The Effect of Novel Azoles, Pyrimidone Analogues And Acetohydrazone Derivatives On The Life Cycle Of The Malaria Parasite

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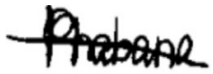
Declaration

I, Puleng Agnes Thabana, 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.



Puleng Thabana

Signed at.....Johannesburg..... on the ...23rd..... day of .....October.....2024.

Dedication

To my amazing and loving parents, Mpho and Andrew Thabana, thank you for always being there for me. I am forever grateful.

To my brother, Mahloko Thabana, thank you for believing in me.

Publications and Congress Attendance

Publications:

- Sabahat Samreen, Jyoti Gupta, Puleng A. Thabana, Priyanka Agarwal, Robyn L. van Zyl, Afreen Inam. (2024) Synthesis, Antiprotozoal activity, Molecular Docking and ADME study of 2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N phenylacetamide derivatives. African Journal of Biological Sciences, 6(15):7. https://doi.org/10.48047/AFJBS.6.15.2024.6660-6689. Accepted).
- Afreen Inam, Puleng A. Thabana, Robyn L. van Zyl, Jyoti Gupta, Amir Azam., Synthesis and antiprotozoal evaluation of indole-acetamide based Quinoline conjugates. Rasayan Journal of Chemistry and Asian Journal of chemistry (Submitted).

Congress attendance:

- Puleng Thabana, Nelusha Shunmoogam-Gounden, Amir Azam, Robyn L. van Zyl. Poster presentation: 'Antimalarial and toxicological properties of 6-oxo-1,6 dipyrimidine and 2-(quinolin-8-yloxy)acetohydrazone derivatives' . 2nd Malaria Research Conference, 1-3 August 2016, Pretoria, South Africa

- Puleng Thabana, Shirveen Sewpersad, Neil Koorbanally, Maureen Coetzee, Robyn L. van Zyl Poster presentation: 'Antiplasmodial and toxicological properties of benzimidazoles and imidazole derivatives'. All Africa Congress, 5-8 October 2016, Muldersdrift, South Africa

Abstract

Malaria is a life-threatening disease caused by the *Plasmodium* protozoa; where *P. falciparum* is one of the most common and deadliest species responsible for malaria infections in humans. The goal to eradicate malaria is threatened by parasite resistance to antimalarial drugs and *Anopheles* vector resistance to insecticides. As a result, new and safe antimalarials and insecticides are needed. This study aimed to evaluate the effects of three sets of novel compounds, quinoline-2-benzimidazole (**QB1**-**QB12**), 6-oxo-1,6 dihydropyrimidine (**P1**-**P10**) and 2-(quinolin-8-yloxy)acetohydrazone (**QC1**-**QC11**), as well as known benzimidazoles and imidazoles against *P. falciparum* and *Anopheles* life cycles. Five azoles displayed promising activity against the *P. falciparum* NF54 strain using the *Plasmodium* lactate dehydrogenase assay, where the IC_{50} values ranged between 4.90 ± 0.41 and 77.81 ± 0.71 µM, in comparison to quinine (0.18 ± 0.01 µM). The two most active compounds (tioconazole and itraconazole) displayed antagonistic and additive interactions when combined with quinine, respectively. In comparison to quinine-treated parasites, haemozoin in the trophozoites of both tioconazole-treated and itraconazole-treated parasites appeared more scattered rather than compact units. None of the tested compounds displayed toxicity against the human epithelial cells (HEK-293) as determined by the tetrazolium viability assay and negligible haemolysis was displayed. Toxicity against *Artemia franciscana* after 72 hours of treatment was observed for compounds **QB1**, **QB5**, **QB9**, **QB11**, **P3,** mebendazole, albendazole, ketoconazole, tioconazole, clotrimazole and itraconazole. Unfortunately, none of the tested compounds showed potential as larvicidal or ovicidal agents against *Anopheles arabiensis* (KWAG) compared to formalin and DDT. This study highlighted the potential repurposing of known azole compounds against malaria *P. falciparum* NF54 strain and warrants further investigation.

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AA	Ascorbic acid
ACT	Artemisinin-based combination therapy
APAD	3-Acetylpyridine adenine dinucleotide
CDC	Centers for Disease Control and Prevention
°C	Degree Celsius
CNS	Central nervous system
cytb	Cytochrome b
DDT	Dichlorodiphenyltrichloroethane
DHA	Dihydroartemisinin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
$DPPH^{\bullet}$	2,2-Diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
∑FIC	Sum of Fractional inhibitory concentration
FBS	Fetal bovine serum
g	Gram
g	Gravitational constant/ G-force
g/mol	Grams per mole
GIT	Gastrointestinal tract
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
HEPES	N-2-Hydroxyelthlypiperazine-N'-2-ethane-sulfonic acid
HIV	Human immunodeficiency virus

IC_{50}	Half maximal inhibitory concentration
IRS	Indoor residual spraying
ITN	Insecticides treated net
L	Litre
LC50	Lethal concentration required to kill 50% larvae/nauplii
LDH	Lactate dehydrogenase
LLINs	Long-lasting insecticidal nets
mg	Milligram
mL	Millilitre
mM	Millimolar
µL	Microlitre
µM	Micromolar
M	Molar
mol	Mole
MTT	3-(4, 5-dimethyl-2-thiazol-2-yl)-2, 5- diphenyl-2H-tetrazolium bromide
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide
NBT	Nitro blue tetrazolium
ND	Not determined
%	Percentage
PBS	Phosphate buffered saline
PES	Phenazine ethosulfate
pH	Potential of hydrogen
pLDH	Parasite lactate dehydrogenase
RBC	Red blood cell
RDT	Rapid diagnostic test
ROS	Reactive oxygen species

rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institution number 1640
RNA	Ribonucleic acid
SA-DOH	South Africa Department of Health
s.d.	Standard deviation
TB	Tuberculosis
TRIS	Trisaminomethane
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization

Chapter 1: Introduction

1.1 Malaria – The disease

Malaria is a global infectious disease caused by the *Plasmodium* protozoan parasite. *Plasmodium vivax*, *P. malariae*, *P. ovale*, *P. knowlesi* and *P. falciparum* are the five species that infect humans and in Sub-Saharan Africa, more than 90% of the malarial infections are due to *P. falciparum* (South African National Department of Health (SA-DOH), 2019). According to the World Health Organization (WHO) World Malaria Report 2023, most countries in Sub-Saharan Africa with moderate to high transmission contribute to the malaria global burden due to lack of or weak surveillance and healthcare systems (WHO, 2023). In 2021, 247 million malaria cases were estimated globally, equating to a 2 million case increase in comparison to 2020. This surge was first seen between 2019 and 2020, with an increase of 13 million cases globally. In 2020, the mortality rate increased, before slightly decreasing in 2021 (from 15.1 to 14.8 deaths per 100 000 population) (WHO, 2022). In children under 5 years of age, the percentage of total malaria death decreased from 87% in 2000 to 76% in 2020, and has remained the same over the years (WHO, 2022). In addition to the transmission of malaria by the *Anopheles* vector, malaria can spread through infected blood products (transfusion exposure), needle sharing and by congenital transmission (Trampuz *et al.,* 2003). It is an acute illness with the ability to progress to severe disease and death, especially if the population is non-immune, diagnosis is not done early, and treatment not started promptly (CDC, 2019).

There have been major reductions in malaria transmission and infection rates in Africa in the past years due to investments towards malaria control across the continent and artemisinin-based combination therapy (ACT) (Hemingway *et al.,* 2016). Insecticides-treated nets (ITNs) are said to be the main vector control method in Sub-Saharan Africa (WHO, 2022). Access to ITNs and the ability to use them is one of the strategies that can be capitalised upon in malaria control. Olapeju *et al.* (2018) showed that the more access people have to ITNs, the more they are used (Olapeju *et al.,* 2018). In addition, it was also observed that the number of females and males using accessible ITNs is comparable, whereas more females compared to males use ITNs in areas where they are not

accessible. In 2021, 200 million ITNs were distributed globally in malaria endemic countries, and 176 million of these were distributed in Sub-Saharan Africa (Democratic Republic of Congo, Côte d'Ivoire, Kenya, Ghana and Nigeria) (WHO, 2022).

Malaria continues to be a major concern and contributor to women and children's deaths that could be avoided (WHO, 2023). Children between the ages of 1 month to 5 years of age are at a greater risk of being infected in tropical countries (Trampuz *et al.,* 2003). *P. falciparum* accounts for the main burden of malaria in pregnant women due to prevalence and other *Plasmodium* species do not parasitise the placental blood to the same extent (Schlagenhauf and Petersen, 2008; WHO, 2022). Pregnant women face a greater risk of this disease due to their reduced immunity and if not treated properly, can lead to maternal deaths, underweight babies, miscarriages and stillbirths (Asante and Asenso-Okyere, 2003; WHO, 2022). Furthermore, it has been estimated that 2% of children who recover from cerebral malaria suffer from neurological disorders including epilepsy (Asante and Asenso-Okyere, 2003). There has been an increase in malaria exposure during pregnancy over the years with 13.3 million estimated pregnant women exposure to malaria in the WHO African Region in 2021 (WHO, 2022).

1.2 Malaria endemic areas in Africa and South Africa

In terms of social and economic aspects, malaria has an intimate relationship and detrimental effect on economic development perpetuating poverty on the African continent (Maharaj *et al.,* 2013). In South Africa, malaria is endemic to the north-eastern part of the country, mainly in the low-altitude areas of Limpopo, Kwa-Zulu Natal and Mpumalanga (Figure 1.1) and is mostly seasonal, with the high risk of transmission in the wet summer months (South African DOH, 2019).

1.3 Malaria life cycle

The life cycle of the malarial parasite is complex and involves the *Anopheles* mosquito and human host (Figure 1.2 (1-9)) (CDC, 2019). The liver stage is initiated by the injection of *Plasmodium* sporozoites by an infected female mosquito into the human host (1), invading hepatocytes in which they differentiate into schizonts (2), that divide into thousands of hepatic merozoites (3) (CDC, 2019).

These merozoites enter the blood stream, where they initiate the erythrocytic cycle (4). This cycle begins with the infection of the red blood cells (RBCs) by hepatic merozoites, which develop into rings, thereafter trophozoites and finally schizonts. The infected RBC lyses to release daughter merozoites that initiate a new cycle. Some of the parasites develop into gametocytes (sexual cycle (5)), which are responsible for parasite transmission to the next feeding mosquito to continue the cycle (6). The life cycle of *P. falciparum* within its human host is approximately 48 hours (24 hours in *P*. *knowlesi* and 72 hours in *P*. *malariae*) (CDC, 2019).

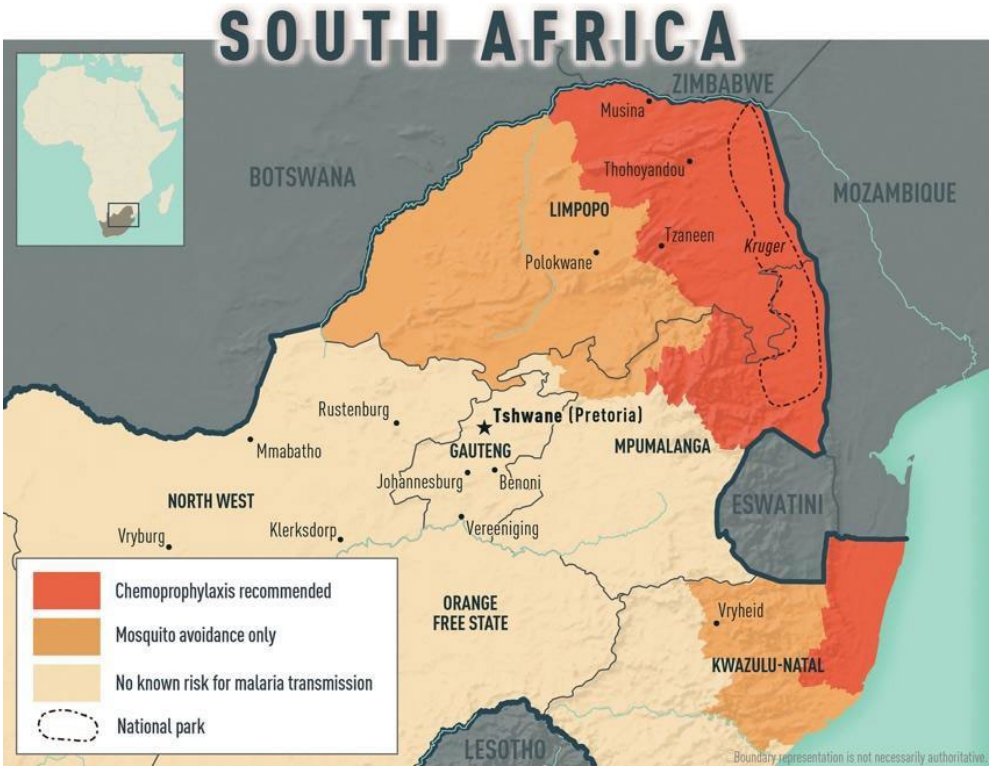


Figure 1.1: South African Malaria Risk Map (CDC, 2023).

The male (microgametocytes) and female gametocytes (macrogametocytes) are ingested by the *Anopheles* mosquito during a blood meal and while in the mosquito's stomach, the microgametocytes fuse with the macrogametocytes to produce zygotes (6 and 7). The zygotes in turn become ookinetes, which invade the midgut wall of the mosquito and develop into oocytes (8). The oocytes then release sporozoites (9) which travel to the mosquito's salivary glands ready for

injection into the next host during the blood meal to continue the parasites' life cycle (1) (CDC, 2019) (Figure 1.2).

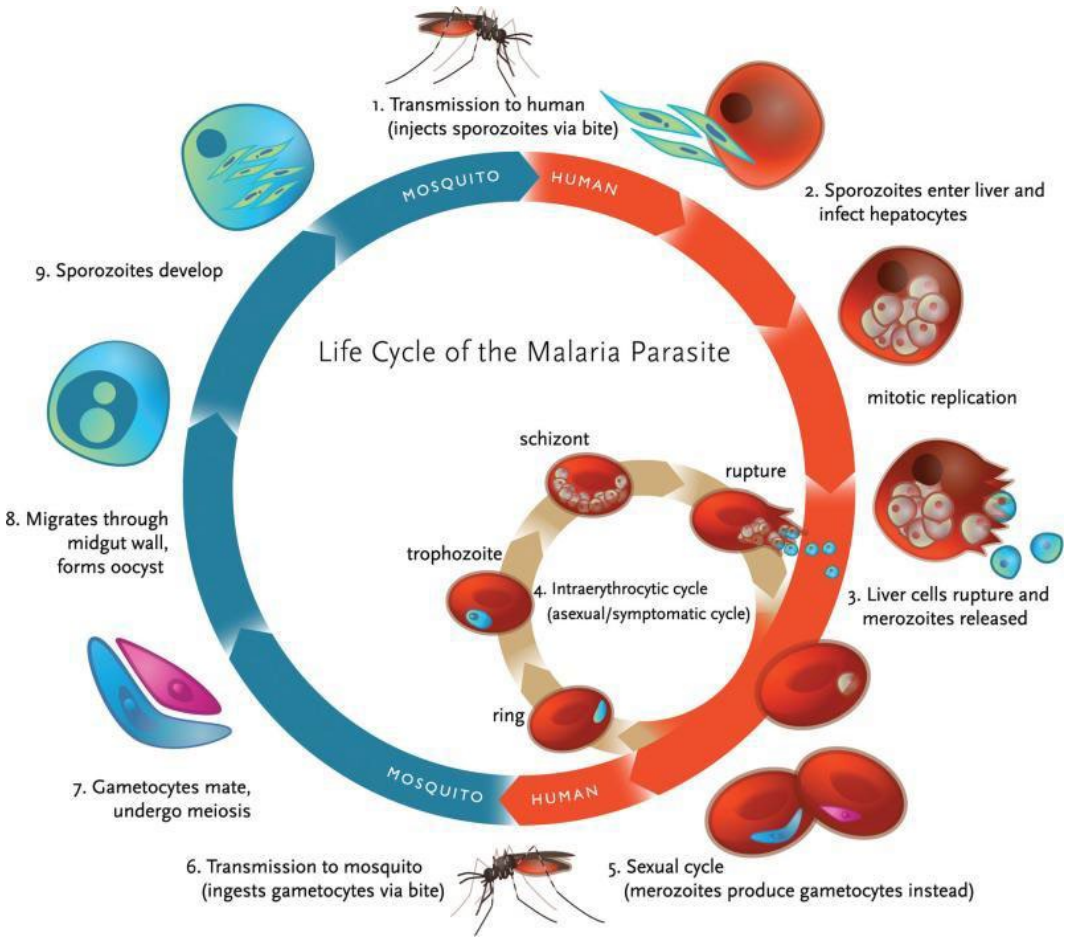


Figure 1.2: The life cycle of malaria parasite showing the transmission (1-3), asexual (4) and sexual (5) intra-erythrocytic and mosquito (6-9) stages (Klein, 2013).

1.4 Symptoms of malaria

The asexual blood stages are responsible for the pathogenesis of malaria with the intensity of the clinical manifestations of malaria depending on the previous immune status of the host, inoculum size or parasitaemia (CDC, 2023). The first symptoms of malaria are nonspecific and include headache, fatigue, abdominal discomfort, muscle and joint aches, which are usually followed by perspiration, chills, fever, anorexia, vomiting and worsening malaise (WHO, 2018). Without prompt and effective treatment, malaria can rapidly progress to severe malaria with potentially fatal complications. Complications associated with severe malaria normally co-exist and include severe

organ failure, with the parasite accumulating in the brain capillaries, leading to coma and death (South African DOH, 2019). Hypoglycaemia and metabolic acidosis may also manifest as systemic complications, especially in pregnant women (Trampuz *et al.,* 2003; South African DOH, 2019).

1.5 The malarial vector

1.5.1 Invertebrate *Plasmodium* host

Mosquitoes are invertebrate arthropods and are classified into the family *Culicidae*, with approximately 3,500 species grouped into 41 genera belonging to this family (Xia *et al.,* 2018). For successful transmission of the parasite from person to person the main pathway is through the *Anopheles* vector, and it is a critical step in the life cycle of the *Plasmodium* parasite (Figure 1.2; 1,6-9). Unlike the human host, the vector does not suffer from any harmful or fatal effects due to the malarial infection (CDC, 2019).

1.5.2 Distribution of vector species

Anopheles gambiae complex and *An. funestus* group are the main *Anopheles species* found in South Africa (Dahan-Moss *et al.,* 2022). *An. arabiensis* which belongs to the *An. gambiae* complex is present in all three affected South African provinces and is found mainly in dry, savannah environments with sunlit, temporary, fresh-water pools as breeding sites (Brooke *et al.,* 2013) and adult *An. arabiensis* rests both outdoors and indoors. They cannot survive at temperatures below 10°C and above 40°C, and water is important for the larval stage, accounting for increased transmission during the warm, wet season (South African National Department of Health, 2018). Generally, most female mosquitoes need at least one blood meal before laying fertilized eggs, allowing them to transfer protozoan or viral infection to their vertebrate hosts (Nikbakhtzadeh *et al.,* 2016). *Anopheles* mosquitoes tend to feed close to ground level and are selective to the lower legs rather than upper body or arms (South African National Department of Health, 2018).

1.5.3 Life cycle of *Anopheles*

The *Anopheles* mosquito has four stages to its life cycle, namely egg, larva, pupa and adult (Childs *et al.,* 2016) (Figure 1.3). Eggs are laid horizontally and singly on the water surface by adult female mosquitoes (1) and hatch into larvae within 48 hours (2). The developing larvae feed on microorganisms and organic matter in water, and eventually develop into pupae during the fourth instar (3). Pupae are known to be non-feeding, but mobile and responsive to light changes. The pupae then develop into adult mosquitoes (4), which rest on the water surface to allow the body parts to harden before flying (CDC, 2023). During the egg production, mature females feed on blood, and lay a batch of eggs prior to taking the next blood meal. Eggs are laid on water in batches of 50-200, and the time taken for eggs to hatch into larvae depends largely on temperature. At temperatures of 30°C, eggs hatch in 2-3 days and at temperatures of 16°C in 7-14 days. The larvae use 'mouth brushes' found on their heads for feeding and have four developmental stages known as instars. In normal tropical temperatures, the larva takes 5-10 days to develop into pupa depending on the species. The pupa is shaped like a comma, does not feed on anything but undergoes radical metamorphosis, normally stays at the water surface and this stage lasts for 2-5 days (CDC, 2023). The pupa then develops into an adult which consists of the head, thorax and abdominal segments. Both male and female *Anopheles* mosquitos feed on nectar and other sources of sugar for energy (CDC, 2023).

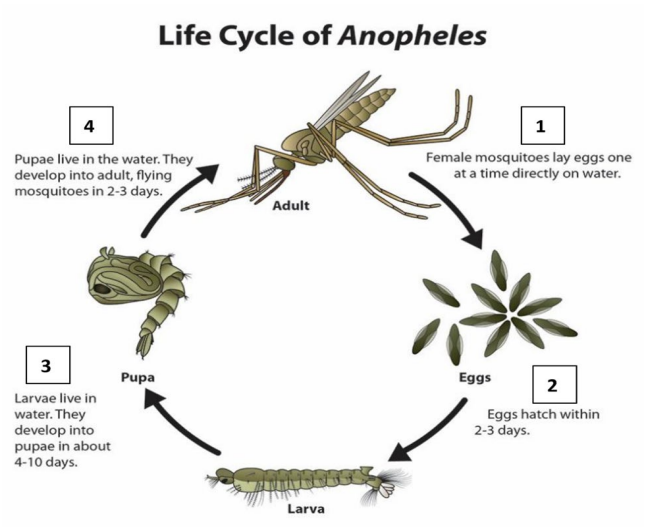


Figure 1.3: Life cycle of *Anopheles* mosquito (CDC, 2023).

1.6 Malaria elimination

The main goal of all interventions targeting malaria is malaria eradication. From 2000 to 2021, the number of countries endemic to malaria was reduced from 108 to 84 countries (WHO, 2022). Malaria elimination requires strategies that include a combination of vector control, development of new antimalarials, insecticides and vaccines. Studies show that the malaria reduction in Africa has been attributed to using various combination of these control strategies (WHO, 2023). To support the intense efforts directed at controlling *Anopheles* mosquito vectors through the development of insecticide-impregnated bed nets and indoor residual spraying (IRS) of insecticides, there is a need for the parallel developmental efforts to ensure elimination of the malaria parasite (Cui *et al.,* 2015).

