012). Some chalcones may also induce apoptosis through generation of reactive oxygen species (Motani *et al*., 2008), notwithstanding being known as anti-oxidant molecules. The pro-oxidant activity of chalcones may be conferred by their strong reactivity with thiol groups in living organisms, by the induction of a series of reactions with

hydroxyl radicals in living cells, or by reducing the antioxidant mechanisms, such as the glutathione content (Miranda *et al*., 2000).

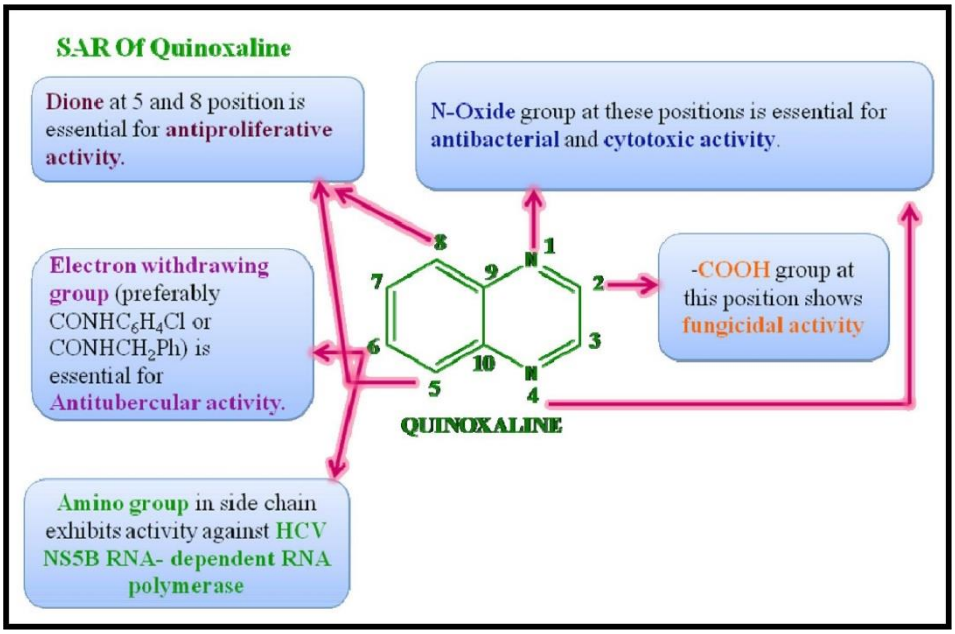


Figure 4.4: Structure activity relationship of quinoxaline compounds for various biological activities (Kaushal *et al*., 2019).

Studies on synthetic methoxylated chalcones suggested that the number and the position of methoxy substituents on the aromatic rings appeared to be critical for their anticancer activity. Their antimitotic effect was dependent on the substitutions at the B ring, especially in 2-, 4-, and 6-positions. However, in the case of dimethoxylated derivatives, the methoxy groups showed activity when linked to carbons 2 and 6 in the B ring (Boumendjel *et al*., 2008). In case of the trimethoxylated chalcones, methoxylation at 3rd, 4th and 5th position of the B ring was favoured.

Combining the active metabolite of artemisinin, namely dihydroartemisinin with a substituted chalcone moiety linked by either ether or ester, Yang *et al*. (2009) reported that the derivatives exhibited greater anti-proliferative and cytotoxic effects than dihydroartemisinin, against human leukaemia HL-60 and mouse lymphoma P388 cells. The dihydroartemisinin chalcones linked by ether were found to have more cytotoxic than dihydroartemisinin chalcones linked by ester with apoptosis induction abilities.

Quinolines have also been found to have anticancer activity against various cell cells such as lung carcinoma, breast cancer cell lines and leaukemia cells through inhibition of tyrosin kinase, tubulin polymerazation, topoisomerase II inhibition (Solomon and Lee, 2011; Bingul *et al*., 2016). The quinoline scaffold plays an important role in anticancer drug development as their derivatives have shown excellent results through different mechanism of action such as growth inhibitors by cell cycle arrest, apoptosis, inhibition of angiogenesis, disruption of cell migration, and modulation of nuclear receptor responsiveness (Afzal *et al*., 2014). From fluorinated 2-phenyl-4-quinolone derivatives, it was noted that the ketone moiety was crucial for the activity with fluoro-6-pyrrol-2-phenyl-4-quinolone exhibiting potent cytotoxic activity (log GI_{50} < 8.00) against renal and melanoma tumor cell lines (Xia *et al*., 2001).

The standard control used in this study, camptothecin, is a cytotoxic quinoline alkaloid exerted its anticancer activity by binding to the topoisomerase I and DNA complex, resulting in a ternary complex, and thereby stabilizing it. This prevented DNA re-ligation and damaged the DNA, which resulted in apoptosis (Redinbo *et al*., 1998).

4.4 Anti-oxidant activity

The reactive oxygen species, which include superoxide anions ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH), and lipid peroxidation, which involves a series of free radical mediated chain reactions processes, are associated with several types of biological damage. Anti-oxidants are compounds that delay autoxidation by inhibiting free radical formation or by interrupting propagation of the free radical by one (or more) of several mechanisms: (1) scavenging species that initiate peroxidation, (2) chelating metal ions such that they (iron or copper) are unable to generate reactive species or decompose lipid peroxides, (3) quenching $O_2\bullet^-$ preventing formation of peroxides, (4) breaking the autoxidative chain reaction, and/or (5) reducing localized O_2 concentrations (Brewar, 2011). The most effective anti-oxidants are those that interrupt the free radical chain reaction. Usually containing aromatic or phenolic rings, these anti-oxidants donate H● to the free radicals formed during oxidation becoming a radical themselves (R- + ROO- → ROOR). These radical intermediates are stabilized by the resonance delocalization of the electron within the aromatic ring and formation of quinone structures (Brewar, 2011). Chelation of the bivalent transition metal ions of iron and copper effectively reduces oxidation, preventing catalyzes of oxidative processes,

formation of hydroxyl radicals, and decomposing hydroperoxides via Fenton reactions (Halliwell, 1987).

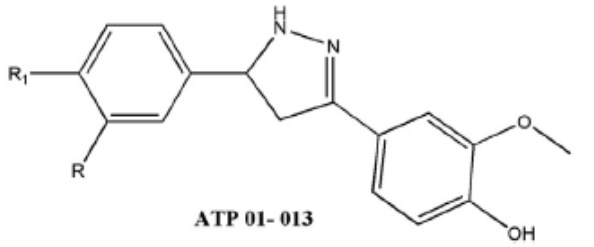
The anti-oxidant activity has contrasting benefits in the human host and the malaria parasite. Where in the human host, anti-oxidative properties of the test compounds can be beneficial to the sensitive membranes and enzymes in the host, especially with free iron in the infected patient (Zhang *et al.*, 2018). Whilst in the malaria parasite, anti-oxidants can protect the parasite from redox stress, which is an excellent pathway to kill the parasite via lipid peroxidation. Antimalarial drugs such as primaquine and artemisinin induce the formation of free radicals as their mechanism of action and the addition of anti-oxidants, such as ascorbic acid have been shown to eliminate the inhibitory properties of these drugs and compromised the rates of parasite clearance in *P. berghei* malaria infection in mice (Ganiyu e*t al.*, 2012). This is proposed to occur mostly in glutathione-deficient red cells, as often happens in parasitized red blood cells due to oxidative stress, where ascorbic acid reacts with iron or iron-containing compounds to generate hydrogen peroxide or hydroxyl radical and accentuate the haemolytic mechanisms in malaria (Li *et al*., 2006).

All components of the investigated quinoxaline hybrids have previously been reported to possess anti-oxidant activities with varying degrees of potency (Anto *et al*., 1995; Kumar *et al*., 2013; Haripriya *et al*., 2016). However, the hybrids in this study did not significantly inhibit oxidative pathways by scavenging free radicals or chelating redox active ferrous ions (Table 3.6 and 3.7).

4.4.1 Anti-oxidant activity of pyrazoline derivatives

The 3, 5-disubstituted-2-pyrazoline analogues (IC_{50} value: <40 µg/ml) have been reported to possess promising anti-oxidant activity when compared with ascorbic acid (IC_{50} value: <20 µg/ml) (Kumar *et al.*, 2013). A series of 3-[(4-hydroxy-3-methoxy) phenyl]-5-phenyl substituted phenyl-2-pyrazoline (ATP) derivatives (Figure 4.5) were found to possess anti-oxidant activity with ATP-1, ATP-2 and ATP-3 showing the most potential. With the 4-methyl derivative (ATP-2) showing the most activity to scavenge the DPPH free radical, nitric oxide, superoxide and hydrogen peroxide. On comparing the various derivatives, Kumar *et al.* (2013) proposed that the electron releasing group, -CH_3 at the *meta*-substituted group of 2-pyrazoline, was important for the inhibition of free radicals. This was observed with compound **QCP10** (4-CH_3; 15.10%) (Table 3.7). Whilst, electron withdrawing groups and high electron releasing