The challenge facing malaria in the move from control to elimination, is the development of resistance to the artemisinin drug class as well as insecticides. To overcome these challenges, is to implement active surveillance programmes, use sensitive diagnostic tests to detect very low parasitaemia, improve communication with health care professionals and monitor parasite drug and vector resistance (Blumberg *et al.,* 2014). One other aspect that needs to be taken into consideration when targeting elimination, is the fact that malaria can be an asymptomatic infection in endemic areas among semi-immune populations who are more likely to be untreated and carry the parasite, thereby increasing the risk of transmission (Gosling *et al.,* 2011). As much as elimination is possible, it is critical that various strategic efforts need to be maintained in South Africa and neighbouring countries before the artemisinin-based combinations fails.

In the efforts to control and eradicate malaria, targeting gametocytes has become a possible strategy (D'Alessandro *et al.,* 2013) and therefore drugs inhibiting gametocyte development are needed. From 2020 in the WHO African Region, countries such as Ghana, Rwanda, Gambia, Ethiopia, South Africa and Zimbabwe were either classified as malaria free or on track to meet the eradication plan by 2025 (WHO, 2022). Although countries such as Mozambique, Senegal, Kenya, Togo and Tanzania are not on track with malaria elimination, they have made progress in the reduction of malaria transmission. Financial constraints also play a significant role in malaria elimination, as well as ensuring sustainable funding to maintain the *status quo* even after elimination. This further

emphasises the urgent need for alternative, cost-effective and potent drugs with the potential to target both asexual and sexual stages of malaria. Thereby, improving symptoms, preventing relapse due to hepatic stages and recrudescent parasites, and importantly provide protection by inhibiting the transmission of the sexual stages (Biamonte *et al.,* 2013). To align with elimination initiatives by Botswana, Eswatini and Namibia, the National Department of Health in South Africa had a malaria elimination strategic plan from 2019 to 2023, as well as a target of zero local malaria cases and malaria deaths by 2030 (South African DOH, 2019).

1.7 Diagnosis

To effectively control the disease and give proper treatment to any patient, proper diagnosis is of utmost importance. Diagnosis of malaria is complicated due to the non-specific presenting symptoms and delayed diagnosis can lead to poor outcomes (Tangpukdee *et al.,* 2009). In diagnosing malaria, microscopic examination of Giesma-stained blood films is still the most used technique (Blumberg *et al.,* 2014) and this technique relies on laboratory personnel training, experience and quality of the blood smear (Trampuz *et al.,* 2003), making proper personnel training an important aspect of the diagnosis. One other technique that can be used is the malaria rapid diagnostic test which is simple and allows for rapid treatment. However, this technique is limited by specificity, sensitivity, cannot quantify parasite density and is more sensitive for falciparum than non-falciparum species (Blumberg *et al.,* 2014). Proper diagnostics prevent unnecessary treatments and free up resources so that health care professionals can focus on other health issues.

1.8 Management of malaria in South Africa

The South African Department of Health has put in place measures to manage malaria, and these include non-pharmacological vector control measures, use of repellents, chemoprophylaxis and treatment of malaria, which is the general order of trying to prevent an infection (South African DOH, , 2019).

1.8.1 Malaria vector control

The use of insecticides for indoor residual spraying and larviciding remains important in malaria vector control and more research must be done to ensure their continued success. Over the years, two methods have been shown to be effective in malaria vector control, namely IRS with insecticides to potentially kill the mosquito before the parasite develops into an infective stage and using ITNs for protection against mosquitoes as per WHO recommendations (WHO, 2022).

1.8.1.1 Indoor residual spraying and insecticide-treated nets

The advantage of controlling malaria by targeting the vectors allows for the reduction of infections and drug-related side effects, contraindications and resistance associated with antimalarial drugs used in the management of malaria. There are four classes of insecticides used in vector control: organophosphates (e.g., malathion), carbamates (e.g., bendiocarb), organochlorines (e.g., dichlorodiphenyltrichloroethane (DDT)) and pyrethroids (e.g., permethrin) (WHO, 2022). Organophosphates and carbamates act by inhibiting acetylcholinesterase (AChE1), an enzyme responsible for the hydrolysis of acetylcholine in the central nervous system which leads to its accumulation, impairing the nerve function and ultimately leading to the insect's death. (Lee and Barron, 2016). Organochlorines act by preventing the activation of the sodium-gated channels, which causes the insect to go into spasms and eventually die (Coats, 1990). Currently, only pyrethroids are recommended for use on long-lasting insecticidal nets (LLINs) due to low mammalian toxicity and are used extensively against the adult. Pyrethroids are known to achieve this by causing permanent opening of the voltage-gated sodium channels and lead to a decrease in peak sodium ions, causing pyrethroid-induced paraesthesia. In addition, the voltage-gated chloride channels have been found to be sensitive to pyrethroids (Martin-Reina *et al.,* 2017)**.**

1.8.1.2 Larviciding

Larviciding is an important strategy in malaria control and involves killing the larval stage before it develops into adults at their potential breeding sites. Larvicides that are currently in use are mostly synthetic, pose other challenges such as toxic effects to the environment, humans and non-target-organisms (Mavundza *et al.,* 2013). Chemical larvicides have received less attention over the years,

with microbial larvicides receiving preference (Walker and Lynch, 2007). Unlike adult mosquitoes, larvae do not change their behaviour to avoid interventions towards larval habitants, allowing high and effective coverage (Killeen *et al.,* 2002). The integrated control of both the larvae and adult mosquitoes is a good strategy to control *Anopheles* larvae (Fillinger and Lindsay, 2011). The four larvicides in use include (1) surface oils and films, such as biodegradable ethoxylated alcohol surfactants which act by suffocating larvae or pupae; (2) synthetic organic chemicals, such as organophosphates which act on the nervous system of immature stages, (3) microbials, such as *Bacillus* species which kill larvae by producing toxins that attack the gut lining of the insect and finally (4) insect growth regulators, such as methoprene that interferes with the metamorphoses of the pupae into adult (Fillinger and Lindsay, 2011).

1.8.2 Non-pharmacological prevention of malaria infections

Advising travelers to low-risk areas on malaria prophylaxis is essential, as is the traveler's understanding of the risk of malaria. Advice should be tailored to the risk depending on age and health status of the travelers where several non-drug measures can be recommended for protection against a malaria infection. These include impregnated nets with insecticides, using treated mosquito screens on the doors and windows, visiting malaria areas during winter and dry seasons, burning insect repellent coils containing diethyltoluamide (DEET), wearing light-coloured clothing and long sleeves, as well as avoidance of mosquito bites (South African National Department of Health, 2018). The use of DEET is not recommended in infants less than two years old, with citronella oil the most effective oil in this age group. Not all repellents available commercially have been approved and some only deter the mosquito for a short period, such are citronella wrist bands and devices emitting ultrasonic sounds with many not offering adequate protection (Van Zyl, 2018).

1.8.3 Chemoprophylaxis of malaria

Over the past decades, the development of parasite resistance has necessitated changes in chemoprophylaxis and treatment policies in South Africa. Malaria chemoprophylaxis is recommended for people travelling to endemic areas within, as well as outside the country and

during the warm and wet seasons (South African National Department of Health, 2018). Chemoprophylaxis can either be causal i.e., works on the pre-erythrocytic liver forms or suppressive i.e., works on erythrocytic blood forms (Castelli *et al.,* 2010). It is important to consider various factors before recommending chemoprophylaxis, such as the risk of contracting malaria in the specific area being visited, as well as the age of the patient, health status, concurrent medication and adverse effects of chemoprophylaxis as no chemoprophylaxis is 100% safe (South African DOH, 2019). Chemoprophylaxis should also be tailored to the individual as blanket guidelines are not recommended (Table 1.1).

Initially, chloroquine was the recommended antimalarial for chemoprophylaxis until *P. falciparum* developed resistance to it which spread throughout the endemic areas (Schwartz, 2012). Chloroquine and its replacement sulfadoxine-pyrimethamine were abandoned due to development of resistance and South Africa registered mefloquine. Currently, mefloquine, doxycycline and the atovaquone-proguanil combination are recommended in South Africa for chemoprophylaxis (South African DOH, 2019).

Mefloquine is highly effective against *P. falciparum* and acute infections of *P. vivax* and provides advantages such as long-term use with simple weekly dosing which makes adherence easier (Table 1.1). The drug is relatively well tolerated and possesses a good pharmacokinetic profile in pregnant women and children (SAMF, 2023). Doxycycline is effective against *P. falciparum* and this regimen is taken daily from day one before entering a malaria area and four weeks after leaving the area (Table 1.1). People taking doxycycline are advised to avoid sunlight (ultraviolet) exposure and take it after a meal with a glass of water (South African DOH, 2018). The Atovaquone-proguanil combination is effective against *P. falciparum,* acute infections of *P. vivax, P. ovale* and *P. malariae* and this regimen is started at least a day before entering the malaria area, taken daily while there and for seven days after leaving the area (Table 1.1) (South African National Department of Health, 2019). The pharmacological properties of these prophylactic drugs are summarized in Table 1.1.

1.8.4 Treatment of malaria in South Africa

The South African DOH Guidelines for the treatment of malaria in South Africa recommend artemether-lumefantrine, quinine-doxycycline/clindamycin and artesunate to treat uncomplicated

or complicated malaria in South Africa (Table 1.2), while primaquine is recommended for *P. vivax* and *P. ovale* infections following treatment with artemether-lumefantrine (South African DOH, 2019).

Artemether-lumefantrine, is an artemisinin-based combination therapy (ACT), first-line therapy recommended for uncomplicated malaria caused by *P. falciparum*, *P. malariae* and *P. knowlesi* (South African DOH, 2019). This combination should always be taken with a fat-containing meal or drink to ensure adequate absorption of lumefantrine (Table 1.2). The combination has been shown to be better tolerated than a quinine combination, along with having a shorter treatment course. Less than 10% treatment failure with this combination has been reported in Malawi, Gambia and Angola between 2010 and 2015 (WHO, 2018). Quinine is normally used with clindamycin or doxycycline to ensure complete parasite clearance and the second agent (clindamycin or doxycycline) should be initiated two to three days after commencing quinine. This combination is only used if artemether-lumefantrine is contraindicated or unavailable for uncomplicated malaria infections. Intravenous artesunate is the first-line treatment recommended for severe malaria caused by all *Plasmodium* species and is known to have a good safety index and tolerability (South African DOH, 2019).

1.9 The malaria vaccine update

Efforts towards developing a vaccine against malaria have shown great success, with two vaccines currently recommended for use in preventing *P. falciparum* malaria in children (RTS,S/AS01 and R21/Matrix-M) (WHO, 2023).

Table 1.1: Chemoprophylaxis of malaria in South Africa including their mechanism of action, side effects and considerations in pregnancy and children. [1] Mungthin *et al.,* (1998); [2]Castelli *et al.,* (2010); [3]South African DOH, 2018; [4]Tan *et al.,* (2011); [5]Overbosch (2006); [6]Baggish and Hill (2002), [7] Skinner-Adams *et al.,* (2019), [8]SAMF (2020)

Chemoprophylaxis of malaria in South Africa			
Drugs	**Mechanism of action**	**Adverse effects at therapeutic levels**	**Considerations (children and pregnancy)**
Mefloquine	Inhibits the formation of haemozoin, leading to the accumulation of haem, which induces lipid peroxidation, leading to parasite death[1]	Neuropsychiatric and gastrointestinal effects, anecdotally ototoxic, nausea, vomiting, dizziness, chest pain oedema, sinus, bradycardia, insomnia and seizures[2]	Not for use in children <5 kg Category B - drug of choice in pregnant women, however large amounts are excreted in breast milk[8].
Doxycycline	Inhibits protein synthesis associated with the parasitic apicoplast[4]	Vaginal candidiasis in women (long term-use), skin photosensitivity, gastrointestinal effects[4]	Not used in children <8 years Contraindicated in pregnancy[3]
Atovaquone-proguanil	**Atovaquone:** Acts by inhibiting the mitochondrial electron transport in cytochrome *bcl* in the parasite[6] **Proguanil:** Acts by inhibiting plasmodial dihydrofolate reductase through its active metabolite, cycloguanil which inhibits folate formation in both pre-erythrocytic and erythrocytic parasites[7]	Well tolerated. Headache, vomiting, nausea, diarrhoea, abdominal pain[3,5]	Safety not established in pregnancy and lactation[8]

Table 1.2: Treatment of malaria in South Africa including their mechanism of action, side effects and considerations in pregnancy and children. [1]Meshnick *et al.,* (1993); [2]Asawamahasakda *et al.,* (1994); [3]Berman and Adams (1997); [4]Rogerson (2017); [5]South African DOH, 2019; [6]Mungthin *et al.,* (1998). [7]WHO, 2015; [8]Parola *et al.,* (2001); [9]Schlitzer (2007); [10]Tan *et al.,* (2011); [11]Luzzi and Peto (1993), [12] Skinner-Adams *et al., (*2019),[13]SAMF (2020)

Treatment of malaria in South Africa			
Drugs	**Mechanism of action**	**Adverse effects at therapeutic levels**	**Considerations (children and pregnancy)**
Artemisinin derivatives	Acts by damaging the intraparasitic organelles and alkylation of parasite proteins through the generation of reactive oxygen species (ROS) by its endoperoxide bridge cleavage[1-3]	Palpitations, sleep disorders, headache, dizziness, cough, pruritus, gastrointestinal disturbances, anorexia, rash, asthenia, myalgia, fatigue[5]	Recommended in second and third trimester of pregnancy and during lactation[13]
Quinine (with)	Causes accumulation of haem leading to death of the parasite[6]	Nausea, blurred vision, severe quinine toxicity may cause cardiovascular disturbances and central nervous system (CNS) alterations, quinine induced-hypoglycaemia in pregnant women and children, severe thrombocytopenia, angio-oedema[5]	Recommended in the 1st trimester of pregnancy and considered safe at normal therapeutic doses[13]
Clindamycin OR	Inhibits protein synthesis[12]	Nausea, vomiting, tinnitus, hypacusis, diarrhoea, abdominal pain[8]	Preference in pregnant women and children under 8 years[13]
Doxycycline	Inhibits protein synthesis associated with the parasitic apicoplast[4,10]	Photosensitivity, dental staining by chelating with calcium ions, pseudomembrane colitis, skin eruptions[11]	Avoid in pregnancy and children <8 years[5]

1.10 Resistance to malaria control measures

There has been increasing reports of resistance to currently approved antimalarials and insecticides, threatening the control measures in place and eventually the eradication plan (WHO, 2023).

1.10.1 Resistance to antimalarials

Unfortunately, the effectiveness of most antimalarial drugs is reduced by the development of resistance which can be due to transporter mutations, i.e., polymorphisms in the *P. falciparum* multidrug resistance-1 (*pfmdr1*) gene has an impact on multiple antimalarial drugs, such as chloroquine, quinine, lumefantrine, halofantrine, atovaquone and artemisinin (Sidhu et al., 2006; Cui *et al.,* 2015). The normal function of this gene in *P. falciparum* is not known, however its protein localizes to the membrane of the food vacuole that is the site of action for some drugs (Cui *et al.,* 2015). Multidrug resistance commonly occurs in malaria and cross-resistance can occur with drugs that are not structurally related (Duraisingh and Cowman, 2005) such as sulfoxamide-pyrimethamine and dihydroartemisinin-piperaquine that is attributed to mutations of KEL1/PLA1 strain (Hamilton *et al.,* 2019).

Interestingly, the Southeast Asia parasites developed drug resistance 100-fold higher when compared to South America or West Africa (Rathod *et al.,* 1997). Resistance to chloroquine was due to point mutations in the gene encoding for the *P. falciparum* chloroquine-resistance transporter (*Pf*CRT) protein (Wellems and Plowe, 2001) and resistance to quinine has been associated with transporter polymorphism such as single nucleotide polymorphisms (SNPs) (Cowman *et al.,* 1994). Atovaquone-resistant parasites were found to be resistant to the proguanil synergistic effects normally observed in atovaquone-sensitive parasites (Srivastava *et al.,* 1999). Resistance to atovaquone is mainly due to a point mutation in the mitochondrial and multiple copies of the cytochrome b (*cytb*) gene that encodes a subunit of cytochrome bc_2 complex (Kessl *et al.,* 2005). There have also been reports of resistance to artemisinin in South-East Asia (Cambodia, Thailand, Lao PDR, Viet Nam, Myanmar and Yunnan) (WHO, 2023) with no resistance being detected in South Africa as of 2023 (Centre for Emerging Zoonotic and Parasitic Diseases, 2023). The mechanism of

artemisinin resistance has been linked to several mutations in the K13-propeller domain, which leads to delayed parasite clearance both *in vitro* and *in vivo* (Ariey *et al.,* 2014; Fairhurst and Dondorp, 2016; WHO, 2018). Elevated phosphatidylinositol 3-phosphate levels have also been found to lead to vesicle expansion and as a result proteostasis due to increased interaction with unfolded protein response (Suresh and Haldar, 2018). The *P. falciparum* parasite uses phosphatidylinositol 3-phosphate to modulate cellular stress responses and to stabilise the parasite's digestive vacuole exposed to heat stress at clinically relevant temperatures as a survival mechanism (Lu, *et al.,* 2020).

Due to increased drug resistance, monitoring the efficacy of these drugs using passive surveillance, *in vitro* assays and molecular markers when possible has become important to measure the burden of malaria (Bacon *et al.,* 2009). On the other hand, measuring the impact of resistance to antimalarial drugs is not easy because it may not be recognised until several deaths are reported and strains evaluated, and this is mostly due to underestimation of the magnitude of the problem by routine health information systems (Yeung *et al.,* 2004).

1.10.2 Resistance to insecticides

In South Africa, five *Anopheles* species have been implicated in the *P. falciparum* malaria transmission *(Anopheles funestus, Anopheles arabiensis, Anopheles merus, Anopheles vaneedeni and Anopheles parensis).* The introduction of dichlorodiphenyltrichloroethane (DDT) and permethrin insecticides against *Anopheles funestus* led to high levels of resistance, which was later observed with *Anopheles arabiensis* (Munhenga *et al.,* 2022). It remains a challenge to identify other insecticides possessing the characteristics of good efficacy versus good safety profile and low cost. As a result, it has been suggested that novel non-pyrethroid insecticides be mixed with pyrethroids on bed nets to delay the development of mutations or resistance (Ngufor *et al.,* 2017). Mechanisms of resistance by the mosquito vectors to the insecticides in use have been categorized as either:

- metabolic – when the insecticide is broken down by the internal enzymes in the vector before exerting its toxic effect;
- target site – when there is a genetic mutation of the protein receptor within the vector that acts as a target site for the insecticide, reducing the effect of the insecticide;

- reduced penetration – when changes to the outer cuticle of the vector reduces the absorption of the insecticide into the mosquito or,
- behavioral resistance – involves changes in the behavior of the mosquito such as avoidance of insecticide-treated surfaces or feeding changes (WHO, 2018).

There have been new insecticides of dual ingredient ITNs recently recommended by WHO for use: Namely, :

- Pyrethroid-chlorfenapyr nets: combines a pyrethroid and a pyrrole insecticide which enhance the killing effect of the net,
- Pyrethroid-pyriproxyfen nets: combines a pyrethroid with an insect growth regulator that disrupts reproduction and growth (WHO, 2023).

Resistance by *P. falciparum* has highlighted the need for the development of new, cost-effective chemotherapeutic agents that maintain the high efficacy of artemisinin (Bloland *et al.,* 2000) and target both the asexual and sexual stages of the parasite. In the same manner, alternative agents that can be used in malaria vector control are needed due to resistance that has emerged to currently approved insecticides.

1.11 Potential new antimalarial agents

Three groups of compounds (quinoline hydrazones, dihydropyrimidine and azoles) were investigated for their antimalarial activity potential to combat the resistance and pressure on the currently commercialized antimalarials. The scaffold of all three groups has been used over the years in anticancer, antiplatelet, antimicrobial and antibacterial treatment options, amongst others.

1.11.1 Quinoline hydrazones

Quinolines are nitrogen-based heterocyclic aromatic compounds (Figure 1.4) known for their broad activity against bacteria and some of the characteristics of quinolines include high bioavailability and

favourable pharmacokinetics (Gao and Zhou, 2017). The quinoline scaffold is also found in structures of certain antiviral drugs (Rajabalian *et al.,* 2007), anti-cancer drugs (Idowu and Schweizer, 2017) and has been documented to possess antimalarial, anti-inflammatory, analgesic, antiplatelet and anti-asthmatic properties (Rajesh, 2018). Quinoline hydrazones are among some of the molecules that have received attention in targeting cancer and tuberculosis, and the synthetic flexibility of quinolone, as well as the polar and non-polar properties of quinoline hydrazones has played a major role in this regard (Mandewale *et al.,* 2017). Hydrazones have also been found to have anticancer, antiviral, anti-inflammatory, antimicrobial, analgesic, antiplatelet, antifungal, anticonvulsant, antihelmintic, antiprotozoal, antitrypanosomal, cardio-protective and antimalarial activity (Verma *et al.,* 2013). Among others, bisquinolines (Raynes *et al.,* 1996), such as the derivatives of ferrochloroquine, were designed such that the ferrocene group replaced the carbon skeleton of chloroquine (Chibale *et al.,* 2000) showing promising antimalarial activity. Similarly the 4-anilinoquinolines showed activity against chloroquine-sensitive *P. falciparum* strains (McNulty *et al.,* 2014).

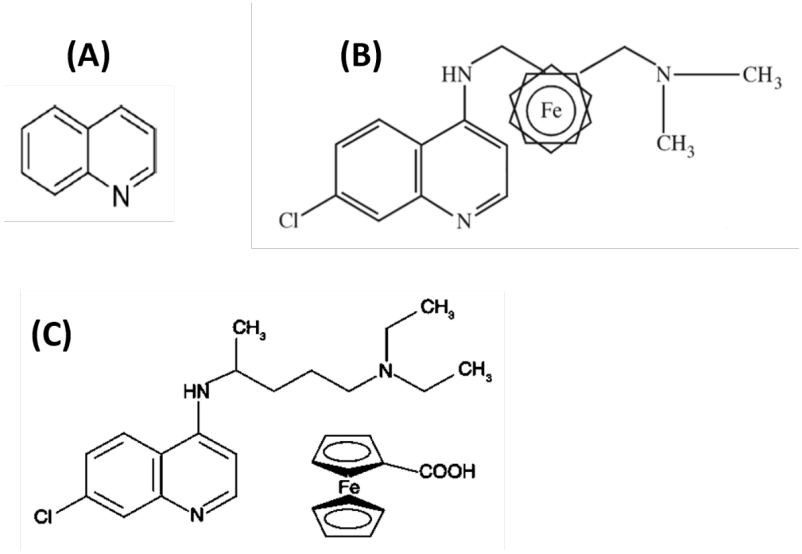


Figure 1.4: Quinoline - A (Rajesh, 2018); Ferrochloroquine - B (Pradines, 2001) and Chloroquine and Ferrocene - C (Roux and Biot, 2012) structures.

1.11.2 Dihydropyrimidine

Dihydropyrimidines have been reported to possess antimicrobial, anticancer, anti-inflammatory, antihypertensive, anti-human immunodeficiency virus, anticonvulsant, antibacterial and antimalarial activity (Sadanandam *et al.,* 1992; Chikhale *et al.,* 2009; Kumar *et al.,* 2009; Chiang *et*

al., 2009, Chitra *et al.,* 2010; Andrews and Ahmed, 2015) (Figure 1.5). Padmashali *et al.,* (2019) synthesized pyrimidine derivatives linked to thiophene moieties and found these to have promising antibacterial and antifungal activity (Padmashali *et al.,* 2019). In cancer, they act as an antimetabolite, disrupting ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) synthesis and as a result combating cancerous cells (Mahapatra *et al.,* 2021). Pyrimidine-containing molecules are continuously expanding their application in heteroaromatic chemistry due to their unique structures and the unique properties of pyrimidines are attributed by their electron-attracting nitrogen atoms. The pyrimidine nucleus also occurs in nature in nucleotides and nucleic acids as well as in alkaloids from coffee, tea and cocoa (Bhat *et al.,* 2017).

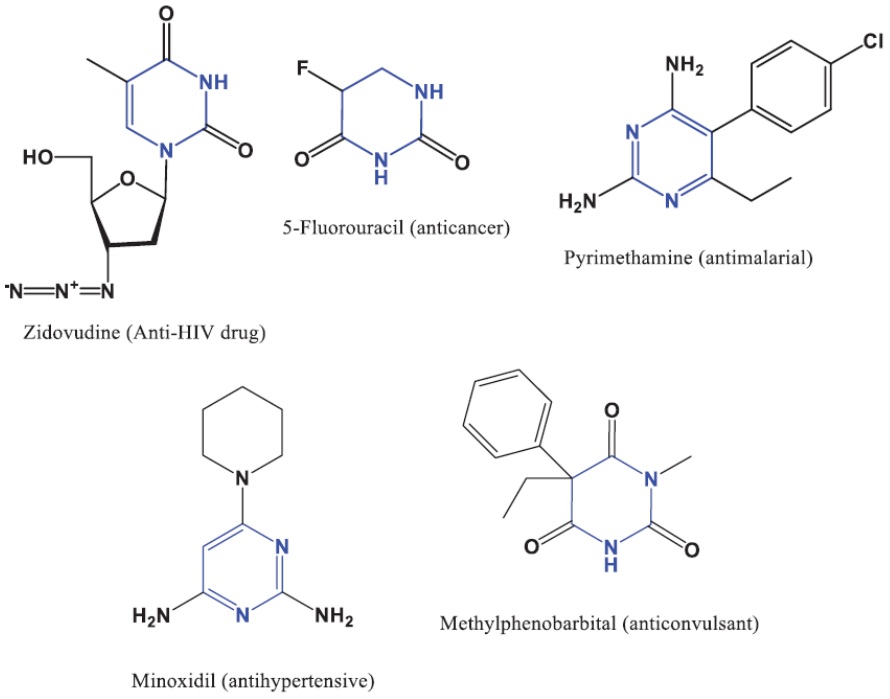


Figure 1.5: Active pharmaceutical molecules (Zidovudine used as an anti-HIV drug; 5-Fluorouracil used as an anticancer; Pyrimethamine used as an antimalarial; Minoxidil used as an antihypertensive and Methylphenobarbital as an anticonvulsant) having pyrimidine pharmacophore (Padmashali *et al.,* 2019).

1.11.3 Benzimidazoles and imidazoles

Benzimidazoles are a group of heterocyclic, aromatic compounds and the benzimidazole nuclei is one of the most important strategies in drug discovery as it resembles purine-like structures (Figure 1.6) (Woolley, 1944). Benzimidazoles are not only important in medicine, but have recently been

used in supramolecular chemistry, photochemistry, bioinorganic chemistry, catalysis, biomedical and analytical applications and in material science (Keri *et al.,* 2009; Rehman *et al.,* 2013; Tao *et al.,* 2016). An imidazole is a nitrogen-containing heterocyclic ring with pharmaceutical and biological importance (Figure 1.6C) (Rani *et al.,* 2013).

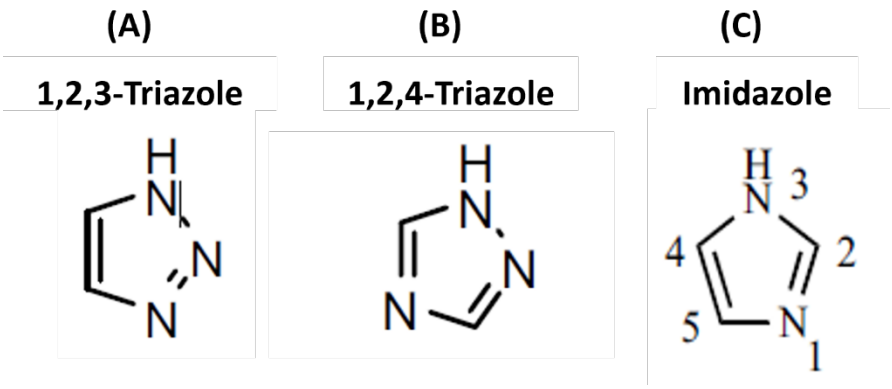


Figure 1.6: Triazole isomers (A and B) and imidazole (C) structures: 1,2,4-triazole (B) has more activity in comparison to 1,2,3-triazole (A), which is rather more stable (Asif, 2015; Rani *et al.,* 2013).

The heterocyclic azoles such as imidazole and benzimidazole have shown great potential in treating various diseases and their derivatives possess a diverse spectrum of pharmacological properties (Figure 1.7) due to their structural features and electron-rich environment (Gaba and Mohan, 2016). The azole compounds have also been found to have variable antifungal activity (Khabnadideh *et al.,* 2012) and have a role in the management of cystic fibrosis lung transplant patients by replacing nephrotoxic agents such as amphotericin B (Billaud *et al.,* 2010). The imidazole-containing derivatives synthesized by Verma *et al.* (2013) showed this group to have anti-human immunodeficiency virus type-1 properties and antioxidant activity (Boiani and González, 2005). There has also been interest in the activity of azoles, particularly triazoles (Figure 1.6A and B) due to their diverse functional structure in medicine (Ali *et al.,* 2015; Asif, 2015). Some of the triazoles include tioconazole, fluconazole, itraconazole, voriconazole and posaconazole.

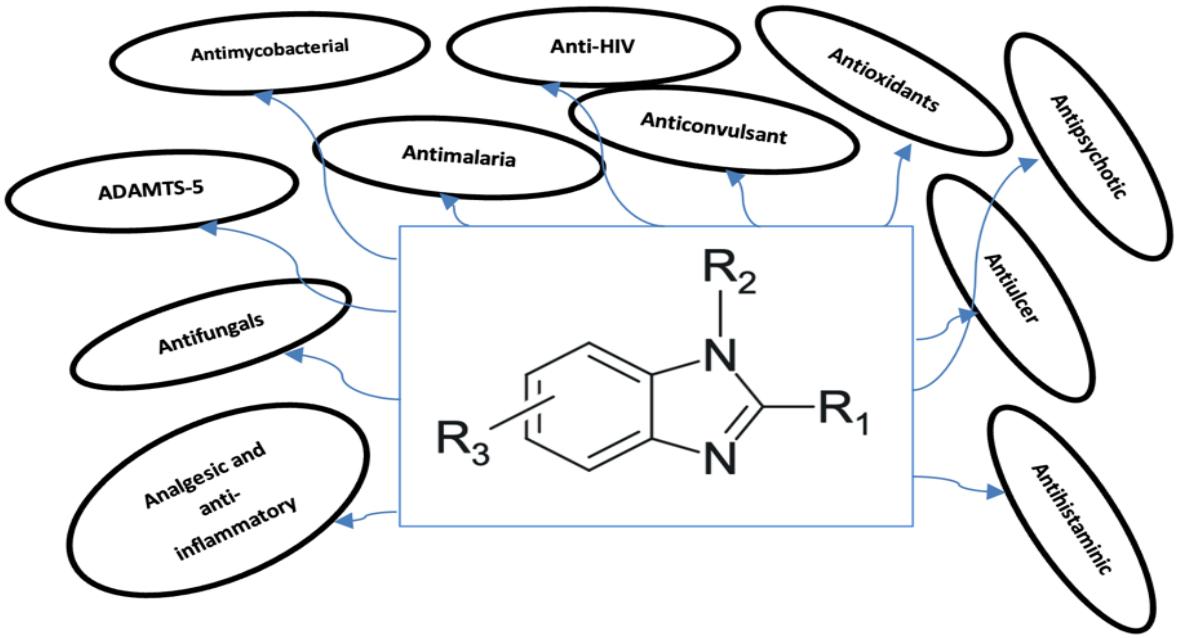


Figure 1.7: Pharmacological profile of benzimidazole nucleus adopted from Thatikayala *et al.,* 2022.

In malaria, clotrimazole and ketoconazole have showed affinity for haem (Huy *et al.,* 2002a). Clotrimazole has also shown activity by interfering with parasite replication and modifying the morphology of the parasites (Tiffert, 2000). Itraconazole inhibits cytochrome P450 enzymes, particularly 14-α-demethylase (Lestner and Hope, 2013) and tioconazole has been described as an analogue of nicotinamide adenine dinucleotide (NADH), occupying the same position as NADH in the active site pocket of *P. falciparum* enzyme lactate dehydrogenase (*Pf*LDH) (Penna-Coutinho *et al.,* 2011).

Despite current achievements in malaria control, resistance to antimalarials and insecticides continues to threaten these measures and as a result, alternative strategies should be continuously sought. The scaffolds of these latter compounds, namely the quinoline hydrazones, pyrimidines and azoles show promise and these offer opportunities to develop new derivatives that could provide lead compounds for potential alternatives in the search for new antimalarial drugs.

1.12 Aims & Objectives

The aim of this study was to determine the antimalarial and pharmacological properties of novel 6-oxo-1,6 dihydropyrimidine (**P** compounds), 2-(quinolin-8-yloxy)acetohydrazone (**QC** compounds), benzimidazole quinolones (**QB** series) compounds and known benzimidazoles and imidazoles.

This aim was achieved through the following objectives:

- To determine the *in vitro* antimalarial activity of the compounds using the parasite lactate dehydrogenase (pLDH) assay.
- To determine the combined interaction between the two most active compounds with known antimalarial drug, quinine.
- To evaluate the effects of the two most active compounds on the morphology of the *P. falciparum* parasites to elucidate their possible mechanism of action.
- To determine the larvicidal and ovicidal effects of the compounds on *Anopheles* mosquitoes.
- To determine toxicological properties of the compounds on host red blood cells, *Artemia fransiscana*, human kidney epithelium (HEK-293), human erythroleukemia (K562) and/or human neuroblastoma (SH-SY5Y) cell lines.
- To determine the combined interaction between the two most active compounds with known anticancer drug, cisplatin using the tetrazolium cell viability assay.
- To determine the selectivity index of the compounds on *P. falciparum* parasites compared to human epithelial cells.
- To investigate possible mechanisms of action of the compounds as possible antioxidants or iron chelating agents.

Chapter 2: Methodology

2.1 Test compounds and controls

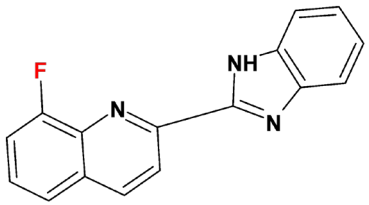
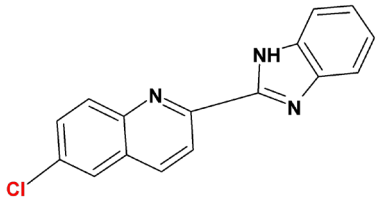
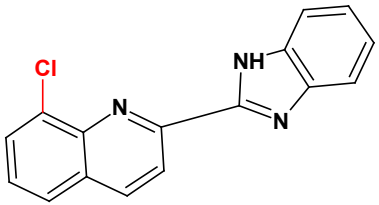
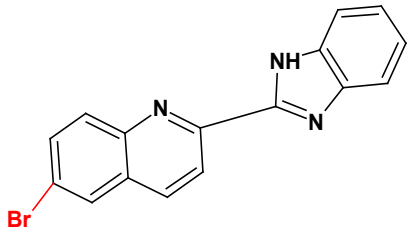
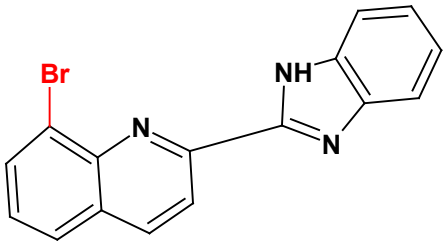
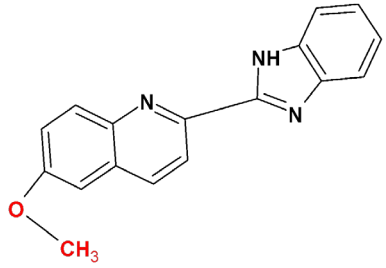
All control compounds and reagents were purchased from reputable commercial suppliers (Merck, Sigma-Aldrich®, Thermo Scientific, Prolabo®, Fluka). The novel quinoline-2-benzimidazole (**QB1-QB12**) compounds (Table 2.1) were synthesized by Professor N. Koorbanally from the Department of Chemistry, University of KwaZulu Natal. The novel 6-oxo-1,6-dihydropyrimidine (**P1-P10**) (Table 2.2) and 2-(quinolin-8-yloxy)acetohydrazone (**QC1-QC11**) derivatives (Table 2.3) were synthesized and characterized by Prof A. Azam from Department of Chemistry, Jamia Millia Islamia, Jamia Nagar, India.

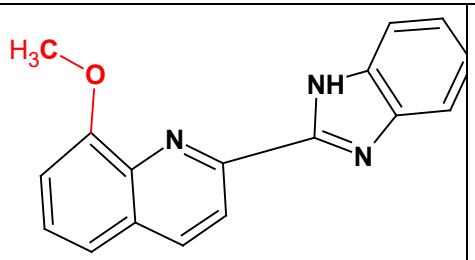
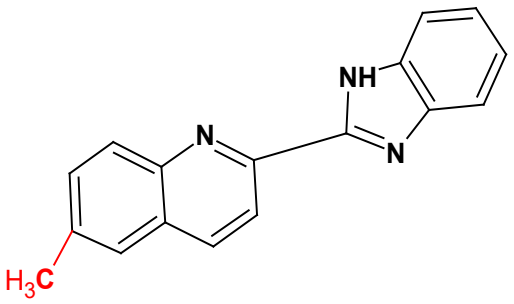
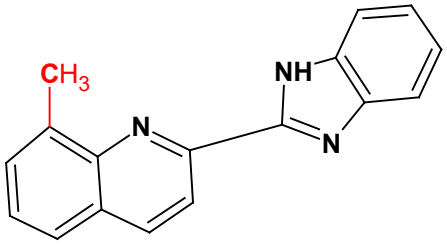
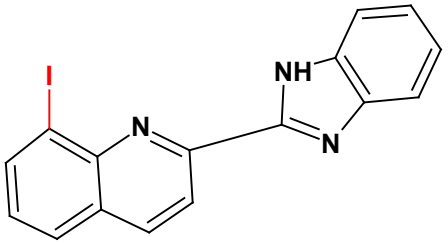
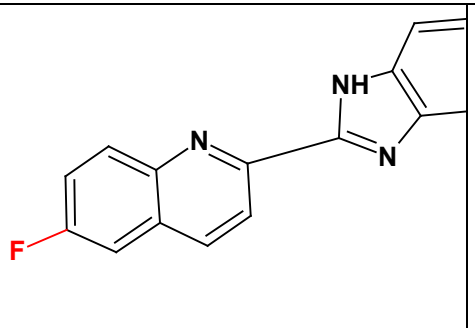
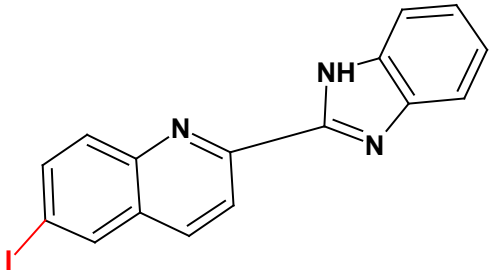
All test and control compounds were readily dissolved in dimethyl sulfoxide (DMSO) to make up a stock of 10 mM and stored at -70°C to optimize their stability when stored in solution. All 43 compounds and controls (quinine, cisplatin, Triton™ X-100, ascorbic acid and ethylenediaminetetraacetic acid (EDTA)) were screened at 50 µM for all assays except for larvicidal and ovicidal assays, where they were screened at 0.5 µM, along with the positive controls, potassium dichromate, dichlorodiphenyltrichloroethane (DDT) and 1% formalin. The most active compounds were used to obtain log sigmoid dose response curves to determine the concentration required to inhibit 50% cell/parasite growth (IC_{50} values).

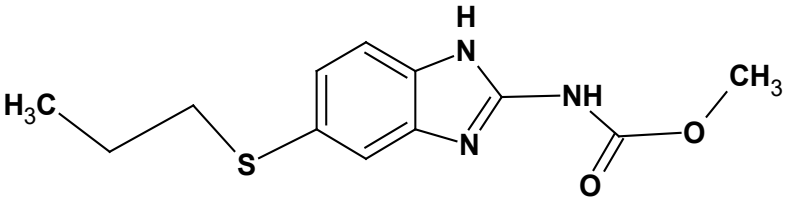
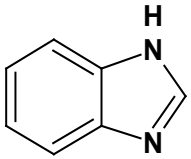
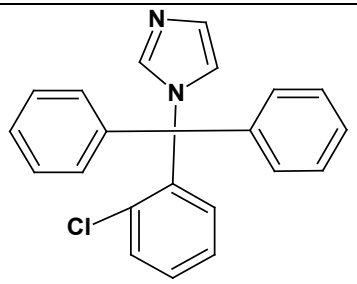
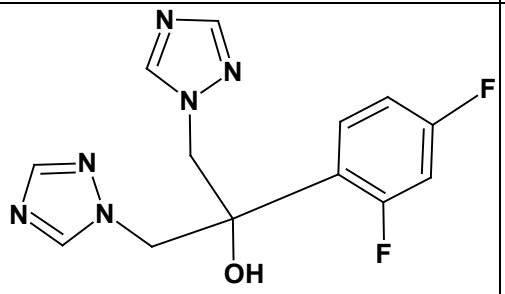
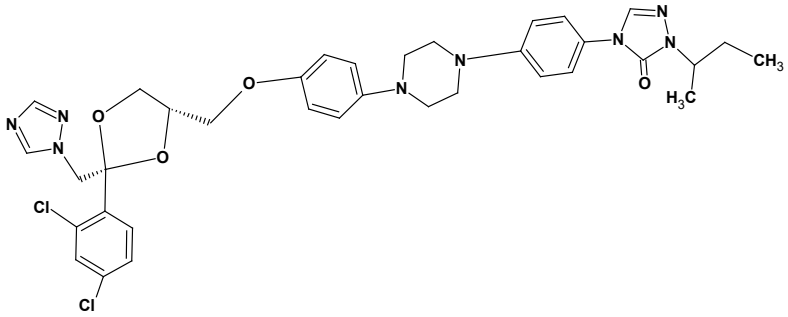
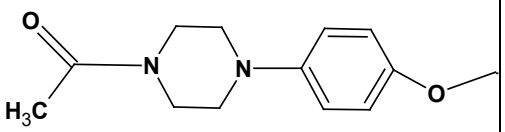
2.2 Malaria culturing

A chloroquine-sensitive strain of *P. falciparum* (NF54) malaria parasites were cultured and maintained according to methods described by Jensen and Trager (1977), Freese *et al.* (1998) and protocols from the department.

Table 2.1: The chemical structures of novel quinoline-2-benzimidazole (QB) derivatives, well characterized benzimidazoles and therapeutically used imidazoles.