groups, such as methoxy (ATP-8), were not facilitators of free radical inhibition; as observed with **QPP9** (4-OCH_3; 6.90%) (Table 3.7). Similarly, it was found that electron withdrawing groups reduced the efficiency of hydrogen released from the amino group of pyrazoline of the ATP compounds. This was seen with all the pyrazoline compounds in the **QPP** and **QPQ** series, where only the 8-Cl substitution inhibited 19% free radical scavenging at 50µM; whilst the remaining bromine and fluorine derivatives were ineffective (Table 3.6 and 3.7). Such that if halogens were the substituents on the 3-phenyl of pyrazoline, anti-oxidant activity was greatly reduced revealing the inappropriateness of halogens in anti-oxidant compounds.



Compound	R	R1
ATP-1	H	H
ATP-2	H	CH_3
ATP-3	CH_3	H
ATP-4	H	Br
ATP-6	H	Cl
ATP-8	H	OCH_3

Figure 4.5: 3,5-Disubstituted-2-pyrazoline analogues reported to have promising anti-oxidant activity (Kumar *et al*., 2013).

4.4.2 Anti-oxidant activity of chalcones

The anti-oxidant potential of the chalcone derivatives was proposed to be related to its *(i)* hydrogen or electron donation capacity, *(ii)* its ability to stabilize and delocalize the unpaired electron, and *(iii)* potential to chelate transition metal ions. These actions were achieved either by the hydrogen atom or single electron transfer (Go, 2003; Ren *et al*., 2003). Generally, compounds with $–SCH_3$ and $–OCH_3$ in the *para* position of the ring A and –OH in the ring B were more active. But in this study, none of the quinoxaline-chalcone hybrids had anti-oxidant activity (Table 3.6 and 3.7). Several studies have shown that the anti-oxidant abilities of chalcones is mainly due to polyhydroxylation of the phenyl ring (Cheng *et al*., 1998; Ren *et al*., 2003; Grazul and Budzisz, 2009). However, none of the compounds with chalcone linker (**QCP** and **QCQ** compounds) contained hydroxyl- substitutions (Table 2.1 and 2.2).

The quinoxaline hybrids did possess some degree of iron chelating ability with compound **QPQ8** and **QPP6** the more active (36.13 ± 3.74 and 33.18 ± 1.38% chelation, respectively) (Table 3.6 and 3.7). Due to its high reactivity, Fe^{2+} is the most powerful pro-oxidant among the various species of transition metal ions. Iron can stimulate lipid peroxidation by the Fenton

reaction and accelerates peroxidation by decomposing lipid hydroperoxides in to peroxyl and alkoxyl radicals, that can themselves abstract hydrogen and propagate the chain reaction of lipid peroxidation (Halliwell, 1987). Isaac *et al*. (2012) reported that (2,4-dichloro-4'-methoxy chalcone) was found to be an efficient ferrous ion chelator with 76.0% iron chelated at 325 µM. It was proposed that the –CO group present in the synthesized compound may have chelated the ferrous ions, thus reducing the concentration of catalysing transition metal available for lipid peroxidation. Chelating agents are also an effective secondary anti-oxidants due to their ability to reduce the redox potential and thereby stabilizing the oxidized form of the metal ion. Potent inhibition of lipid peroxidation has been observed with dihydroxy chalcone, but not for those with -Cl and -O--CH, -O- substitutions. From the chalcones synthesised by Anto *et al*. (1995), the data inferred that substitution of electron donating groups at the *ortho*- or *para*- positions of the benzene ring could increase anti-oxidant activity of the chalcones, as well as improve their anticancer properties.

4.5 Insecticidal activity

The activity of the quinoxaline hybrids compounds was more directed towards the malaria parasite than the malaria vector as none of the compounds exhibited high ovicidal and larvicidal activity against *An. arabiensis* (KWAG) eggs and larvae in comparison to the relevant controls, formalin and DDT (Section 3.6.1 and Figure 3.9). Both eggs and larvae of *An. arabiensis* eggs seemed to have high tolerance to all test compounds over 72 hour exposure at 0.05 µM. In addition, the eggs also showed moderate tolerance to DDT with 72% hatching rate after 72 hours.