No.	STRUCTURE	MW (g/mol)	No.	STRUCTURE	MW (g/mol)
QB1	 2-(1*H*-benzimidazol-2-yl)-8-fluoroquinoline	263.27	**QB7**	 2-(1*H*-benzimidazol-2-yl)-6-chloroquinoline	279.72
QB2	 2-(1*H*-benzimidazol-2-yl)-8-chloroquinoline	279.72	**QB8**	 2-(1*H*-benzimidazol-2-yl)-6-bromoquinoline	324.18
B3	 2-(1*H*-benzimidazol-2-yl)-6-bromoquinoline	324.18	**QB9**	 2-(1*H*-benzimidazol-2-yl)-6-methoxyquinoline	275.30

QB4		275.30	**QB10**		259.30
	2-(1*H*-benzimidazol-2-yl)-8-methoxyquinoline			2-(1*H*-benzimidazol-2-yl)-6-methylquinoline	
QB5		259.30	**QB11**		371.18
	2-(1*H*-benzimidazol-2-yl)-8-methylquinoline			2-(1*H*-benzimidazol-2-yl)-8-iodoquinoline	
QB6		263.27	**QB12**		371.18
	2-(1*H*-benzimidazol-2-yl)-6-fluoroquinoline			2-(1*H*-benzimidazol-2-yl)-6-iodoquinoline	

 Albendazole	265.33	 Benzimidazole	118.14
 Clotrimazole	344.84	 Fluconazole	306.27
 Itraconazole	705.64	 Ketoconazole	531.43

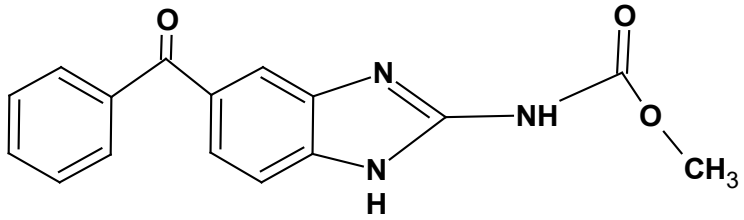
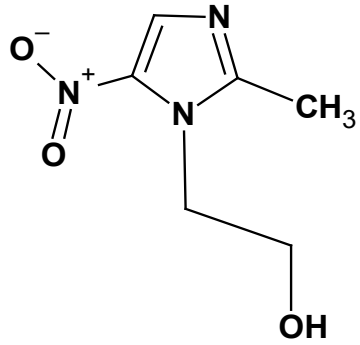
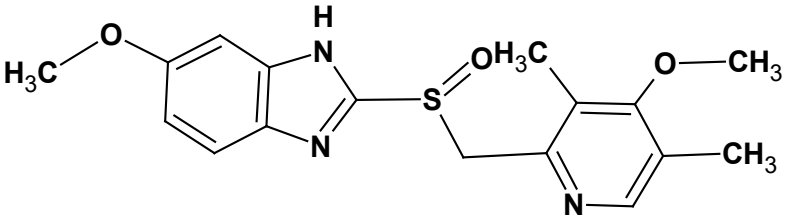
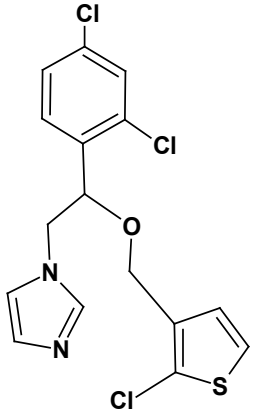
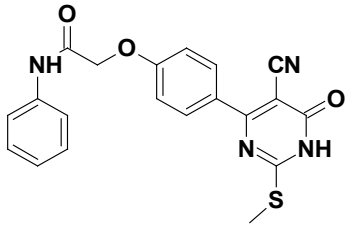
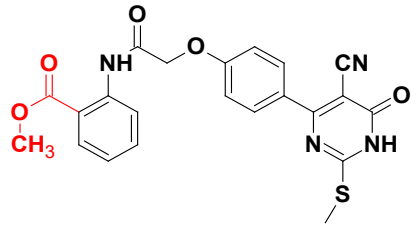
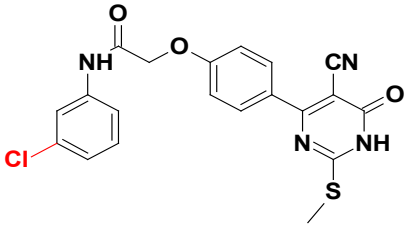
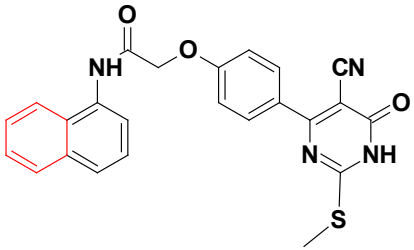
 Mebendazole	295.29	 Metronidazole	171.15
 Omeprazole	345.42	 Tioconazole	387.71

Table 2.2: The chemical structures of novel 6-oxo-1,6 dihydropyrimidine (**P**-series) derivatives.

No.	STRUCTURE	MW (g/mol)	No.	STRUCTURE	MW (g/mol)
P1	 2-{4-[5-cyano-2-(methylsulfanyl)-6-oxo-1,6-dihydropyrimidin-4-yl]phenoxy}-*N*-phenylacetamide	392.43	**P6**	 methyl 2-(2-{4-[5-cyano-2-(methylsulfanyl)-6-oxo-1,6-dihydropyrimidin-4-yl]phenoxy}acetamido)benzoate	450.47
P2	 *N*-(3-chlorophenyl)-2-{4-[5-cyano-2-(methylsulfanyl)-6-oxo-1,6-dihydropyrimidin-4-yl]phenoxy}acetamide	426.88	**P7**	 2-{4-[5-cyano-2-(methylsulfanyl)-6-oxo-1,6-dihydropyrimidin-4-yl]phenoxy}-*N*-(naphthalen-1-yl)acetamide	442.49

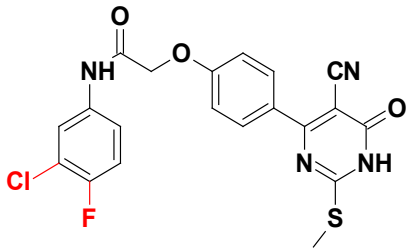
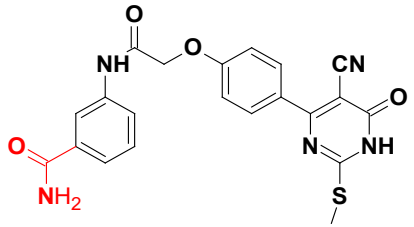
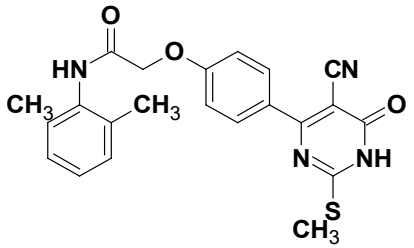
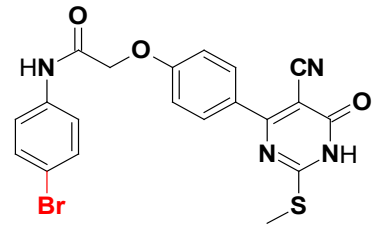
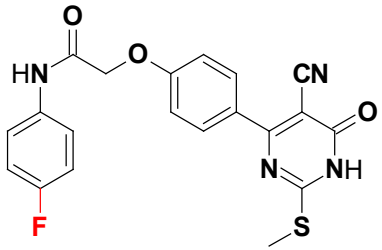
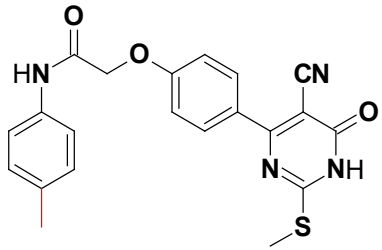
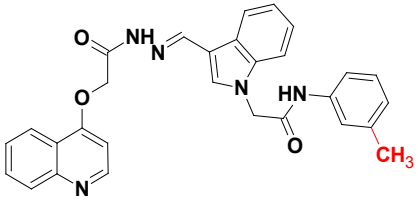
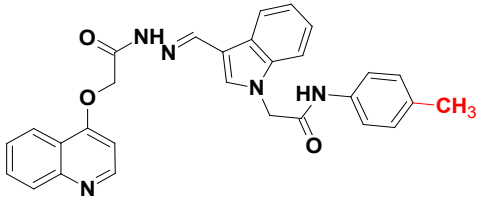
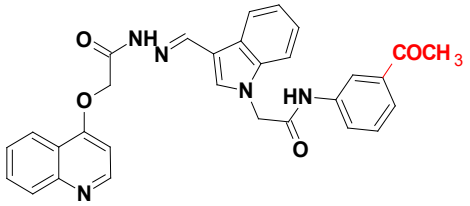
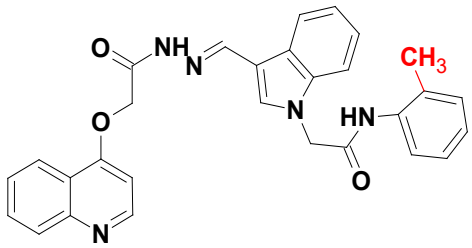
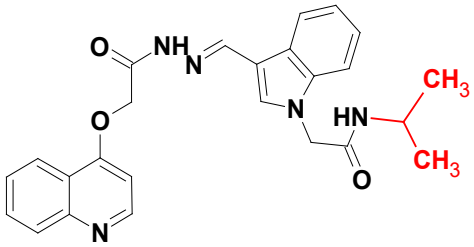
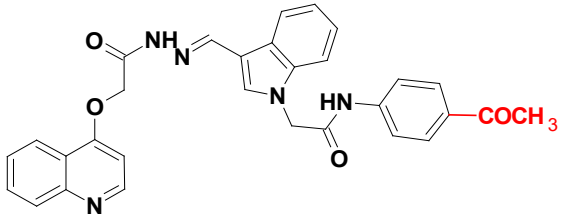
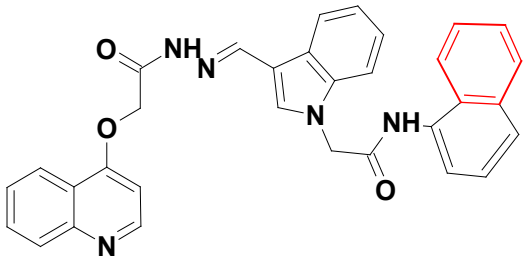
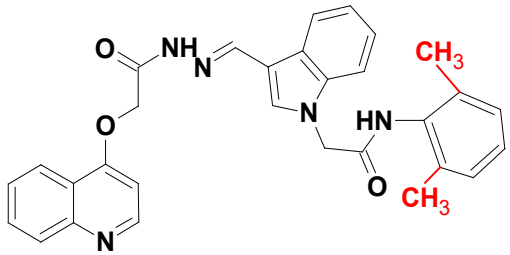
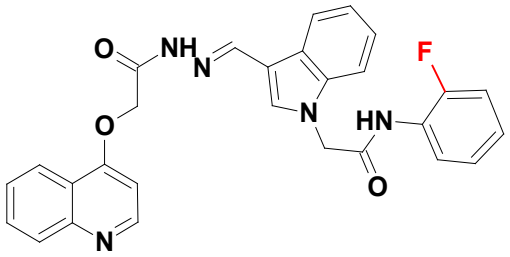
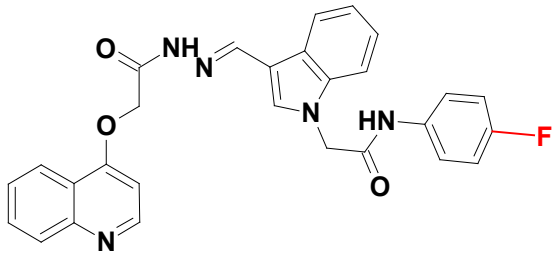
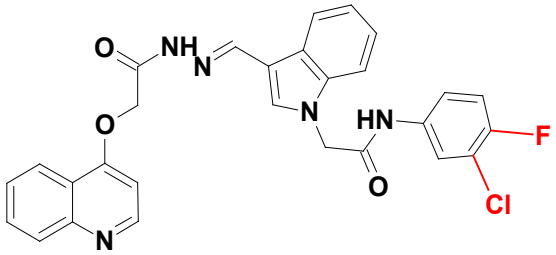
P3		444.05	**P8**		435.46
	N-(3-chloro-4-fluorophenyl)-2-{4-[5-cyano-2-(methylsulfanyl)-6-oxo-1,6-dihydropyrimidin-4-yl]phenoxy}acetamide			3-(2-{4-[5-cyano-2-(methylsulfanyl)-6-oxo-1,6-dihydropyrimidin-4-yl]phenoxy}acetamido)benzamid	
P4		420.48	**P9**		471.33
	2-{4-[5-cyano-2-(methylsulfanyl)-6-oxo-1,6-dihydro-pyrimidin-4-yl]phenoxy}-*N*-(2,6-dimethylphenyl)acetamide			*N*-(4-bromophenyl)-2-{4-[5-cyano-2-(methylsulfanyl)-6-oxo-1,6-dihydropyrimidin-4-yl]phenoxy}acetamide	
P5		410.42	**P10**		406.46
	2-{4-[5-cyano-2-(methylsulfanyl)-6-oxo-1,6-dihydro-pyrimidin-4-yl]phenoxy}-*N*-(4-fluorophenyl)acetamide			2-{4-[5-cyano-2-(methylsulfanyl)-6-oxo-1,6-dihydro-pyrimidin-4-yl]phenoxy}-*N*-(4-methylphenyl)acetamide	

Table 2.3: The chemical structures of novel 2-(quinolin-8-yloxy)acetohydrazone (**QC**-Series) derivatives.

No.	STRUCTURE	MW (g/mol)	No.	STRUCTURE	MW (g/mol)
QC1		491.54	**QC 7**		491.54
	N-(3-methylphenyl)-2-{3-[(E)-{[2-(quinolin-4-yloxy)acetamido]imino}methyl]-1H-indol-1-yl}acetamide			N-(4-methylphenyl)-2-{3-[(E)-{[2-(quinolin-4-yloxy)acetamido]imino}methyl]-1H-indol-1-yl}acetamide	
QC2		519.56	**QC8**		491.54
	N-(3-acetylphenyl)-2-{3-[(E)-{[2-(quinolin-4-yloxy)acetamido]imino}methyl]-1H-indol-1-yl}acetamide			N-(2-methylphenyl)-2-{3-[(E)-{[2-(quinolin-4-yloxy)acetamido]imino}methyl]-1H-indol-1-yl}acetamid	
QC3		443.51	**QC9**		519.56
	N-(propan-2-yl)-2-{3-[(E)-{[2-(quinolin-4-yloxy)acetamido]imino}methyl]-1H-indol-1-yl}acetamide			N-(4-acetylphenyl)-2-{3-[(E)-{[2-(quinolin-4-yloxy)acetamido]imino}methyl]-1H-indol-1-yl}acetamide	

QC4		527.59	**QC10**		505.58
	N-(naphthalen-1-yl)-2-{3-[(E)-{[2-(quinolin-4-yloxy)acetamido]imino}methyl]-1H-indol-1-yl}acetamide			N-(2,6-dimethylphenyl)-2-{3-[(E)-{[2-(quinolin-4-yloxy)acetamido]imino}methyl]-1H-indol-1-yl}acetamide	
QC5		495.50	**QC11**		495.52
	N-(2-fluorophenyl)-2-{3-[(E)-{[2-(quinolin-4-yloxy)acetamido]imino}methyl]-1H-indol-1-yl}acetamide			N-(4-fluorophenyl)-2-{3-[(E)-{[2-(quinolin-4-yloxy)acetamido]imino}methyl]-1H-indol-1-yl}acetamide	
QC6		529.96			
	N-(3-chloro-4-fluorophenyl)-2-{3-[(E)-{[2-(quinolin-4-yloxy)acetamido]imino}methyl]-yl}acetamide				

2.2.1 Preparation of reagents

2.2.1.1 Preparation of incomplete culture and experimental media

Incomplete media used for culturing the malaria parasite consisted of 10.4 g Roswell Park Memorial Institute (RPMI)-1640 (Sigma-Aldrich®), 5.9 g N-2-hydroxyethyl-piperazine-N-2-ethane-sulfonic acid (HEPES, Sigma-Aldrich®), 44 mg hypoxanthine (Sigma-Aldrich®) and 4 g glucose (Merck®) dissolved in 1 L sterile Millipore™ water. Incomplete experimental media was made in the same way except it did not contain gentamicin. Both media were stored at 4°C.

2.2.1.2 Preparation of complete culture and experimental media

Before use of the incomplete culture and experimental media, AlbuMAX™ (5% v/v, Gibco®) and sodium bicarbonate ($NaHCO_3$, 5% w/v, Sigma Aldrich®) was added to incomplete culture media (Section 2.2.1.1). AlbuMAX™ (5% w/v) stock solution was prepared in incomplete experimental media and sterilised through a 0.22 µm filter (Sterivex™) and stored at -20°C until required.

2.2.2 Uninfected red blood cells preparation

Whole blood was collected in anticoagulant tubes with acid citrate dextrose-B solution from healthy, drug free volunteers (Appendix 1, clearance certificate number M140669) and stored at 4°C. The whole blood was washed by centrifuging at 400 *g* for 5 minutes (Thermo Scientific Megafuge 8 centrifuge) with sterile phosphate buffered saline (PBS, pH 7.4) and the supernatant discarded between each of the three washes. The resultant red blood cell (RBC) pellet was then suspended in PBS and stored at 4°C for up to two weeks. PBS (pH 7.4) was prepared by dissolving: 0.3 g potassium chloride (Sigma-Aldrich®), 8 g sodium chloride (Sigma-Aldrich®), 0.73 g sodium phosphate dibasic dihydrate (Sigma-Aldrich®), 0.2 g potassium phosphate monobasic (Sigma-Aldrich®), in 1 L of Millipore™ water, autoclaved for sterility and stored at 4°C until needed.

2.2.3 Maintenance of *P. falciparum* NF54 strain culture

Bioethics clearance (protocol number: 20180104) was obtained from the University of the Witwatersrand Human Research Ethics committee to culture *P. falciparum* parasites (NF54 strain) (Appendix 2). The parasites were cultured in a tissue culture flask (Sigma-Aldrich®) at a

maximum of 5% parasitaemia and 5% haematocrit. Stained, thin blood smear slides were prepared (Section 2.2.4) and examined microscopically using a Nikon Optiphot microscope at 1000x magnification (oil immersion). Blood (1 ml) was added to the flask when the parasitaemia was >5% trophozoites/schizonts and synchronized when in the ring stage (Section 2.2.5). Spent media from the flask was replaced with complete medium (Section 2.2.1), before the culture was gassed for 30 seconds with 3% oxygen, 4% carbon dioxide, 93% nitrogen and incubated at 37°C in a motionless position.

2.2.4 Giemsa stain

Thin blood smears of the malaria culture were prepared daily on alcohol cleaned glass slides to assess the percentage parasitaemia, stage and morphology of the parasites (Figure 1.2). The Rapi-diff stain set (Clinical Sciences Diagnostics) was used to stain the parasites. Air dried smears were fixed with solution 1 (100% methanol) and stained firstly with solution 2, which was used to stain the RBCs (5 dips of 1 second each) and then solution 3 that stained the parasites (7 dips of 1 second each). The slides were rinsed with water, dried and microscopically examined at 1000x magnification under oil immersion. The percentage parasitaemia was calculated by counting 10 fields of the thin blood smear (Equation 2.1) to determine the appropriate action to take to maintain the parasite culture or to use in an experiment.

$$\% \text{ Parasitaemia} = \frac{Total\ number\ of\ parasitized\ RBCs}{Total\ number\ of\ uninfected\ RBCs + Parasitised\ RBCs}\ X\ 100$$

Equation 2.1: The equation used to calculate percentage parasitaemia (Thurber *et al.,* 2013).

2.2.5 Synchronization of the culture

It is important to have parasites predominantly in the ring stage to carry out experimental protocols and the degree of synchrony varies from study to study (Lambros and Vanderberg, 1979). The sorbitol synchronization method allows for continued growth of ring stage of the parasite while lysing the trophozoites and schizonts (Lambros and Vanderberg, 1979).

D-sorbitol 5% (w/v) working solution was prepared by dissolving 50 g D-sorbitol (Sigma-Aldrich®) into 1 L sterile water, filtered using 0.22 µm filter and stored at 4°C. Every second day when the parasites were predominantly in the ring stage, D-sorbitol was added to the culture to induce synchronization. The culture was centrifuged at 400 *g* for 5 minutes at room temperature and the supernatant discarded. To the pellet, 10 mL sorbitol was added, incubated at 37°C for 8 minutes. Thereafter, centrifuged at 400 *g* for 5 minutes and the supernatant discarded. The culture was washed twice in PBS (pH 7.4), before being resuspended in complete culture media, gassed and incubated at 37°C.

2.3 *Plasmodium* lactate dehydrogenase assay

The *p*LDH assay is based on the principle that *Plasmodium* lactate dehydrogenase (*p*LDH) isoforms can be distinguished from the human ones by its ability to use the NAD analog 3-acetyl pyridine adenine dinucleotide (APAD) at a higher rate, making the *p*LDH easily distinguishable in blood samples (Makler *et al.,* 1998). The *p*LDH oxidizes lactate to pyruvate while reducing APAD to 3-Acetylpyridine adenine dinucleotide (reduced form) [APADH], which reduces the yellow nitro blue tetrazolium (NBT) to purple formazan salts with the assistance of phenazine ethosulfate (PES) (Markwalter *et al.,* 2016) (Figure 2.1).

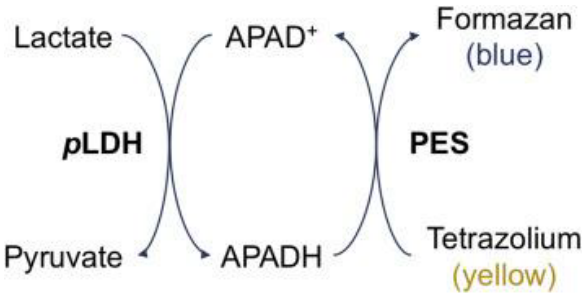


Figure 2.1: The *p*LDH assay measuring *Plasmodium* lactate dehydrogenase enzyme (Markwalter *et al.,* 2016). Where: pLDH: *Plasmodium* lactate dehydrogenase; $APAD^+$: 3-Acetylpyridine adenine dinucleotide; APADH: 3-Acetylpyridine adenine dinucleotide (reduced form); PES: Phenazine ethosulfate.

2.3.1 Preparation of *p*LDH reagents

The Malstat™ reagent (pH 9.1) was prepared with the trisaminomethane (Tris) base (0.055 M, Prolabo®), calcium-L-lactate (0.055 M, Fluka), APAD (0.037 mM, Sigma-Aldrich®) and 200 µL Triton™ X-100 (1 mL/L, Sigma-Aldrich®) made up to 100 ml in Millipore™ water, filtered using 0.22 µm filter and stored at 4 °C for up to a week. The 1.96 mM NBT (Sigma-Aldrich®) and 0.24 mM PES (Sigma-Aldrich®) stocks were prepared, filtered (0.22 µm) and mixed in a ratio of 1:1. NBT was stored at 4°C for 1 week and PES was stored at 4°C for 6 six months.

2.3.2 Experimental protocol

A blood smear of the culture was prepared to make sure that the parasites were predominantly in the ring stage. If this was the case, the culture was treated with sorbitol to remove any trophozoite staged parasites (Section 2.2.5). Thereafter, the parasites were washed three times with 10 mL PBS at 400 *g* for 5 minutes each. After the last wash step, another blood smear was prepared, stained (Section 2.2.4), the % parasitaemia determined (Section 2.2.4), adjusted to a 2% parasitaemia and 2% haematocrit with washed uninfected RBCs (Section 2.2.2) and complete experimental media (Section 2.2.1.2).

A 96 well plate was prepared by plating 100 µL of this latter parasite suspension to all but the 0% parasite control wells. The 0% parasite control consisted of a 2% haematocrit suspension of RBCs alone with complete experimental media and the 100% untreated parasite control consisted of 2% parasitaemia and 2% haematocrit suspension. The plates were then incubated at 37°C in a humidified candle jar for 24 hours. Thereafter, 12.5 µL of the test and control (quinine, Sigma-Aldrich®) compounds at 50 µM with 9 times dilution factor were added in duplicate wells and incubated for a further 48 hours in the humidified candle jar. After incubation, the plates were placed in a -70°C freezer for 1 hour, thawed for 30-45 minutes at 37°C and shook at 1200 rpm for 5 minutes. From the lysate, 25 µL was then added to a new sterile plate, to which 100 µL Malstat™ solution (Section 2.3.1) was added along with 20 µL PES/NBT (1:1) (Section 2.3.1). This was incubated for a further 40 minutes at 37°C, 5% (v/v) acetic acid (50 µl) added to end the reaction and absorbance read at 620 nm. The % parasite growth was calculated as per Equation 2.2.

$$\% \text{ Parasite growth} = \frac{ABS_{test\ compound} - ABS_{blank}}{ABS_{100\%\ control} - ABS_{blank}}\ x\ 100$$

Equation 2.2: Percentage parasite growth calculation. Where, ABS= Absorbance (Mustapha, 2017).

2.3.3 Combination studies

With reports of resistance to the current antimalarial drugs, new regimens for combination therapy are needed (WHO, 2020). Drug combination is also used to reduce toxicity and dose in addition to minimization of drug resistance. Combination analysis is designed to determine the combined effect of compounds thus classifying their interaction as synergistic, additive or antagonistic when compared with their individual activity (Gessner, 1995) (Figure 2.2). In a simple definition, a synergistic interaction is more than the individual effects of two drugs, an additive interaction is equal to the sum of the effect of the two drugs and the antagonistic interaction is less than the sum of the effect of the two drugs independently (Chou, 2010).

The two most active compounds to inhibit parasite growth were used in combination with quinine to assess their activity and potential use in combination with quinine. Solutions of the two most active compounds and quinine were prepared at concentrations 18 times higher than the required concentration for the experiment, which takes into consideration the 9 times dilution factor of the experiment and the 2 times dilution factor after combining the solutions in equal volumes. To determine this interaction, the fractional inhibitory concentration (FIC) was used to generate an isobologram using GraphPad® Prism Version 5.02.

$$\text{FIC} = \frac{IC_{50}\ of\ test\ compound\ in\ combination}{IC_{50}\ \ of\ test\ compound\ alone}$$

Equation 2.3: Calculation to determine the FIC value IC_{50} (Nunes *et al.,* 2017).

Table 2.4: The concentration ratios used to determine the interaction between the test compounds and quinine. Where these are the final concentrations in the well after taking a nine fold dilution factor into account.

Control / Test compound	Concentration (µM)							
	A	B	C	D	E	F	G	H
Quinine	50	40	20	15	0.1	0.01	0.001	0
Test compound	0	0.075	0.75	7.5	50	100	150	200

To verify the results obtained from the isobologram in this study, the ∑FIC value was calculated for quinine and the test compounds and interpreted as follows: <0.75 as synergistic, 0.75-1.25 as additive and >1.25 as antagonistic (Van Zyl *et al.,* 2010) (Figure 2.2).

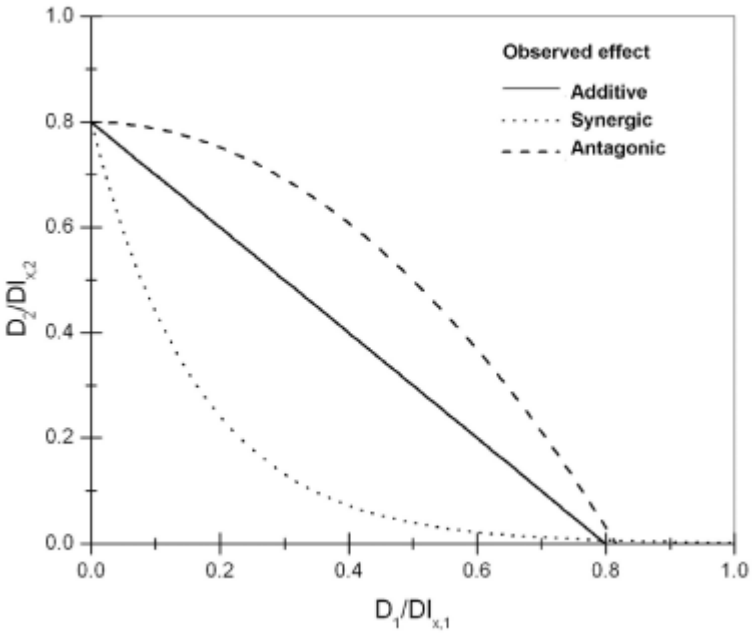


Figure 2.2: Isobologram showing possible pharmacological interactions between quinine and the two most active compounds (Biavatti, 2009).

2.4 Morphological evaluation of the parasite stage

This evaluation was done to determine the specific effects of the two most active compounds on the asexual stage in the life cycle of the malaria parasite (Lee *et al.,* 2009). The IC_{50} values of these compounds were determined from the log sigmoidal dose-response curves. A synchronized ring-staged parasite suspension (Section 2.2.5) at a 2% parasitaemia and 2% haematocrit suspended in complete culture media (7 mL) was placed in each flask with the test or control compounds at their IC_{50} concentrations. An untreated control was prepared in the absence of any drug at 2% haematocrit and 2% parasitaemia. The cultures were gassed for 15 seconds in an atmosphere of 4% carbon dioxide, 3% oxygen and 93% nitrogen, sealed and incubated at 37°C. Every 8 hours the flasks were removed from the incubator and agitated gently to re-suspend the contents and 200 µL of the centrifuged sample (671 *g* for 5 minutes) used to make thin blood smears (Section 2.2.4). The cultures were re-gassed and incubated after each time interval before preparing a blood smear. The blood smears were analyzed microscopically at 1000x magnification (Nikon Optiphot microscope) to note any morphological changes of the parasites, to determine total parasitaemia and percent parasitaemia at each stage.

2.5 Vector studies

2.5.1 Ovicidal activity

One of the strategies important in malaria elimination is that of controlling the malaria vector by targeting the initial stages of the mosquito populations (Fillinger and Lindsay, 2011). The purpose of this assay was to assess the potential of the test compounds to inhibit the mosquito eggs from hatching into larvae (Pineda-Cortel *et al.,* 2019). The eggs (*An. Arabiensis,* KWAG strain) were obtained from the National Institute for Communicable Diseases (NICD), Johannesburg, South Africa. The clearance certificate (reference number 07-11-2017-O) permitting the use of *Anopheles* eggs was granted by the Animal Research Ethics Committee, University of the Witwatersrand (Appendix 3).

2.5.1.1 Experimental protocol

KWAG eggs plated onto filter paper were counted and 20 eggs plated per well of a 24 well-plate, along with 990 µL of distilled water. The eggs were then exposed to the test compounds (10 µL) at 0.5 µM and 1% (v/v) formalin (Sigma Aldrich®) was used as the positive control and DMSO (10 µL, 10% (v/v)) as the negative control. The eggs were placed on a shaker for 72 hours at 25°C and mortality recorded at 24, 48 and 72 hours. The compounds were considered active if the percentage mortality was above 60%. Percentage mortality was calculated as according to Equation 2.4.

$$\% \text{ Egg mortality} = 100 - \left(\frac{Hatched\ larvae\ at\ 24/48\ and\ 72\ hours}{Total\ eggs}\ x\ 100 \right)$$

Equation 2.4: Determination of percentage *Anopheles* egg mortality at time points of 24, 48 and 72 hours (Reegan *et al.,* 2015).

2.5.1.2 Morphological evaluation of the KWAG eggs

Morphology of the KWAG eggs was evaluated microscopically (Zeiss©) under 8x magnification to assess the effect of the test compounds after 24, 48 and 72 hours.

2.5.2 Larvicidal assay

Though larvicides threaten non-target organisms, larvicidal agents are important in controlling mosquitoes at their breeding sites and with the development of resistance to the current insecticides, new alternatives need to be investigated (Antonio-Nkondjio, *et al.,* 2018). The aim of this assay was to investigate the potential use of the compounds in scope against *An. arabiensis* larvae (KWAG strain). This assay was conducted according to the WHO protocol with slight modifications (WHO, 2005). Larva was obtained from the National Institute for Communicable Diseases (NICD), Johannesburg, South Africa. The clearance certificate (reference number 07-11-2017-O) permitting the use of *Anopheles* larva was granted by the Animal Research Ethics Committee, University of the Witwatersrand (Appendix 3).

2.5.2.1 Larval food

Larval food was obtained from the NICD whereby 500 g Epol unflavoured dry dog biscuits and 10 g Brewer's yeast were pulverized to a fine powder. It was stored at room temperature.

2.5.2.2 Experimental protocol

The mosquito larvae were exposed to the test compounds and control (dichlorodiphenyltrichloroethane, DDT; Sigma Aldrich® at 0.05 µM, taking the 100 times dilution factor into account) to determine their inhibitory activity. Batches of 20 third instar larvae were transferred into small disposable cups with 25 mL deionized water along with 250 µL of the test compounds (0.5 µM) or DDT (0.05 µM). Any small or damaged larvae were removed and replaced with viable healthy larvae. DMSO was used as the negative control. The larval food was added evenly over the surface of the water of each cup and the cups then covered with a fine meshed net and elastic band to secure the net in place. The larvae were kept in an environment of 25-29°C.

The number of dead larvae was counted and recorded at 24, 48 and 72 hours. Larvae were considered dead if it showed no movement when nudged in the cervical region. Moribund larvae were counted and added to the dead larvae when calculating percentage mortality (Equation 2.5).

$$\% \text{ Larval mortality} = 100 - \left[\left(\frac{Y_{Treated\ at\ 24,48,72\ hours}}{X_{Untreated\ at\ 24,48,72\ hours}}\right) \times 100\right]$$

Equation 2.5: Determination of percentage larvae mortality. Where X = larvae % survival in untreated control, Y = larvae % survival in the treated control at time points of 24, 48 and 72 hours (WHO, 2005).

2.6 Toxicological profile

To ensure the compounds were not toxic to the host RBCs, they were incubated with the RBCs to monitor haemolysis. The *Artemia* lethality assay was performed to determine the toxicity

against the whole organism and the cell viability assay against the human embryonic kidney epithelial (HEK-293), myelogenous leukemia (K562) and neuroblastoma (SH-SY5Y) cell lines.

2.6.1 Red blood cell lysis assay

This assay was done to investigate the ability of the compounds to affect the integrity of the RBC membrane which could induce lysis of the uninfected RBCs thereby being detrimental to the host. The protocol according to Hayat *et al.,* (2011) was used.

2.6.1.1 Preparation of test compounds and positive control

Stock solutions (10 mM) of test compounds and quinine were diluted in incomplete experimental media (Section 2.2.1) to a screening concentration of 50 µM.

2.6.1.2 Experimental protocol

A 2% haematocrit RBC (180 µL) suspension and 25 µL incomplete experimental media was plated in a sterile 96-well plate with 25 µL of the test compounds and quinine at 50 µM taking the dilution factor of ten into account. The last row of the plate (Row H) was used for controls where 2% (v/v) Triton™ X-100 was the 100% haemolytic control and incomplete media used as the 0% haemolytic control.

The plates were placed in a humidified candle jar and incubated at 37°C for 48 hours, before being gently agitated and centrifuged at 377 *g* for 5 minutes. In a second non-sterile 96 well-plate, the supernatant (50 µL) from each well was added and diluted with water (150 µL) and the absorbance read at 412 nm to determine the percentage haemolysis (Equation 2.6).

$$\% \text{ Haemolysis} = \frac{ABS_{(test\ compound)} - ABS_{(0\%\ haemolysis\ control)}}{Average\ ABS_{\ (100\%\ haemolysis\ control - 0\%\ haemolysis\ control\ )}}\ X\ 100$$

Equation 2.6: Determination of the percentage haemolysis caused by the compounds on the red blood cells. Where, ABS= Absorbance (Mustapha, 2017).

2.6.2 Cell viability assay

The 3(-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay is based on the ability of living cells to convert MTT into formazan crystals, which regulates their mitochondrial activity (Van Meerloo *et al.,* 2011). This assay was conducted according to the method described by Mosmann (1983) and Van Zyl *et al.* (2010) on the transformed human cell line, human kidney epithelial (HEK293), myelogenous leukemic (K562) and the neuroblastoma (SH-SY5Y) cell lines. The clearance certificate (reference number W-CJ-160129-2) permitting the use of these cells was granted by the Human Research Ethics Committee (Medical) from the University of the Witwatersrand (Appendix 4). The toxicity profile of **QB**, benzimidazole and imidazole compounds was not tested against the human neuroblastoma (SH-SY5Y) cell line.

2.6.2.1 Preparation of reagents

- **Incomplete and complete medium**

The incomplete medium was prepared with 13.53 g/L Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich®) and 44 mM sodium bicarbonate ($NaHCO_3$, Sigma Aldrich®) in MilliQ® water, that was stirred until it dissolved. The solution was then filter sterilised through a 0.22 µm filter and stored at 4°C.

To prepare the complete medium, incomplete DMEM and Ham's F-12 medium (Thermo Scientific) were supplemented with 10% (v/v) foetal bovine serum (FBS) and equal volumes of the two media was used for maintaining the cells and stored at 4°C.

- **MTT solution**

The MTT solution (12 mM, Sigma Aldrich®) was prepared in PBS (pH 7.4) and filter sterilised through a 0.22 µm filter and stored at 4°C.

2.6.2.2 Maintenance of cells

Three cell lines were used to investigate the cytotoxicological properties of the test compounds compared to the control compounds: the adherent normalized human kidney epithelial (HEK293), neuroblastoma cell line (SH-SY5Y) and a suspension cell line (K562).

The cells were grown in complete medium supplemented with 10 µM carbenicillin. The cultured cells were kept in an incubator containing 3% oxygen, 92% nitrogen and 5% carbon dioxide at 37°C and were maintained under sterile conditions with growth being monitored microscopically at 1000x magnification (Nikon Optiphot microscope). Sub-culturing was done every second day depending on the confluency. If cells were >70% confluent, the HEK-293 and SH-SY5Y cells were trypsinised as follows. Spent media was discarded, cells washed once with 10 mL sterile PBS (pH 7.4), 2 mL trypsin (10%) added for 1 minute, flask agitated to lift cells and 2 mL of complete media added to inactivate the trypsin. The cell suspension was transferred to a 50 mL tube, re-suspended to 10 mL with complete medium for use in cell viability experiments. If the K562 cell suspension was densely populated, the cell density was determined as described for the trypsinised cells. Cell suspension (1 mL) was used to re-establish the K562 cell culture in fresh complete media (9 mL) supplemented with carbenicillin and incubated (37°C, 5% carbon dioxide).

2.6.2.3 Experimental protocol

The cell suspension (Section 2.5.2.2) was adjusted to 10,000 cells per well for all cell viability experiments. The cell suspension was stained with 0.2% (w/v) trypan blue (Sigma Aldrich®) at a 1:1 ratio (20 µL each) and the number of cells microscopically counted at 1000x magnification to determine the average cell density (viable cell number per mL = average number of viable cells per square X dilution factor X 10^4). To calculate the number of cells, four fields of cells from the haemocytometer were counted and the average number of cells determined. Cells in the four corner squares and center square of the haemocytometer were counted. Cells touching an edge were only counted if touching the top and left edges and excluded if touching bottom and right edges. The cell suspension was adjusted in complete media such that 10,000 cells per 180 µL

plated per well, except for the blank control wells in which 180 µL complete media without cells was added. To the cell suspension/control wells, 20 µL of the test compounds or control (50 µM, cisplatin, Sigma Aldrich®) were added, except to the untreated control wells where 20 µL complete medium was added. The plate was incubated for 48 hours at 37°C in a humidified environment with 3% oxygen, 92% nitrogen and 5% CO_2. Following the 48 hours incubation period, 40 µL MTT (12 mM) was added to each well and the plate further incubated for 4 hours. After which, the plates were centrifuged at 671 *g* for 5 minutes and 150 µL supernatant replaced with 200 µL DMSO to solubilize the formazan crystals. The plates were agitated at 1000 rpm for 5 minutes on a MRC Thermo-Shaker and the absorbance read at 540 nm (purple formazan colour) and 690 nm (reference wavelength for the untreated yellow MTT) using a UV-Vis plate spectrophotometer (Labsystems Multiskan LC). Percentage cell viability was calculated according to Equation 2.7.

$$\%\text{Cell viability} = \frac{ABS\ test\ compound_{(540\ nm)} - ABS\ test\ compound_{(690\ nm)}}{Mean\ ABS\ (100\%\ control_{(540\ nm)} - 0\%\ control_{(690\ nm)})}\ X\ 100$$

Equation 2.7: Percentage cell viability calculated with appropriate controls taken into account Where ABS= absorbance, 100% control = cells only control, 0% control = blank control without cells (Karakaş and Ulukaya, 2017).

2.6.2.4 Combination studies

The two most active compounds against the K562 cells based on their *in vitro* individual IC_{50} values were identified and combined in various ratios (Table 2.5 and Table 2.6) with the standard anticancer drug, cisplatin to investigate their potential use in cancer combination therapy. 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. The data and graphs were analysed and interpreted as described in Section 2.3.3.

Table 2.5: Concentration ratios used for tioconazole and cisplatin combination.

Control / Test compound	Concentration (µM)							
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Cisplatin	1000	500	250	100	50	5	0.5	0
Tioconazole	0	0.05	0.5	5	25	50	100	200

Table 2.6: Concentration ratios used for mebendazole and cisplatin combination.

Control / Test compound	Concentration (µM)							
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Cisplatin	1000	500	250	100	50	5	0.5	0
Mebendazole	0	0.1	0.5	5	10	25	100	200

2.6.2.5 Safety Index

As an initial indicator of safety, a safety index ($SI_{HEK293/Pf}$) was calculated (Equation 2.8) to determine whether the test compounds were more selective towards the malarial parasite or the human kidney epithelial cells and considered to have a preliminary favourable safety profile if the $SI_{HEK293/Pf}$ was greater than 10 (Badisa *et al.,* 2009; Manohar *et al.,* 2012). The higher the ($SI_{HEK293/Pf}$) value, the greater the selectivity of the test compounds towards the parasite.

$$\text{Safety Index } (SI_{HEK293/Pf}) = \frac{Cytotoxicity\ (IC50)}{Antimalarial\ activity\ (IC50)}$$

Equation 2.8: Safety index for *P. falciparum* parasites in comparison to HEK293 cells (Badisa *et al.,* 2009).

2.6.3 *Artemia* lethality assay

Artemia lethality assay is a cytotoxicity test designed for bioactive compounds based on the killing ability of test compounds on *Artemia franciscana* (Wu, 2014). This assay was performed to assess the toxicity of the test and control compounds on a whole organism in comparison to human cell lines and was adapted according to Asaduzzaman *et al.* (2015). The clearance certificate permitting the use of *A. franciscana* was granted by the Animal Research Ethics Committee of the University of the Witwatersrand (Appendix 5: reference number 07-11-2017-O).

2.6.3.1 Preparation of reagents

- **Salt water**

Salt water was prepared by dissolving 32 g sea salt (Aquarium Systems®) per 1 L deionized water.

2.6.3.2 Hatching of *Artemia* eggs

To hatch *Artemia* eggs, 0.5 g of the cysts were incubated for 24 hours at 25°C in 500 mL salt water with a continuous artificial source of light (60 Watts) and vigorously aerated using a rotary pump to disperse the eggs (Figure 2.3). After 24 hours, newly hatched *Artemia* nauplii were decanted into a container with 200 mL salt water for 20 minutes under the artificial source of light (60 Watts), concentrating the phototactic nauplii to a suitable density, thus separating the viable nauplii from the dead nauplii and unhatched eggs (Figure 2.3).

2.6.3.3 Experimental protocol

Water (50 µL) containing 20-35 live nauplii was pipetted into each well of a 24 well plate. Salt water (197.5 µL) was added to each well, as well as 2.5 µL of the test or control compound at 0.5 µM for screening taking the 100 times dilution factor into account. Potassium dichromate (0.5 µM; 125 µL) was plated as the positive control and drug-free salt water as the negative control. Dead nauplii were counted microscopically.