The embryo of *Anopheles* is surrounded by chorion and studies by Ramar *et al*. (2014) found that the ability of ovicides to act on embryo inside the egg shell was dependent on successful penetration through the chorion of the egg and this is influenced by the exposure time and dose of the compound. Therefore, from the results obtained, the compounds did not have the ability to penetrate through the eggshell to act on the embryo inside. The emerged new larvae also seemed to tolerate the compound and were able to swim normally like the untreated control and there was no notable morphological effect (Table 3.10). In contrast, while DDT did not seem to prevent hatching of the eggs, the emerged larvae died within 24 hours of hatching (Section 3.6.1).

It was observed that the morphological effect of DDT on *An. arabiensis* larvae (Table 3.10) and new larvae from treated eggs, was mainly between the head and the thorax, causing them to dissociate (Table 3.10). This could have been due damages in nerves. DDT is an organochlorine insecticide that exerts its mechanism of toxicity on sodium ion channels through prolonging the Na^+ conductance causing hyper-excitability of the nerve which eventually leads death (Van den Berg, 2009).

A similar effect was noted with compounds **QCP8** and **QCP10** (Table 3.10) where there were alterations of the head and thorax, possibly also indicating that these compounds might have acted on the nervous system. Compounds **QCP8** (23%) **QCP10** (18%), as well as **QCP9** (12%) exhibited low larvicidal activity in comparison to DDT, 100% after 72 hours. Kathrada (2017), found similar results where there was a lack of larvicidal activity (larvae mortality up to 5%) when 7-chloroquinoline derivatives were screened at 0.5 µM against 3rd instar *An. arabiensis* (KGB strain) larvae. The study suggested that the lack of larvicidal activity was due to structural differences between the test compounds and the current insecticides (Kathrada, 2017).

In contrast, chalcone-pyrazoline derivatives (1-(4-methyl cinnoline-3-yl)-3-(substituted phenyl-prop-2-en-1-one [cinnoline based chalcones] were found to possess insecticidal activity against *Periplaneta americanai* (cockroach). A 1% solution of the latter derivatives killed the insects within 6 to 15 minutes compared to 7 minutes for the standard, cypermethrin (Gautam and Chourasia, 2010). All the chloro derivatives of these chalcone-pyrazoline derivatives were found to possess similar insecticidal activity compared to the standard; whilst the bromo and methoxy derivatives showed moderate activity (10-12 minutes).

Chalcone derivatives which have been tested against mosquitoes' larvae of *Culex quinquefasciatus*, demonstrated that at a concentration of 100 ppm between 50 -100% mortality occurred (Das *et al.*, 2005). From a series of chalcone derivatives synthesised by Begum *et al*. (2011), the most potent against *Culex quinquefasciatus* with LC_{50} values of 5 mole.dm^{-3}, were a 4-chlorochalcone [(2E)-3-(1,3-benzodioxol-5-yl)-1-phenylprop-2-en-1-one (8)] and (2E)-3-(4-chlorophenyl)-1-phenylprop-2-en-1-one (11), with a methylenedioxy group at 3,4-position of ring B (Begum *et al*., 2011). Quantitative structure activity relationship analysis revealed that the presence of a hydroxyl group at 2'-position of ring A or replacement of ring B (phenyl) by furan ring in (2E)-3-(furan-2-yl)-1-(2-hydroxyphenyl)prop-2-en-1-one (17), also increased the larvicidal activity (LC_{50} value: 19 mole.dm^{-3}) (Begum *et al*., 2011). In addition, a 3-chlorine

substitution in ring B (compound 11) was also another feature of favourable activity (Begum *et al*., 2011). The presence of methylenedioxy group at 3 and 4 positions of ring B further enhanced the larvicidal activity of chalcone-type compound. However, extension of conjugation and blocking of α, β-unsaturated ketone part of chalcones diminished the activity of these compounds (Begum *et al*., 2011).

The naturally-occurring chalcones, lonchocarpin, derricin, and isocordoin, isolated from *Lonchocarpus neuroscapha*, showed deterrent activity against the larvae of *Spodoptera littoralis* and *S. exempta*, (crop pests) (Simmonds *et al*., 1990). Structure-activity relationship analysis revealed that the presence of two aryl rings separated by a three carbon linkage was necessary for activity. Among the chalcones, compounds possessing a 4'-methyloxy group was more active than derivatives with a 4'-hydroxy moiety (Simmonds *et al*., 1990). The effectiveness of chalcones as insecticides is enhanced by the presence of electron-withdrawing substituents in ring A and electron-withdrawing or electron-releasing substituents in ring B (Díaz-Tielas e*t al.*, 2016).