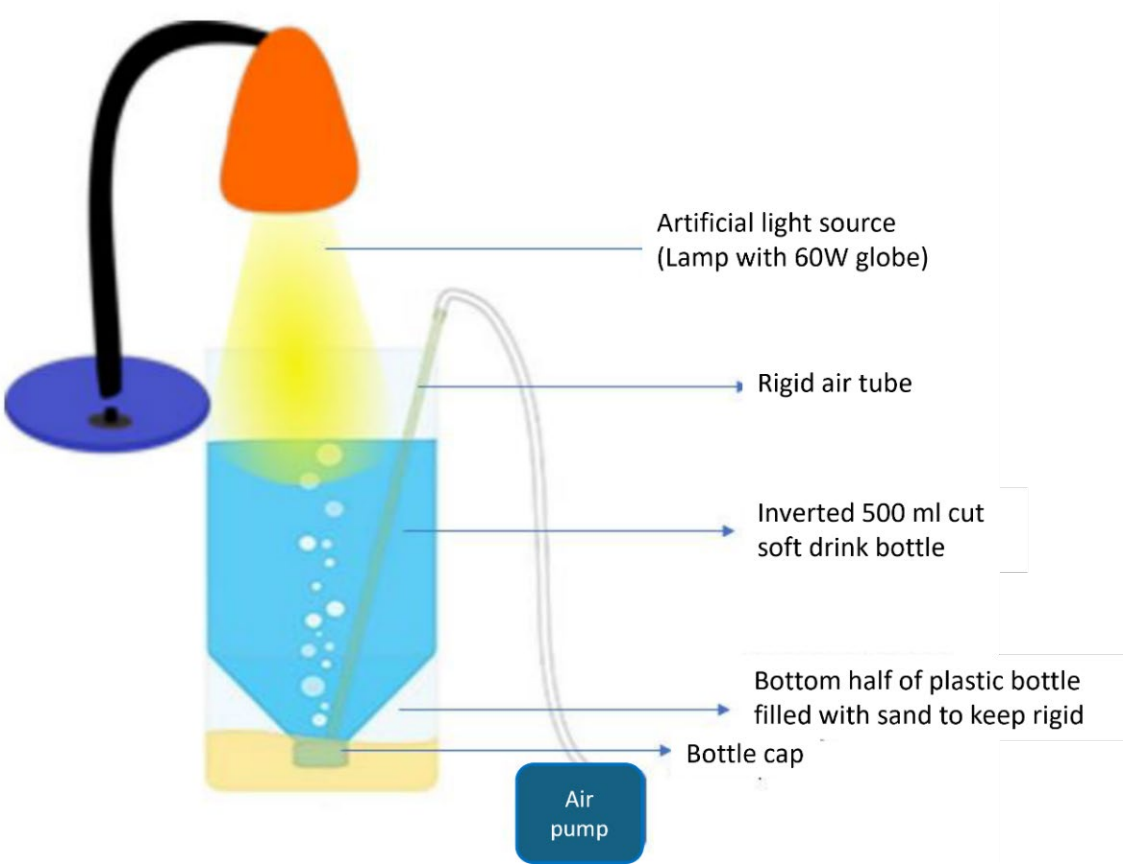


Figure 2.3: The design of the *Artemia* hatchery setup (Kathrada, 2017).

(Zeiss©) under 8x magnification at time 0 and the plates were left for 24 hours at room temperature. Dead nauplii was also counted microscopically under 8x magnification (Zeiss©) after 24, 48 and 72 hours (T_{24}, T_{48} and T_{72}). All nauplii were killed by adding 50 µL 5% (v/v) acetic acid and the total number of nauplii per well counted. Percentage mortality per well was then calculated according to Equation 2.9. The nauplii was further investigated microscopically (8x magnification) (Olympus® CKX41) at 24, 48 and 72 hours for significant morphological changes.

$$\% \text{ Mortality} = \frac{Dead\ nauplii_{T24,T48,T72} - Dead\ nauplii_{T0}}{Total\ nauplii}\ X\ 100$$

Equation 2.9: Percentage mortality of nauplii caused by the compounds over 24, 48 and 72 hours. Where T_{24} = 24 hours, T_{48} = 48 hours and T_{72} = 72 hours (Sahgal *et al.,* 2010).

2.7 Measurement of drug-likeness and drug toxicity

2.7.1 Measurement of drug-likeness

To predict the pharmacokinetic properties of the potential antimalarial compounds in scope, the Lipinski's rule of five (Ro5) was used (Lipinski *et al.,* 1997). According to this rule, a good drug candidate is one with less than 5 hydrogen bond donors, has less than 10 hydrogen bond acceptors, has a molecular weight less than 500 g/mol and its partition coefficient (Log P) is less than 5 (Lipinski *et al.,* 1997). For the purpose of this study, the partition coefficient was used as the predictor of the compound's solubility. ACD/ChemSketch software (freeware version) was then used to draw the two-dimensional structures of the compounds and ACD/iLab software (version 2) was used to predict solubility and permeability of these compounds. Compounds that did not meet more than one of the parameters according to Lipinski's Ro5 were considered as non-drug-like, possessing poor pharmacokinetic properties. The degree of ionization of the compounds was determined using ionization constants (pKa values) as predicted by the ACD/iLab software (version 2) at pH 7.4 (red blood cell cytosol) and pH 5 (parasite digestive food vacuole) using the Henderson-Hasselbalch equation (Po and Senozan, 2001) (Equation 2.10).

$$\% \text{ Ionization} = \frac{100}{1+ antilog\ (pKa-pH)}$$

Equation 2.10: Henderson-Hasselbalch equation used to determine percentage ionization for weak base and acids (Po and Senozan, 2001).

2.7.2 Drug toxicity

To determine the toxicity profile of the test compounds by assessing their toxic hazards characteristics through the application of a decision tree, Toxtree online version (https://toxtree.sourceforge.net/) was used. The Toxtree application determines the Cramer class of a chemical substance and estimates its relative toxic hazard (class I to III, with class III being the most toxic based on the Threshold of Toxicological Concern). A compound having a Threshold of Toxicological Concern value of less than 1 800 µg/day is classified as having the

lowest toxicity, less than 540 µg/day is intermediate and less toxic compared to class III (less than 90 µg/day), which suggests significant toxicity.

2.8 Anti-oxidant activity

2.8.1 The 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH•) assay determines the anti-oxidant activity using a stable free radical 2,2-diphenyl-β-picrylhydrazyl. This assay is simple, rapid, inexpensive and measures the scavenging activity of the anti-oxidants against this free radical (Kedare and Singh, 2011). The free radical of DPPH is characterized as being stable by virtue of the spare electron delocalization over the molecule as a whole, preventing it from dimerizing (Figure 2.4). This delocalization also causes the formation of a deep violet colour at 540 nm. If a compound possesses anti-oxidant activity by donating the hydrogen atom, it reduces the violet colour of DPPH• (Gulcin *et al.,* 2010).

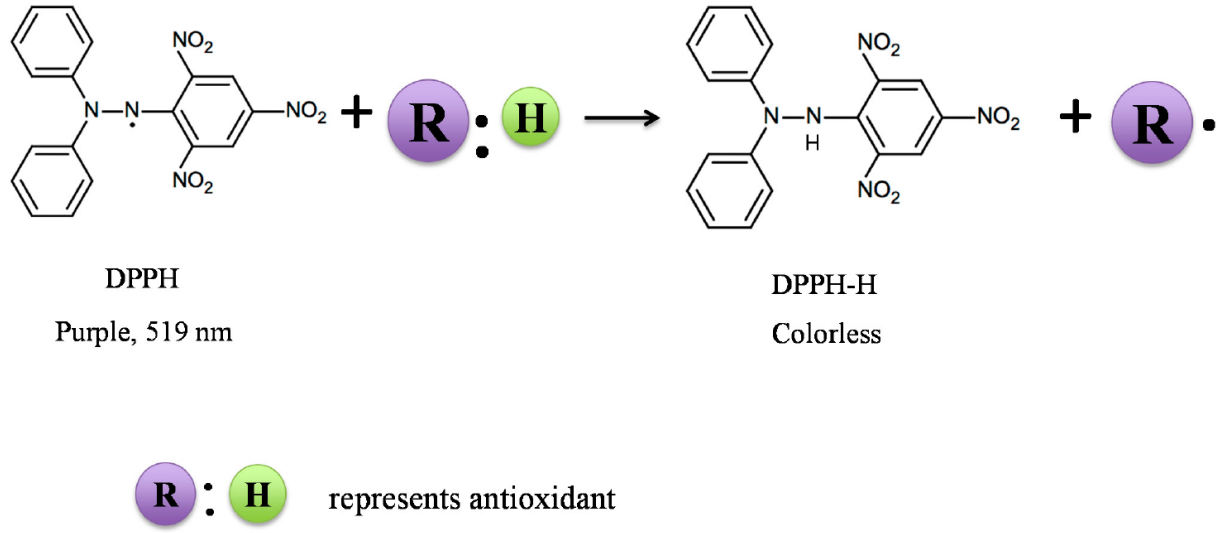


Figure 2.4: Reaction showing the formation of the stable non-radical form of DPPH from its unstable radical form. Where DPPH = the 2,2-diphenyl-1-picrylhydrazyl free radical, R:H = antioxidant, DPPH-H = unstable radical form (Liang and Kitts, 2014).

2.8.2 Preparation of reagents

The DPPH• (96.16 µM) solution was prepared 12 hours before use by dissolving 7.584 mg DPPH in 200 mL HPLC grade methanol. This solution was stored in the dark at 4°C. The DPPH• stock prepared was stored at 4°C in the dark for a week.

2.8.3 Experimental protocol

The test compounds (50 µM) taking the five times dilution factor into account and control (Ascorbic acid™, Sigma Aldrich®) were plated (50 µL) in triplicate in a non-sterile 96-well microtiter plate and DMSO was used as the blank. Thereafter, 200 µL of the 96.16 µM DPPH• solution was added to all wells except the blank, where 200 µL methanol was added. The plate was incubated for 30 minutes in the dark at room temperature and absorbance read at 540 nM. The free radical scavenging effect was determined according to Equation 2.11.

$$\% \text{ DPPH free radical scavenging effect} = \frac{(ABS_{100\%\ control} - ABS_{blank}) - (ABS_{test\ compound} - ABS_{blank})}{ABS_{100\%\ control} - ABS_{blank}} \text{ X } 100$$

Equation 2.11: Determination of percentage DPPH free radical scavenging effect. Where ABS = Absorbance, $ABS_{100\%\ control}$ = 100% DPPH control (Omonhinmin *et al.,* 2015).

2.9 Iron chelation assay

Iron is important for plasmodial malaria and are dependent on their host for iron for growth and development (Thipubon *et al.,* 2015). Iron chelators carry the ability to sequest iron and are cytocidal to the parasite despite the abundance of iron in RBCs (Heppner *et al.,* 1988). Ferrozine reacts with iron (Fe^{2+}) to quantitatively form red coloured metal-complexes. However, in the presence of competing chelating agents, the concentration of the ferrozine-iron complexes is reduced and the absorbance of the red colour can be quantitated and is proportional to the

chelating ability of the test compounds (Bibi Sadeer *et al.,* 2020). This assay therefore aims to investigate the iron chelating effects of the test compounds.

2.9.1 Preparation of reagents

- **Iron chloride ($FeCl_2$)** (0.48 mM, Fluka) solution was freshly prepared in 5% (w/v) ammonium acetate (Fluka) before each experiment.
- **Ferrozine** (1.2 mM, Sigma Aldrich®) solution was prepared by dissolving ferrozine in deionized water.

2.9.2 Experimental protocol

The test compounds (50 µM with 7.5 fold dilution factor) and control (EDTA) were plated in triplicate in a non-sterile 96-well microtiter plate (20 µL) before 30 µL of freshly prepared 0.48 mM $FeCl_2$ solution was added to all wells except the blank, to which Milli-Q® water was added. The solvent (DMSO) was used as the experimental control. The plate was allowed to stand in the dark at 25°C for 30 minutes and 100 µL of 1.2 mM ferrozine solution was added to each well except to the blank wells. Absorbance was then measured at 540 nM. Percentage iron chelation was calculated as follows:

$$\% \text{ Iron chelation} = \frac{(ABS_{100\%\ control} - ABS_{blank}) - (ABS_{test\ compound} - ABS_{blank})}{ABS_{100\%\ control} - ABS_{blank}} \text{ X } 100$$

Equation 2.12: Determination of percentage iron chelation. Where ABS = Absorbance, $ABS_{100\%\ control}$ = 100% iron chelation, i.e., EDTA (Adjimani and Asare, 2015).

2.10 Statistical analysis

The absorbance readings for *p*LDH, RBS lysis, free radical scavenging activity, iron chelation and MTT were read at their respective wavelengths using an ultraviolet-visible (UV-Vis) spectrophotometer (Labsystems iEMS Reader MF) Ascent™ software (version 2.6). Where IC_{50} values were required (*p*LDH and MTT), log sigmoidal dose response curves were generated using

GraphPad Prism® (GraphPad Software, Inc., USA, version 5). For assays which required LC_{50} values (*Artemia* lethality assay), IBM SPSS Statistical Software was used to conduct Probit analysis. The Mann-Whitney U-test was performed to determine statistical similarities/differences between the positive controls and test compounds, whereby a *p*-value ≤0.05 was considered significant. For all assays, the results were reported as mean ± standard deviation of three or more experiments.

CHAPTER 3 – RESULTS

Three sets of novel compounds, quinoline-2-benzimidazole (**QB1**-**QB12;** Section 3.1), 6-oxo-1,6 dihydropyrimidine (**P1**-**P10;** Section 3.2) and 2-(quinolin-8-yloxy)acetohydrazone (**QC1**-**QC11;** Section 3.3), as well as benzimidazoles and imidazoles (Section 3.1) were evaluated for their effects on the malarial parasite, preliminary toxicity profile, pharmacokinetic, mechanistic properties, as well as effects on *Anopheles* mosquito and *Artemia* nauplii assessed.

3.1 Azole compounds

3.1.1 Effects on the malarial parasite

QB, benzimidazole and imidazole compounds were evaluated for their effects on the malarial parasite by determining their *in vitro* antimalarial activity, morphological effects on the parasite and combined effects with quinine.

3.1.1.1 *In vitro* antimalarial activity (pLDH assay)

During screening at 50 µM, the tested compounds displayed variable inhibitory activity (parasite growth range: 0.00% and 72.57%) compared to quinine (14.50%) (Table 3.1). One benzimidazole (omeprazole), four imidazoles (ketoconazole, tioconazole, clotrimazole and itraconazole) and two **QB** (**QB5** and **QB8**) compounds showed potential as antimalarials by inhibiting more than 60% parasite growth. The compounds displayed a dose dependent effect and the IC_{50} values of these compounds were then determined (Figure 3.1). Unfortunately, none of the novel **QB** compounds showed significant activity in comparison to the imidazoles, itraconazole (2.48 ± 0.37 µM) and tioconazole (4.90 ± 0.41 µM) (Table 3.1; Figure 3.1) in comparison to quinine (p value = 0.0286). Itraconazole exhibited close to 2-fold higher activity in comparison to tioconazole and the activity of both imidazoles was less than that of quinine (0.18 ± 0.01 µM). Ketoconazole (16.32

± 3.28 µM) and clotrimazole (16.67 ± 4.39 µM) also showed promising antimalarial potential. Out of the 12 **QB** novel compounds, **QB8** was the most active (35.37 ± 0.67 µM) (Table 3.1).

Table 3.1: The *in vitro* antimalarial activity (pLDH assay) of **QB**, benzimidazole, imidazole compounds after screening at 50 µM.

Compound / Control	50 µM (% viability ± S.D.)	IC_{50} (µM ± S.D.)	Compound /Control	50 µM (% viability ± S.D.)	IC_{50} (µM ± S.D.)
QB1	57.75 ± 7.54	ND	**QB12**	47.96 ± 10.53	ND
QB2	44.57 ± 4.50	ND	**Mebendazole**	63.71 ± 5.08	ND
QB3	42.22 ± 6.93	ND	**Benzimidazole**	65.62 ± 8.95	ND
QB4	57.04 ± 6.01	ND	**Omeprazole**	**16.97 ± 1.38**	**38.80 ± 8.48**
QB5	**30.16 ± 3.51**	**77.81 ± 0.71**	**Albendazole**	52.16 ± 7.09	ND
QB6	52.41 ± 4.73	ND	**Ketoconazole**	**3.75 ± 1.76**	**16.32 ± 3.28**
QB7	66.13 ± 3.86	ND	**Tioconazole**	**0.00 ± 2.32**	**4.90 ± 0.41**
QB8	**31.66 ± 5.70**	**35.37 ± 0.67**	**Metronidazole**	66.79 ± 7.90	ND
QB9	49.84 ± 10.47	ND	**Fluconazole**	65.71 ± 7.02	ND
QB10	72.57 ± 5.13	ND	**Clotrimazole**	**0.00 ± 6.41**	**16.67 ± 4.39**
QB11	48.19 ± 10.12	ND	**Itraconazole**	**20.11 ± 5.42**	**2.48 ± 0.37**
Quinine	**14.50 ± 3.65**	**0.18 ± 0.01**	ND= Not determined		

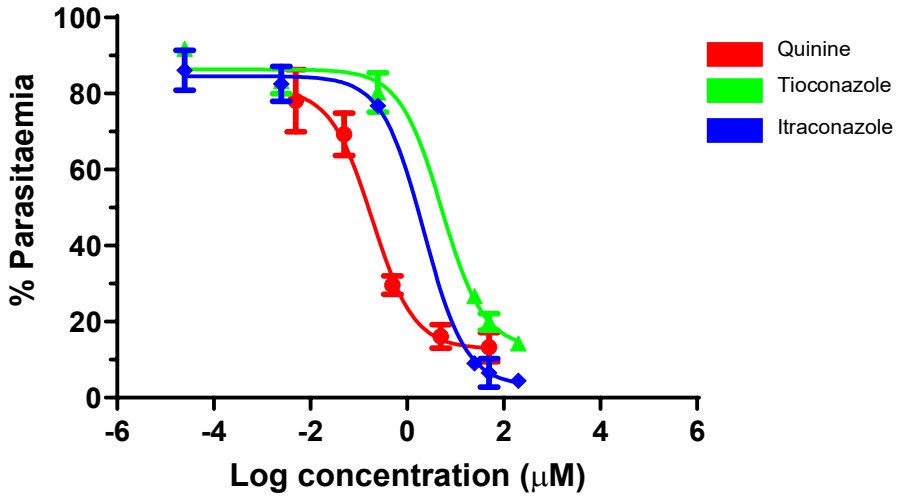


Figure 3.1: Log sigmoidal dose response curve of the antimalarial activity of the two most active compounds, tioconazole and itraconazole against NF54 *Plasmodium* strain, in comparison to quinine.

3.1.1.2 Antimalarial morphological effects

The effects of the two most active compounds (tioconazole and itraconazole, Section 3.1.1.1) were investigated on the intra-erythrocytic life cycle of *P. falciparum* by incubating them with synchronized parasites over 48 hours in comparison to quinine. It was noted that both compounds induced a decline in percentage parasitaemia over 48 hours (Figure 3.2E). Similarly to the untreated control, there was no delay in progression from the ring to the trophozoite stage after 18 hours for tioconazole, itraconazole and quinine flasks (Figure 3.2B) however, quinine and tioconazole-treated trophozoites did not progress into schizonts, and gametocytes were not observed throughout the treatment period in the tioconazole-treated flask.

A small percentage of itraconazole treated parasites only progressed into schizonts after 32 hours (0.10%) (Figure 3.2C) and there were gametocytes observed in comparison to the untreated control and quinine. Tioconazole and quinine treated parasites did not show progression into the next life cycle, shown by 0% new rings after 48 hours; while itraconazole showed 0.14% new rings

when compared to the untreated control (1.28%) (Figure 3.2A), which could imply that its mechanism of action is not on the parasite ring stage.

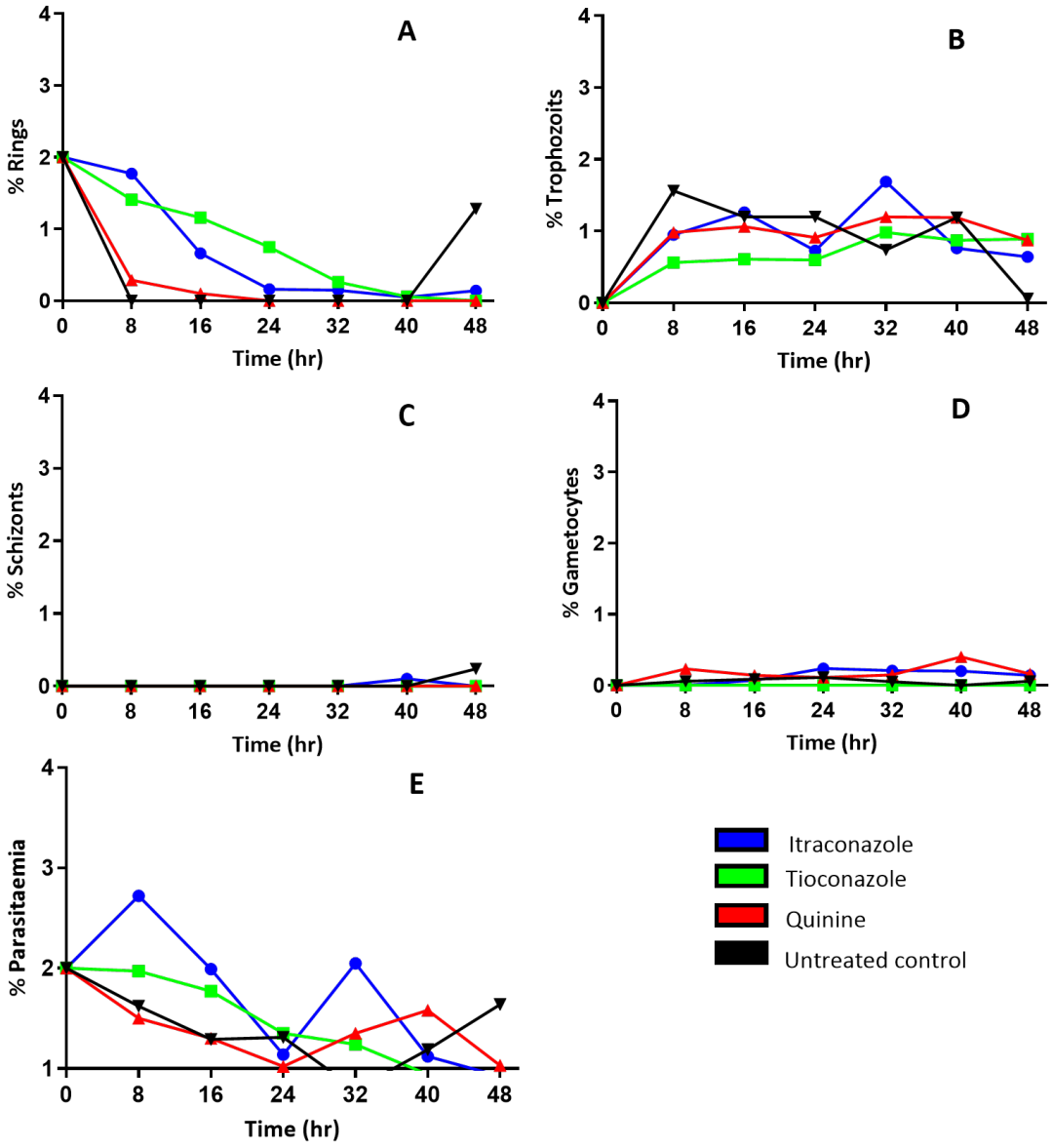
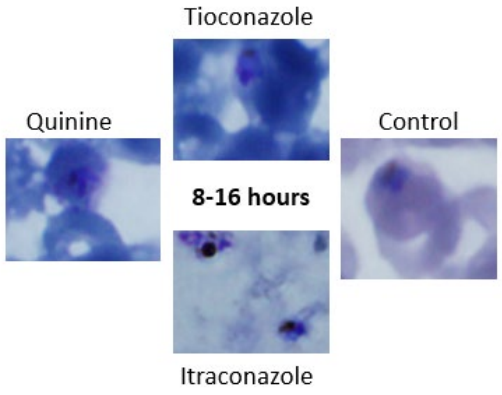
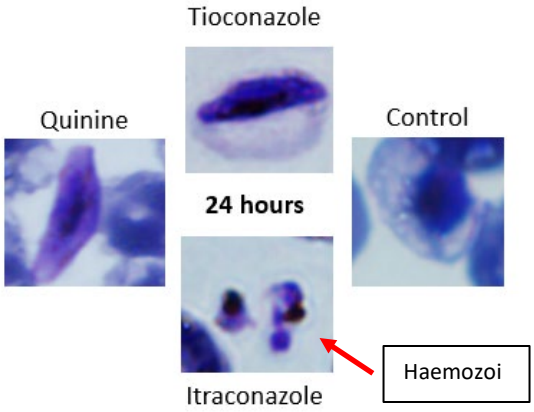
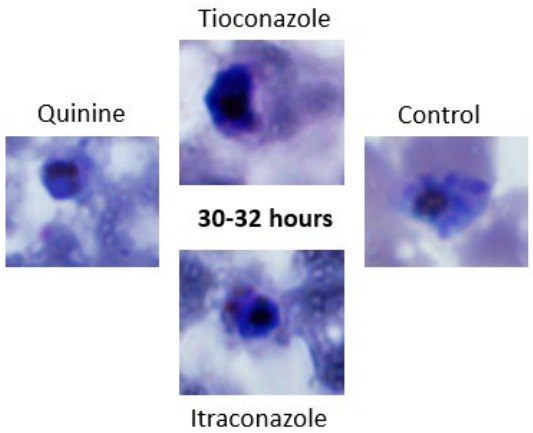
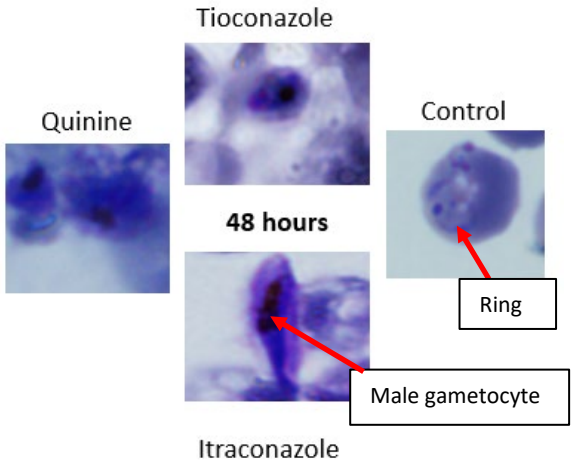


Figure 3.2: Changes in rings (A), trophozoites (B), schizonts (C) gametocytes (D) and total parasitaemia (E) of tioconazole, itraconazole, quinine and untreated control over 48 hours.