Chalcones have been widely investigated due to their insecticidal properties (Pasquale *et al*., 2012; Díaz-Tielas e*t al.*, 2016), and even though pyrazolines have reported insecticidal activity, there is limited research documenting the efficacy of this set of compounds (Shaaban *et al*., 2012). In a comparative study of a series of chalcones with two sets of pyrazoline derivatives (2-pyrazoline-1-carbothioamides and 2-pyrazoline-1-carboxamide), the chalcones (LC_{50} = 2.58 ppm) were found to be more effective as larvicidal agents than the 2-pyrazoline-1-carbothioamides (LC_{50} = 5.69 -15.14 ppm) against 1-day-old *Ae. aegypti* larvae. Similarly, the chalcones were found to have the highest biting deterrent activity against *Aedes aegypti*, but were similarly to the positive control, N, N-diethyl-3-methylbenzamide (DEET) (Koçyiğit-Kaymakçioğlu *et al.*, 2015). However, none of these compounds repelled *Ae. aegypti* even at doses 25-95 times higher than the reference standard, DEET.

All the quinoxaline-derivatives were found to be relatively safe against *A. franciscana* (Appendix F.1 and Appendix F.2), only **QPQ8** was found to be toxic with a LC_{50} of 1.05±1.12 µM compared to potassium dichromate (Figure 3.10). Synthetic chalcones with insecticidal properties have previously been reported to be toxic to freshwater fish, *Orechrois mossambicus*. Chalcones have been found to inhibit essential biochemical enzymes, such as glutathione S-transferase and mitochondrial monoamine oxidase (Tanaka *et al*., 1987; Asif, 2016).

CHAPTER FIVE -
CONCLUSIONS, FUTURE RESEARCH AND STUDY LIMITATIONS

The WHO global technical strategy for malaria has shifted from control of malaria to its elimination (WHO, 2018). However, the current interventions are not enough to achieve the set target to eliminate malarial by 2030. This has therefore, necessitated the search to identify novel active compounds with the potential to act as antimalarial and/or insecticidal agents. As such, eighteen novel quinoxaline-phenyl and seventeen quinoxaline-quinoline hybrids with either a chalcone or pyrazoline linker were successfully synthesized, purified and structures verified by NMR before being tested for their *in vitro* antimalarial and insecticidal activity, as well as preliminary toxicological profile.

The pharmacological properties of both sets of compounds were found to be more directed towards intra-erythrocytic stage of *P. falciparum* NF54 strain rather than the aquatic life stages of *An. arabiensis*. Twenty-four out of the thirty-five quinoxaline-based hybrids displayed antimalarial activity (Figure 3.2 and Figure 3.3), where the inhibitory effect appeared to be influenced by the nature of linker used, as well as the type and position of the substitution on either the phenyl or quinoline ring. Overall, the compounds with chalcone linker in quinoxaline-phenyl hybrid set (**QCP** compounds) and in quinoxaline-quinoline hybrid set (**QCQ** compounds) were on average 3.7 times more active than the compounds with pyrazoline linker (**QPP** and **QPQ** compounds). It has been shown that hybrids compounds with cyclic linkers can decrease antimalarial activity in comparison to linkers with a shorter chain length (Araujo *et al*., 2009; Mott *et al*., 2012; Raj *et al*., 2014). To elucidate the structure-activity relationship more clearly, it would have been advantageous to have simultaneously investigated the individual moieties of the hybrids to assess if the hybrids were more active and less toxic. These base moieties should be obtained along with the complexes/hybrids with future studies.

Regardless of the lower antimalarial activity of compounds **QCP7** and **QCP8** compared to quinine and DHA, these two compounds are worth further studying due to their good antimalarial activity and low toxicity against RBCs (0.1 ± 0.01%) and human epithelial kidney

cells (Table 3.3). Further structural modification could increase their antimalarial activities, while maintaining or improving their safety profiles. The introduction of more functional groups such as alkoxy or di-substitution of bromo and chloro groups on the phenyl ring could increase their antimalarial activity (Domínquez *et al*., 2001; Liu *et al.*, 2001). The oxidisation of nitrogens to obtain quinoxaline 1,4-di-N-oxide, could further increase their antimalarial activity (Zarranz *et al.*, 2005). The synergistic interaction with quinine showed the potential of these two compounds to be used in antimalarial combination therapy. In addition, the stage specific action of **QCP7** and **QCP8** at the ring and trophozoite stages indicated that these compounds possibly exerted their antimalarial action through the chalcone or quinoline moieties (Li *et al.*, 1996; Mishra *et al*., 2008). The potential mechanism of action of the compounds should be elucidated to verify if apoptotic or non-apoptotic pathways occurred during parasite death.

Furthermore, the drug-like properties and favourable percentage ionisation at pH 5 indicated that these compounds would accumulate in the parasite digestive vacuole resulting in parasite death. On the other hand, this could lead to toxic side effect within the human host in a similar to the standard antimalarial drugs, chloroquine and quinine (Müller, 2004; Kumar *et al*., 2007). Further experimentation on the mechanisms of action of the test compounds could be explored such as the effect of the compounds on β-haematin formation, folate pathway and parasite cysteine protease enzymes. The main limitation of the present study was the limited supply of compounds to further carry mechanistic studies.

In contrast to the sensitivity of *P. falciparum* NF54 strain to the test compounds, the eggs and larvae of *An. arabiensis* eggs showed high tolerance to compounds over 72 hours of exposure with minimal ovicidal activity (unhatched eggs: 0-14%) and low larvicidal activity (mortality range 1-20%) (Figure 3.9). The low ovicidal and larvicidal on *An. arabiensis* suggested that these compounds should not be investigated further as insecticide agent despite minimal toxicity against the aquatic *Artemia*. Only **QCP2** was found to be highly toxic against *Artemia* (IC_{50} value: 1.05 ± 0.78 µM) (Figure 3.10), but exhibited 11% mortality against *An. arabiensis*, which indicated that **QCP2** was toxic towards non-target organisms and therefore can be harmful to the aquatic species.

The compounds with a chalcone linker (**QCP** and **QCQ** compounds) exhibited promising anti-leukaemic activity, with compounds **QCP6, QCP7**, **QCP8, QCP9** and **QCP10** displaying selectivity towards the chronic myelogenous K562 leukaemia cells.

However, some of the compounds displayed notable cytotoxicity against human epithelial kidney cells (Figure 3.6 and 3.7), which is of concern if taken together with the *in silico* predictions for a high risk of toxicity. This should be taken into consideration along with the structure-activity relationship shown in this study to produce an improved second generation of compounds. This study has provided five compounds; **QCP6, QCP7**, **QCP8, QCP9** and **QCP10** to be used as templates for further derivatization for novel antimalarial and anti-leukaemic compounds.

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APPENDICES

Appendix A: Human ethics waiver for the purchase and use of blood (W-CBP-180420-2)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL

Human Research Ethics Committee (Medical)

Research Office Secretariat:
Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Office 301/2/4, 29 Princess of Wales Terrace, Parktown, 2193
Tel +27 (0)11-717-1252 /1234/2658/2700
Private Bag 3, Wits 2050
Office email: HREC-Medical.ResearchOffice@wits.ac.za
Website: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Ref: W-CBP-180420-2 20/04/2018

TO WHOM IT MAY CONCERN:

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

Investigator: Miss Teboho Malimabe (student no. 1525675) et al

Supervisor: Prof RL van Zyl

Department: Pharmacology

Project title: The effect of novel quinoxaline derivatives on the life cycle of malaria parasite and vector

Reason: Laboratory analysis. Will use blood to grow the malaria parasite *in vitro*, assess the antimalarial effect of novel compounds and for red blood cell cytotoxicity assays. No human participants will be involved in the study.

Professor CB Penny
Chairperson: Human Research Ethics Committee (Medical)

UNIVERSITY OF THE WITWATERSRAND
OFFICE OF THE DEPUTY VICE CHANCELLOR RESEARCH
20 APR 2018
RESEARCH OFFICE HREC (Medical)
JOHANNESBURG

Copy – HREC (Medical) Secretariat: Zanele Ndlovu and Rhulani Mkansi.