The tioconazole-treated trophozoites appeared darker in colour in comparison to the untreated control and quinine after 30 hours (T_{30}). The itraconazole-treated trophozoites appeared

contracted and stressed after 24 hours (T_{24}) in comparison to the untreated control and quinine (Table 3.2). In comparison to quinine, haemozoin in the trophozoites of both tioconazole and itraconazole appeared more scattered rather than being in clumped units. Less effect on the food vacuole of tioconazole and itraconazole treated parasites was observed in comparison to quinine treated parasites, which showed disrupted membranes of the cytosol and food vacuole.

Table 3.2: The morphological effects of tioconazole, itraconazole and quinine (IC_{50} concentration) over 48 hours.

Morphological effects between 8-16 hours	Morphological effects after 24 hours
	
Morphological effects between 30-32 hours	**Morphological effects after 48 hours**
	

Both male and female gametocytes were observed in the untreated control flask and in terms of morphological appearance, the male gametocytes in the itraconazole treated flask appeared enlarged in size in comparison to the untreated control flasks after 40 hours (T_{40}) and no difference was observed with the female gametocytes in comparison to the untreated flasks (Table 3.2).

3.1.2 Preliminary toxicity profile

To establish the toxicity profile of **QB**, benzimidazole and imidazole compounds, their effects on the RBCs, HEK-293 and K562 cell lines were evaluated. The toxicity profile of **QB**, benzimidazole and imidazole compounds was not tested against the human neuroblastoma (SH-SY5Y) cell line.

3.1.2.1 Haemolytic effects

All test compounds and the antimalarial drug control, quinine, displayed negligible haemolysis of the RBCs at 50 µM (0.1 ± 0.01%) in comparison to Triton X-100™ (positive control), which induced 100% haemolysis at 50 µM.

3.1.2.2 Cytotoxic effects on mammalian cells

3.1.2.2.1 *In vitro* cytotoxic activity (MTT assay)

The MTT mitochondrial assay was used to investigate the toxicity of the **QB**, benzimidazole and imidazole compounds on the human embryonic kidney epithelial cells (HEK-293) and myelogenous leukaemic (K562) cell lines. On the HEK-293 cells, the % cellular viability ranged between 32.25 ± 2.70% and 97.86 ± 7.39%, with tioconazole being the most active compound and fluconazole the least active compound. Tioconazole had an IC_{50} of 42.86 ± 3.92 µM in comparison to cisplatin (202.45 ± 11.95 µM) (Table 3.3).

Table 3.3: The cytotoxicity profile of **QB**, benzimidazole, imidazole compounds at 50 µM on HEK-293 and K562 cell lines.

Compound	HEK-293	K562	Compound	HEK-293	K562
	% Cell viability ± S.D.			% Cell viability ± S.D.	
QB1	59.16 ± 5.51	88.51 ± 7.81	**QB12**	70.13 ± 6.92	82.40 ± 7.50
QB2	88.06 ± 3.25	97.00 ± 14.19	**Mebendazole**	**63.14 ± 8.30**	**48.46 ± 7.04**
QB3	80.28 ± 3.77	95.09 ± 6.62	**Benzimidazole**	82.61 ± 8.85	88.17 ± 7.63
QB4	82.62 ± 5.69	85.11 ± 1.90	**Omeprazole**	93.57 ± 11.43	80.47 ± 1.08
QB5	97.09 ± 15.05	84.89 ± 11.28	**Albendazole**	ND	ND
QB6	76.43 ± 9.64	73.73 ± 6.71	**Ketoconazole**	54.19 ± 2.48	58.99 ± 6.39
QB7	80.03 ± 7.28	77.71 ± 9.96	**Tioconazole**	**32.25 ± 2.70**	**27.30 ± 1.66**
QB8	72.63 ± 10.29	59.51 ± 10.02	**Metronidazole**	92.87 ± 4.51	90.57 ± 4.18
QB9	90.72 ± 2.63	72.06 ± 1.80	**Fluconazole**	97.86 ± 7.39	86.36 ± 3.12
QB10	78.26 ± 6.36	74.10 ± 5.13	**Clotrimazole**	41.12± 17.19	ND
QB11	79.57 ± 6.40	72.45 ± 4.89	**Itraconazole**	87.77 ± 10.06	ND
Cisplatin	55.64 ± 15.82	49.27 ± 16.40	**Quinine**	87.40 ± 1.75	100 ± 13.45

ND = Not determined

Similarly, the activity of these compounds on the K562 cell line ranged between 27.30% and 97.00%. Tioconazole and mebendazole were the two most active compounds on K562 cell line, with a % cellular viability of 27.30 ± 1.66% and 48.46 ± 7.04%, respectively. The IC_{50} of tioconazole was 15.01 ± 1.79 µM and that of mebendazole was 3.98 ± 0.49 µM against K562 cells in comparison to the positive control, cisplatin (71.22 ± 7.63 µM) (Figure 3.3). In comparison to

cisplatin, the activity of both tioconazole and mebendazole was not statistically significant (p= 0.1).

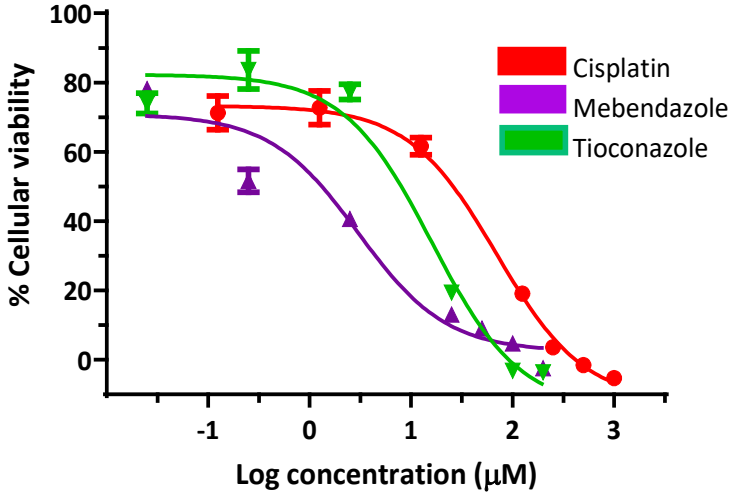


Figure 3.3: Log sigmoidal dose-response curve of the anticancer activity of the two most active compounds, tioconazole and mebendazole against K562 cell line in comparison to cisplatin.

3.1.2.2.2 Selectivity index

The safety indices of **QB**, benzimidazole and imidazole compounds were calculated to determine the selectivity for human epithelial kidney (HEK-293) cells or the malaria parasite (NF54) (Table 3.4). Overall, the compounds displayed cytotoxicity rather than selectivity for the malarial parasite, shown by safety indices ranging between >1.41 and 35.39 in comparison to quinine (620.28). Itraconazole displayed the best safety profile (safety index: 35.39) and proved to be the most potent compound among **QB**, benzimidazole and imidazole compounds in inhibiting *P. falciparum* (NF54).

Table 3.4: The relationship between **QB**, benzimidazole, imidazole compounds toxicity profiles with corresponding antimalarial activity.

Compound	IC_{50} (µM)		Safety index	Compound	IC_{50} (µM)		Safety index
	HEK-293	NF54			HEK-293	NF54	
QB1	>50	>50	1.00	**Mebendazole**	>50	>50	1.00
QB2	>50	>50	1.00	**Benzimidazole**	>50	>50	1.00
QB3	>50	>50	1.00	**Omeprazole**	>50	38.80	>1.29
QB4	>50	>50	1.00	**Albendazole**	ND	>50	ND
QB5	>50	77.81	0.70	**Ketoconazole**	>50	16.32	>3.064
QB6	>50	>50	1.00	**Tioconazole**	**42.86**	**4.90**	**8.75**
QB7	>50	>50	1.00	**Metronidazole**	>50	>50	1.00
QB8	>50	35.37	>1.41	**Fluconazole**	>50	>50	1.00
QB9	>50	>50	1.00	**Clotrimazole**	41.12	16.67	2.47
QB10	>50	>50	1.00	**Itraconazole**	87.77	2.48	35.39
QB11	>50	>50	1.00	**Quinine**	**111.65**	**0.18**	**620.28**
QB12	>50	>50	1.00				

3.1.3 Combination studies

3.1.3.1 Combination effects on malaria parasite

The two most active compounds (tioconazole and itraconazole) against NF54 *Plasmodium* strain were used in combination with quinine to further investigate their potential as antimalarial drugs (Figure 3.4)**.**

The combination of tioconazole and quinine was synergistic at a higher concentration of quinine compared to tioconazole (40: 0.075) (Figure 3.4). As the concentration of quinine was reduced and that of tioconazole increased, the interaction became additive (ΣFIC: 0.75 – 1.25). This

interaction became antagonistic as the concentration of quinine was further reduced and that of tioconazole increased (Ratio of quinine: tioconazole as follows: E = 0.1:50 µM, F = 0.01:100 µM and G = 0.001:150 µM). Overall, tioconazole and quinine demonstrated an antagonistic interaction (ΣFIC = 1.63) (Figure 3.4).

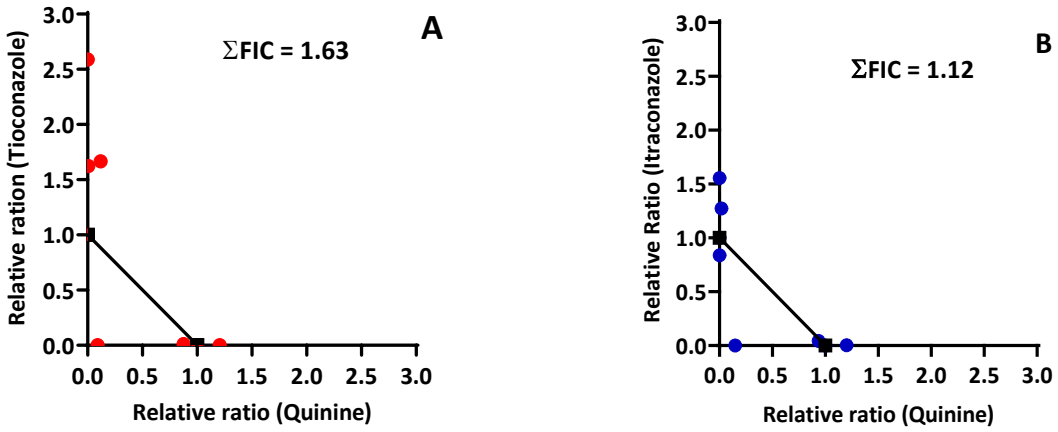


Figure 3.4: Isobolograms showing the drug interactions between quinine and the two most active compounds, tioconazole (A) and itraconazole (B).

The combination of itraconazole and quinine showed an overall additive interaction (ΣFIC = 1.12). A high concentration of quinine versus a low concentration of itraconazole showed a synergistic interaction, which became additive and eventually antagonistic as the concentration of quinine was reduced and that of itraconazole increased (Figure 3.4).

3.1.3.2 Combination effects on mammalian cells

The two most active compounds against K562 cells, tioconazole and mebendazole, were combined with cisplatin to assess their combined effect as synergistic, additive or antagonistic. The combination of tioconazole and cisplatin displayed an overall antagonistic interaction (∑FIC 2.37, Figure 3.5), especially at high concentrations of both compounds (concentration ratio of cisplatin: tioconazole as follows: A = 1000:0 µM, B = 500:0.05 µM, F = 5:50 µM and G = 0.5:100

µM). There was an additive interaction at 20 times cisplatin concentration to that of tioconazole and a synergistic interaction at 2 times cisplatin concentration in one of the two independent experiments done.

The combination of mebendazole and cisplatin on K562 cells displayed an additive interaction overall (∑FIC 0.99; Figure 3.5), however, synergistic, additive and antagonistic interactions across different concentration ratios were observed. Antagonistic interaction resulted from high concentrations of one compound versus the low of another compound (concentration ratio of cisplatin: mebendazole as follows: B = 500:0.1 µM, C = 250:0.5 µM and F = 5:25 µM). Synergistic interactions were observed with concentration ratios where cisplatin was 100 times lower and 20 times higher than that of mebendazole while additive interactions were observed at higher concentrations of quinine to mebendazole.

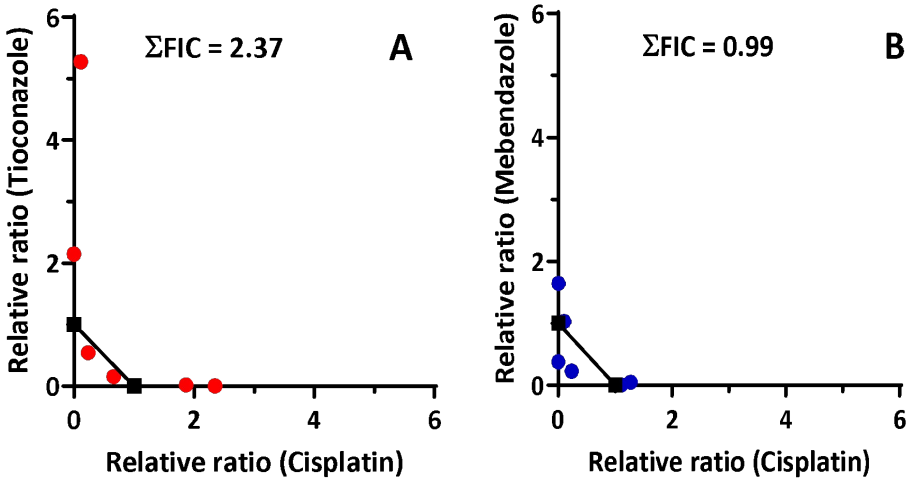


Figure 3.5: Isobolograms showing the drug interactions between cisplatin and the two most active compounds, tioconazole (A) and mebendazole (B) on K562 cell line.

3.1.4 *Artemia* lethality

To further establish the toxicity profile of **QB**, benzimidazole and imidazole compounds, the *Artemia* lethality assay was conducted on *A. franciscana* nauplii as a whole organism in comparison to their effects on *An. Arabiensis* larvae.

3.1.4.1 Effects on *Artemia* nauplii viability

QB5 (86.58 ± 3.17%), **QB9** (92.56 ± 8.42%), **QB11** (56.46 ± 12.96%) and tioconazole (65.30 ± 9.88%) showed more than 50% *A. franciscana* nauplii mortality after 24 hours of treatment at 50 µM. After 48 hours of exposure, more than 60% mortality was observed with **QB5** (97.69 ± 2.10%), **QB9** (100 ± 0.00%), **QB11** (94.29 ± 9.89%), mebendazole (92.94 ± 12.23%), albendazole (92.70 ± 8.85%), tioconazole (99.54 ± 0.80%), clotrimazole (85.78 ± 13.57%) and itraconazole (86.82 ± 2.07%), (Figure 3.6).

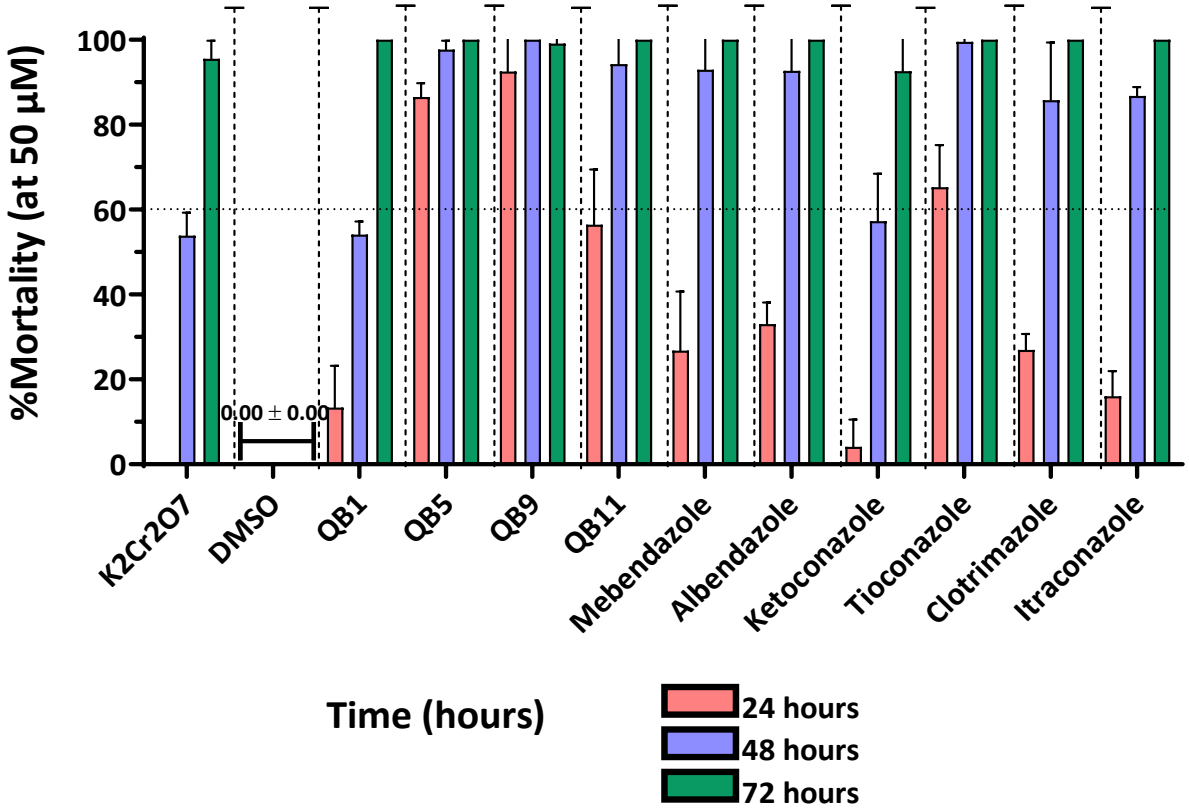


Figure 3.6: The percentage mortality of *A. franciscana* nauplii after 24, 48 and 72 hours treatment with **QB**, benzimidazole and imidazole compounds at 50 µM in triplicate compared to potassium dichromate.

Toxicity on *A. franciscana* nauplii after 72 hours of exposure was observed with **QB1** (100 ± 0.00%), **QB5** (100 ± 0.00%), **QB9** (99.10 ± 1.56%), **QB11** (100 ± 0.00%), mebendazole (100 ± 0.00%), albendazole (100 ± 0.00%), ketoconazole (92.63 ± 11.62%), tioconazole (100 ± 0.00%), clotrimazole (100 ± 0.00%) and itraconazole (100 ± 0.00%). These mortality rates were similar to that of the positive control, potassium dichromate (95.52 ± 4.29%) (Figure 3.6).

Based on the LC_{50} values, itraconazole was the most toxic compound (LC_{50} value: 1.86 µM) while albendazole proved to be the least toxic compound (LC_{50} value: 8.08 µM) in comparison to potassium dichromate (LC_{50} value: 3.04 µM) Table 3.5. In comparison to potassium dichromate, the activity of **QB5, QB9,** mebendazole, albendazole, ketoconazole, clotrimazole and itraconazole was not significant ($p>0.05$).