Appendix B: Biosafety waiver for the culturing of malaria parasite (20180102)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE (IBC)
(R 14/16)

CLEARANCE CERTIFICATE **PROTOCOL NUMBER: 20180102**

BRIEF DESCRIPTION OF APPLICATION:

The effect of novel quinozaline derivatives on the life cycle of malaria parasite and vector

APPLICANT: Miss T Malimbe

SUPERVISOR: Prof RL van Zyl

SCHOOL/DEPARTMENT : Pharmacy and Pharmacology

DATE CONSIDERED: 29 January 2018

EXPIRY DATE: 12 March 2022

DECISION OF COMMITTEE:

1. This clearance certificate expires on 12 March 2022 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
4. Unreported changes to the application may invalidate the clearance given by the IBC

DATE: 12 March 2018

CHAIRPERSON: (Professor J Larkin)

Radiation and Health Physics Unit
University of Witwatersrand
Johannesburg, Republic of South Africa
Tel: +27 (0) 11 717 6301

DECLARATION OF APPLICANT:

To be completed in duplicate and **one copy** returned to the Research Office, Philip Tobias Building, Room 301, 3rd Floor, 29 Princess of Wales Terrace, Parktown, Faculty of Health Sciences, 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:** _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix C: Human ethics waiver for the use of human cell lines (W-CJ-170410-1)

Human Research Ethics Committee (Medical) 50 years 1966 – 2016

Research Office Secretariat: Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Office 301, 29 Princess of Wales Terrace, Parktown, 2193 Tel +27 (0)11-717-1252 / 1234/2656/2700
Private Bag 3, Wits 2050, email: zanele.ndlovu@wits.ac.za
Office email: hrec-medical.researchoffice@wits.ac.za
Website: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Ref: W-CJ-170410-1 10/04/2017

TO WHOM IT MAY CONCERN:

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

Investigators: Prof R van Zyl, Ms Teboho Malimabe (student no 1525675).

Project title: The effect of novel quinoxaline derivatives on the life cycle of the malaria parasite.

Reason: This is an *in vitro* laboratory study using cell lines including Graham, CaCo2, HT-29 K562, SH-SY5Y. There are no human participants.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2017 -04- 10
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkansi, Lebo Moeng

Appendix D.1: Ethics waiver for use of *Anopheles* mosquito eggs and larvae (07-11-2017-0)

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

ANIMAL RESEARCH ETHICS COMMITTEE (AREC)

Registration number: AREC0101210-002

Solomon Mahlangu House
10th Floor, Room 10004
Jorissen Street
Braamfontein, Johannesburg

7 November 2017

To whom it may concern,

Re: **Waiver from Animal Ethics Screening Committee of the University of the Witwatersrand**

This letter serves to confirm that Associate Professor Robyn van Zyl (Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, University of the Witwatersrand) does not require full animal ethics clearance for her studies which use *Anopheles* mosquito species to evaluate known or novel synthetic and natural compounds as potential insecticides against *Anopheles* species.

Reasons: Mosquito eggs and larva are used in the study. No animals or animal products are used in the study.

Conditions: All approved biosafety and biosecurity SOPs should be adhered to.

Should you require any further information, do not hesitate to contact me.

Yours sincerely,

Kennedy Erlwanger

(Chairman: Animal Research Ethics Committee, University of the Witwatersrand)

Reference: R van Zyl –Anopheles Waiver 07-11-2017-O

Assoc Prof Kennedy H. Erlwanger
School of Physiology
Faculty of Health Sciences, University of the Witwatersrand
7 York Road, Parktown, 2193
SOUTH AFRICA
Tel: +27 (0)11 717 2454
Email: Kennedy.Erlwanger@wits.ac.za

Appendix D.2: Approval letter for use of *Anopheles* and *Artemia* for research purposes

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

ANIMAL RESEARCH ETHICS COMMITTEE (AREC)

Registration number: AREC0101210-002

Solomon Mahlangu House
10th Floor, Room 10004
Jorissen Street
Braamfontein, Johannesburg

24 November 2017

To whom it may concern,

Re: Professor R van Zyl Waiver (for MSc students) from Animal Research Ethics Committee of the University of the Witwatersrand

This letter serves to confirm that Associate Professor Robyn van Zyl (Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, University of the Witwatersrand) does not require full animal ethics clearance for her studies undertaken by the students detailed below. She has previously been granted a waiver for the studies as detailed in the documents referenced *R van Zyl (Artemia) 07-11-2017-O and R van Zyl-Anopheles waiver 07-11-2017-O*. The students and studies covered by the waiver letters:

1. Ms Fatima Kathrada [0602240N]: The effect of 7-chloroquinoline derivatives on the life cycle of the malaria parasite.

2. Mr Sahil Lala [358351]: The effect of quinolone heterocyclic derivatives on the malaria parasite and Anopheles vector.

3. Ms Teboho Malimabe [152675]: The effect of novel quinoxaline derivatives on the life cycle of malaria parasite and vector.

4. Ms Natasha Jansen van Vuuren [071867W]: The effect of novel compounds on copper homeostasis in *Plasmodium falciparum* and neuroblastoma cells.

5. Mr Thulani Mahlangu [667999]: The chemotherapeutic properties of novel quinazolines and bis-hydrazones compounds.

6. Ms Puleng Thabana [609529: The effect of novel oxo-1, 6 dihydropyrimidine and 2-(quinolone-8-yloyxy) acetohydrazone derivatives on the life cycle of the malaria parasite.

7. Ms W Ramashia [572354]: Transcription analysis of an immune factor in a main Africa malaria vector *Anopheles funestus*.

Conditions: All previously requisite biosafety and biosecurity SOPs should be adhered to.

Should you require any further information, do not hesitate to contact me.

Yours sincerely,



Kennedy Erlwanger

(Chairman: Animal Research Ethics Committee, University of the Witwatersrand)

Reference: R van Zyl –Anopheles & Artemia PGrad students Waiver 07-11-2017-O

Appendix E: Ethics waiver for the use of *Artemia* eggs and nauplii (07-11-2017-0)

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

ANIMAL RESEARCH ETHICS COMMITTEE (AREC)

Registration number: AREC0101210-002

Solomon Mahlangu House
10th Floor, Room 10004
Jorissen Street
Braamfontein, Johannesburg

7 November 2017

To whom it may concern,

Re: **Waiver from Animal Ethics Screening Committee of the University of the Witwatersrand**

This letter serves to confirm that Associate Professor Robyn van Zyl (Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, University of the Witwatersrand) does not require full animal ethics clearance for her studies which use *Artemia* (Brine shrimp) as a toxicological model to evaluate known or novel synthetic and natural compounds.

Reasons: *Artemia* eggs and *nauplii* are used in the study. No animals or animal products are used in the study.

Conditions: All approved biosafety and biosecurity SOPs should be adhered to.

Should you require any further information, do not hesitate to contact me.

Yours sincerely,

Kennedy Erlwanger

(Chairman: Animal Research Ethics Committee, University of the Witwatersrand)

Reference: R van Zyl Waiver (Artemia) 07-11-2017-O

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Appendix F: Percentage mortality of *A. franciscana* nauplii

Table F.1: Percentage mortality of *A. franciscana* nauplii after 24, 48 and 72 hours treatment with quinoxaline-phenyl hybrids with chalcone linker (**QCP** compounds) and pyrazoline linker (**QPP** compounds) in comparison to control (n = 3).

	Nauplii mortality (%)		
Compound code	**24 hours**	**48 hours**	**72 hours**
QCP1	0.00	2.52	5.66
QCP2	0.35	2.83	5.30
QCP3	0.00	1.13	3.02
QCP5	0.00	2.48	6.20
QCP6	0.69	1.74	2.78
QCP7	0.00	2.48	5.32
QCP8	0.00	0.00	4.84
QCP9	0.00	0.00	5.13
QCP10	0.00	0.00	5.38
QPP1	0.32	13.10	13.10
QPP2	1.07	6.41	7.47
QPP3	0.00	1.82	3.34
QPP5	0.00	0.00	0.00
QPP6	0.00	0.00	0.00
QPP7	0.00	0.00	4.75
QPP8	0.00	0.00	2.58
QPP9	0.00	0.00	5.43
QPP10	0.00	3.91	5.69
Potassium dichromate	0.00	72.52	100.00

Table F.2: Percentage mortality of *A. franciscana* nauplii after 24, 48 and 72 hours treatment with quinoxaline-quinoline hybrids with chalcone linker (**QCQ** compounds) and pyrazoline linker (**QPQ** compounds) in comparison to control (n = 3).

	Nauplii mortality (%)		
Compound code	**24 hours**	**48 hours**	**72 hours**
QPQ1	0.00	2.28	3.36
QCQ2	0.00	0.00	2.26
QCQ3	0.00	0.00	2.12
QCQ5	0.00	0.00	0.00
QCQ6	0.00	0.00	0.00
QCQ7	0.00	0.00	0.00
QCQ8	0.00	0.00	0.00
QCQ9	0.00	0.00	0.00
QCQ10	0.00	0.00	0.00
QPQ1	0.00	0.00	0.00
QPQ2	0.00	70.19	100.00
QPQ3	0.00	11.85	24.01
QPQ5	0.00	30.49	48.48
QPQ6	0.00	4.27	11.74
QPQ7	0.00	0.00	4.03
QPQ8	0.00	5.86	13.27
QPQ10	0.00	4.55	9.44
Potassium dichromate	0.00	72.52	100.00