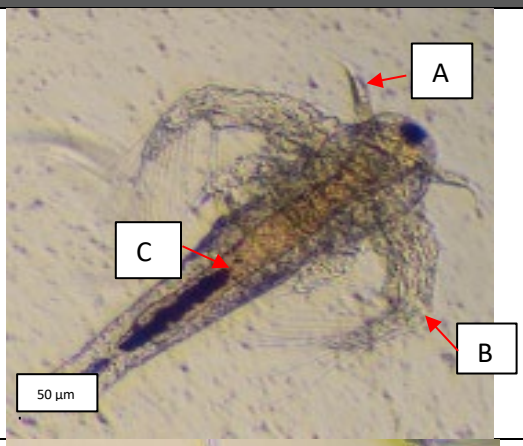
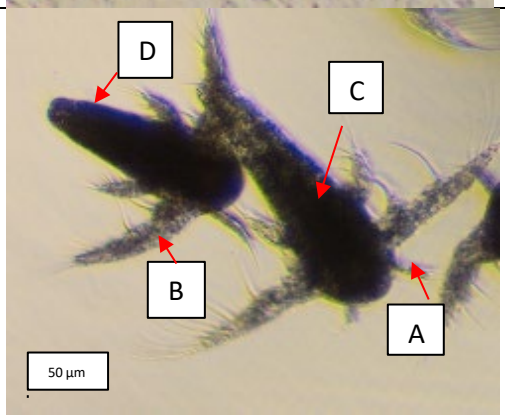
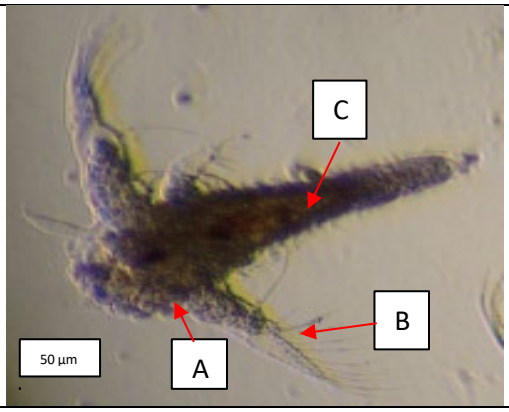
Table 3.5: LC_{50} values of active compounds against *A. franciscana* nauplii after 72 hour exposure.

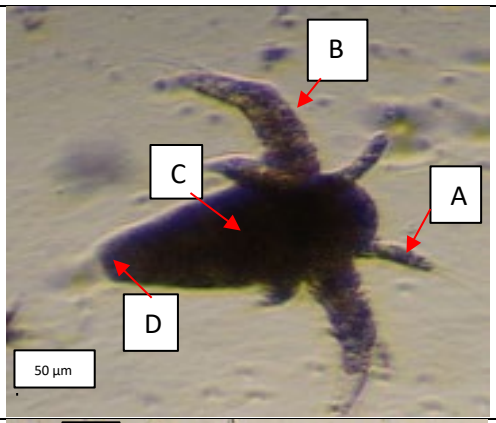
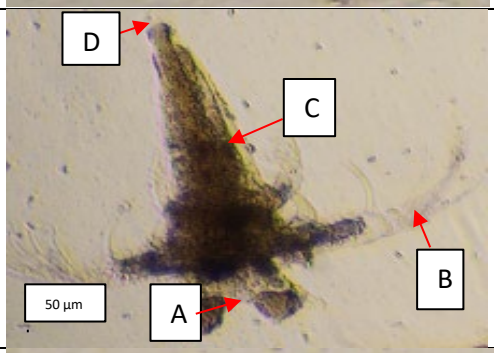
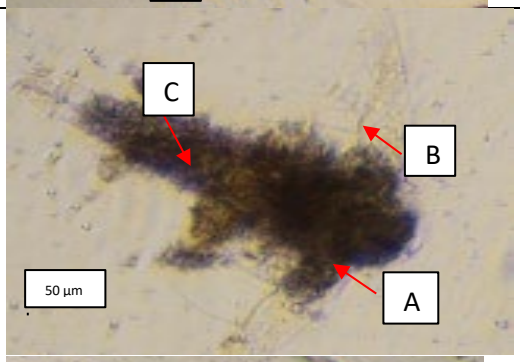
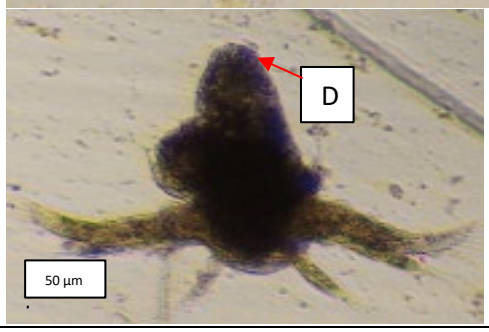
Compound/Control	LC_{50} (µM)	Compound/Control	LC_{50} (µM)
QB1	ND	**Albendazole**	8.08
QB5	5.56	**Ketoconazole**	6.71
QB9	3.80	**Tioconazole**	5.98
QB11	ND	**Clotrimazole**	3.32
Mebendazole	6.00	**Itraconazole**	1.86
Potassium dichromate	3.04	ND = Not determined	

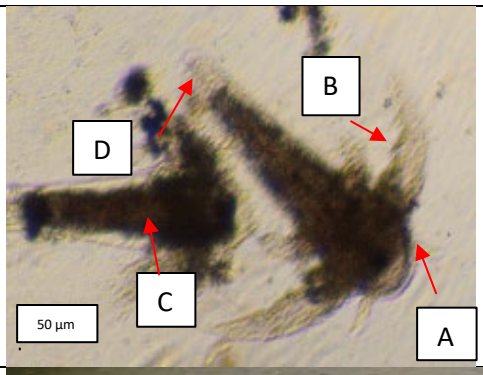
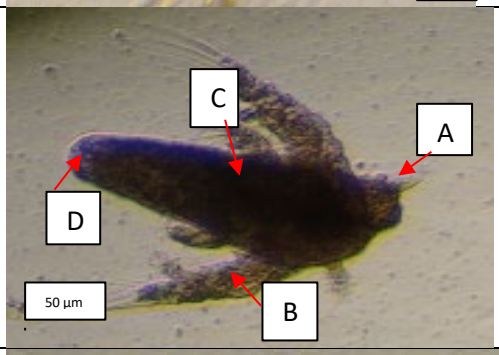
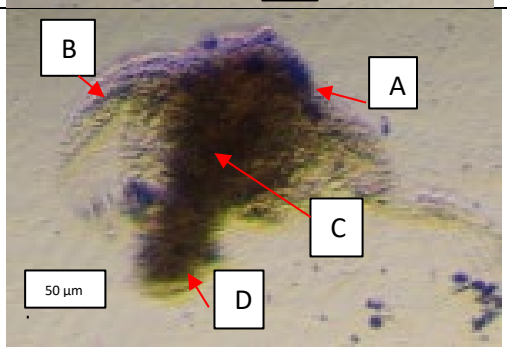
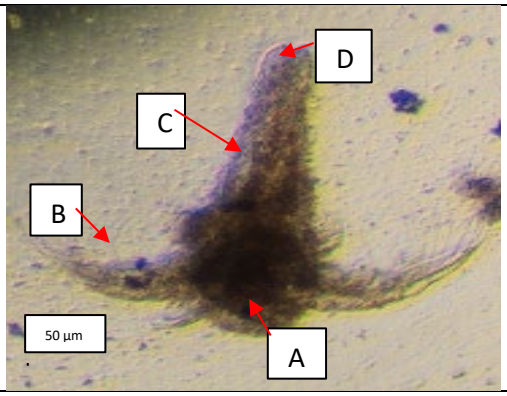
3.1.4.2 Morphological effects on *Artemia*

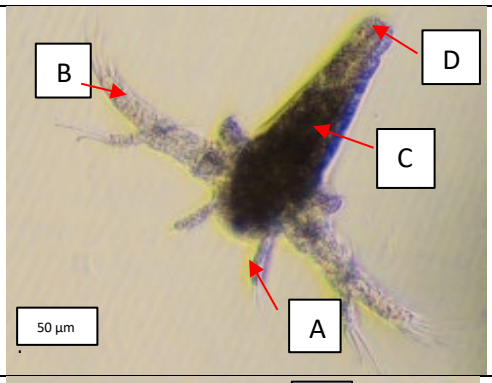
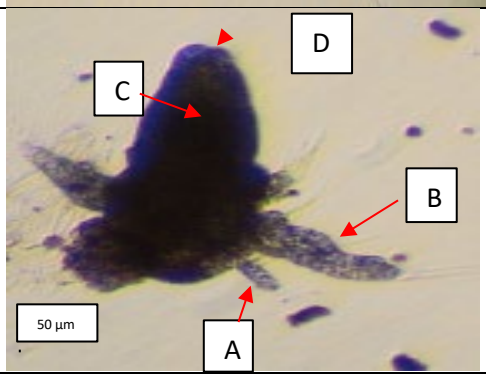
Morphological changes including size of the nauplii, antennae, swimming appendages and pigmentation of the cervical region were seen with the cytotoxic compounds after 72 hours incubation (Table 3.6).

Table 3.6: Morphological effects of **QB**, benzimidazole, imidazole compounds on *A. franciscana* nauplii after 72 hours incubation in comparison to potassium dichromate (positive control) and DMSO (untreated control).

Treatment	Morphological changes	*Artemia franciscana* nauplii
Untreated control	*There was no change in size and colour of the cervical region (C). *Antennae (A) and swimming appendages moved freely (B).	
Potassium dichromate (positive control)	*Nauplii (D), antennae (A) and swimming appendages (B) shrunk. *Colour of the cervical region darkened and thickened (C).	
QB1	* Minimal effect was seen on the swimming appendages (B). *Antennae (A) shrunk. *Colour of the cervical region darkened (C).	

QB5	*Colour of the cervical region darkened and thickened (C). * Nauplii shrunk (D). * Minimal effect was seen on the swimming appendages (B) and antennae (A).	
QB9	*Thickened and darkened the cervical region (C). *Nauplii (D) and antennae (A) shrunk. * Minimal effect was seen on the swimming appendages (B).	
QB11	*Thickened and darkened the cervical region (C) * Nauplii (D) and antennae (A) shrunk. * Swimming appendages shrunk (B).	 

Mebendazole	* Darkened the cervical region (C). * Nauplii (D), appendages (B) and antennae (A) shrunk.	
Albendazole	* Darkened and thickened the cervical region (C). * Nauplii (D) shrunk. *Minimal effect was seen on the swimming appendages (B) and antennae (A).	
Ketoconazole	* Swimming appendages shrunk (B). * Darkened and thickened the cervical region (C). * Nauplii (D) and antennae (A) shrunk.	
Tioconazole	* Antennae (A), swimming appendages (B) and nauplii (D) shrunk. * The cervical region (C) darkened and thickened.	

Clotrimazole	* Minimal effect was seen on the swimming appendages (B) and antennae (A). * Darkened the cervical region (C). * Nauplii (D) shrunk.	
Itraconazole	* Darkened and thickened the cervical region (C). * Antennae (A), swimming appendages (B) and nauplii (D) shrunk.	

3.1.5 Pharmacokinetic and mechanistic properties

The pharmacokinetic properties of **QB**, benzimidazole and imidazole compounds were evaluated using the Lipinski's rule of five. Ionisation and absorption as a function of pH was also used to further evaluate whether these compounds accumulated in the RBCs (pH 7.4) or in the food vacuole of the parasite (pH 5). To evaluate other potential mechanisms of action, the compounds were also evaluated for possible DPPH free radical scavenging and iron chelating activity.

3.1.5.1 Drug-likeness - permeability and solubility predictions of the QB, benzimidazole and imidazole compounds

All **QB** and benzimidazole compounds complied with the Lipinski's rule of five, shown by less than 500 g/mol molecular weight, less than 10 hydrogen bond donors, less than 5 hydrogen bond acceptors and less than 5 log P. These predictions proved all **QB** and benzimidazole compounds to be drug-like when compared to quinine, which has a good solubility.

For imidazoles, ketoconazole, tioconazole, clotrimazole and itraconazole proved to not possess drug-like properties, showed by violation of one of the Lipinski's rule of five parameters (Table 3.7).

Table 3.7: Permeability and solubility predictions of **QB**, benzimidazole and imidazole compounds using the Lipinski's rule of five.

Compound	MW (g/mol) <500	Hydrogen bond donors <10	Hydrogen bond acceptors <5	Log P <5	Rule of five violations
QB1	263.28	1	2	3.97	0
QB2	279.73	1	2	4.43	0
QB3	324.18	1	2	4.60	0
QB4	275.31	1	3	3.67	0
QB5	259.31	1	2	4.34	0
QB6	263.28	1	2	3.97	0
QB7	279.73	1	2	4.43	0
QB8	324.18	1	2	4.60	0
QB9	275.31	1	3	3.67	0
QB10	259.31	1	2	4.34	0
QB11	371.18	1	2	4.76	0
QB12	371.18	1	2	4.76	0
Mebendazole	295.30	2	4	3.26	0
Benzimidazole	118.14	1	1	1.26	0
Omeprazole	345.42	1	5	2.43	0
Albendazole	265.33	2	3	3.21	0
Ketoconazole	531.43	0	6	4.19	2
Tioconazole	387.70	0	2	5.30	1
Metronidazole	171.16	1	4	-0.46	0
Fluconazole	306.28	1	5	0.56	0

Clotrimazole	344.84	0	1	5.84	1
Itraconazole	705.64	0	9	7.31	3
Quinine	324.42	1	4	2.51	0

3.1.5.2 Predicted ionisation and absorption as a function of pH

A pH similar to that of RBC cytosol (pH 7.4) and that of the digestive vacuole of the parasites (pH 5) was used to predict the ionisation of the **QB**, benzimidazole and imidazole compounds through the compound's predicted pKa values (Table 3.8).

Table 3.8: Predicted ionisation and absorption of the **QB**, benzimidazole and imidazole compounds in comparison to quinine (All values predicted using ChemAxon).

Compound	pKa	% ionization - pH 7.4	% ionization - pH 5	% GIT Absorption
QB1	10.53; 2.25	99.93	99.82	0.07
QB2	10.51; 2.23	99.94	99.83	0.06
QB3	10.50; 2.23	99.92	99.83	0.08
QB4	10.56; 2.26	99.93	99.82	0.07
QB5	10.54; 2.26	99.93	99.82	0.07
QB6	10.52; 2.24	99.92	99.83	0.08
QB7	10.50; 2.23	99.92	99.83	0.08
QB8	10.49; 2.23	99.92	99.83	0.08
QB9	10.55; 2.27	99.93	99.82	0.07
QB10	10.54; 2.26	99.93	99.82	0.07
QB11	10.50; 2.23	99.92	99.83	0.08
QB12	10.50; 2.23	99.92	99.83	0.08
Mebendazole	8.44; 3.93	91.56	92.08	8.44
Benzimidazole	12.25; 5.79	97.59	13.9	2.41
Omeprazole	9.29; 4.77	98.51	62.78	1.49
Albendazole	9.51; 4.27	99.16	84.17	0.84

Compound	pKa	% ionization - pH 7.4	% ionization - pH 5	% GIT Absorption
Clotrimazole	6.26	93.19	5.17	6.81
Itraconazole	3.91	99.97	92.41	0.03
Quinine	13.89; 9.05	2.21	90.57	97.79
Ketoconazole	6.42	90.47	3.35	9.53
Tioconazole	6.48	89.33	3.23	10.67
Metronidazole	15.41; 3.03	100	98.95	0.00
Fluconazole	12.68; 2.3	100	99.8	0.00

3.1.5.3 DPPH free radical scavenging activity

None of the compounds (**QB**, benzimidazole and imidazole) exhibited potency for free radical scavenging activity (0.10 ± 0.01%) at 50 µM when compared to ascorbic acid (86.13 ± 1.43%; IC_{50} value: 25.58 ± 2.64 µM).

3.1.5.4 Iron chelating activity

None of the compounds (**QB**, benzimidazole and imidazole) showed potential as iron chelators or chelating iron as a mechanism of action; with activity ranging between 0.10 ± 0.01% and 7.81 ± 1.70% in comparison to EDTA (71.58 ± 4.50%) at 50 µM.

3.1.6 Effects on the *Anopheles* mosquito

The effects of **QB**, benzimidazole and imidazole compounds on the *Anopheles* mosquito were investigated against third instar *An. arabiensis* larvae and eggs.

3.1.6.1 Larvicidal effects

None of the compounds (**QB**, benzimidazole and imidazole) exhibited inhibitory potential when screened at 0.5 µM against third instar *An. arabiensis* larvae (KWAG) between 24 and 72 hours in comparison to (Figure 3.7; Figure 3.8).

Clotrimazole showed the most activity after 24 hours (11.67 ± 5.77%), while omeprazole showed the most activity after 48 and 72 hours, killing 53.33 ± 2.89% of *An. arabiensis* larvae after both time intervals in comparison to DDT (100 ± 0.00%). Among the known azoles, **QB10** showed the most activity with % mortality of 48.33 ± 2.89% and 50 ± 0.00% after 48 and 72 hours respectively among the **QB** compounds.

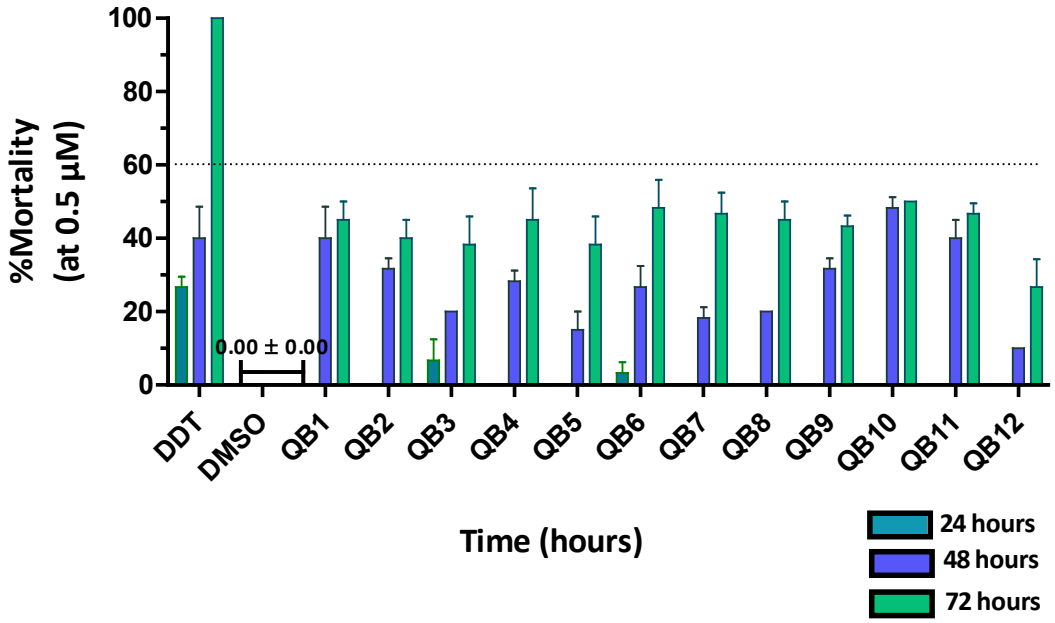


Figure 3.7: The percentage mortality of *An. arabiensis* larvae between 24 and 72 hours after treatment with **QB** compounds at 0.5 µM compared to DDT.

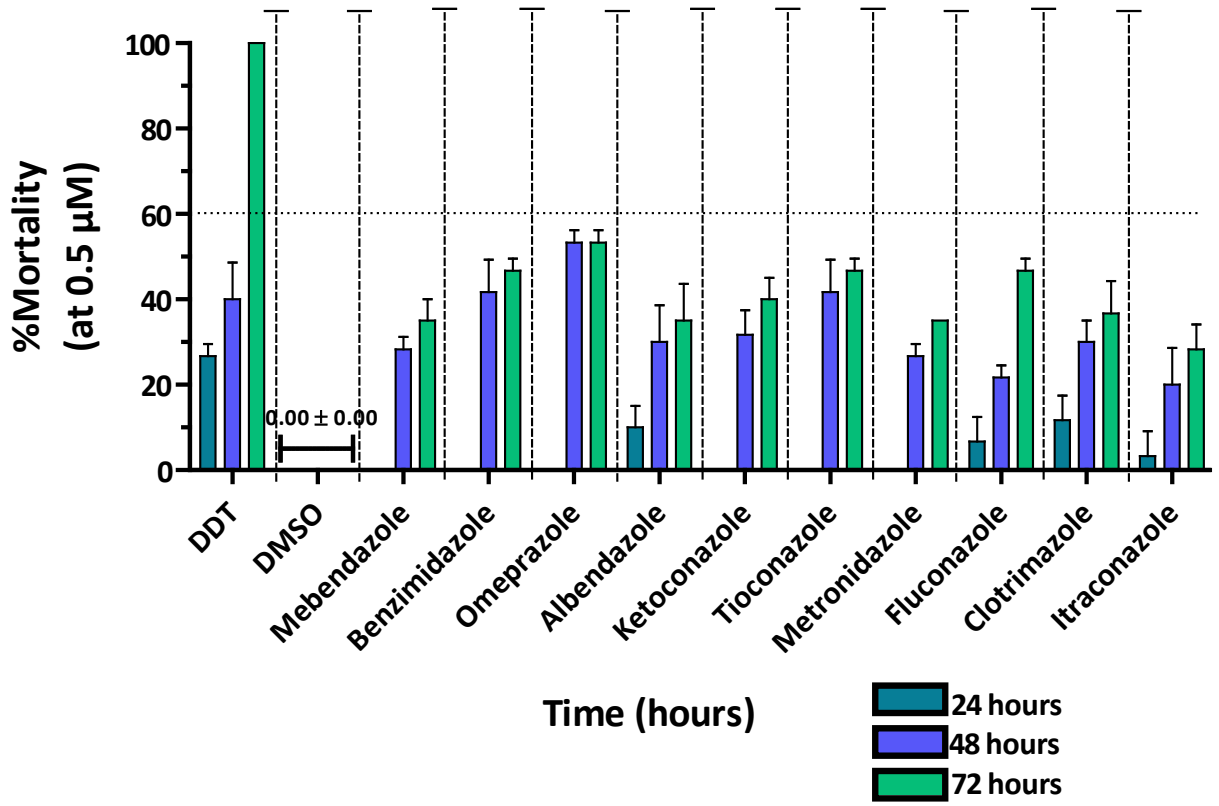
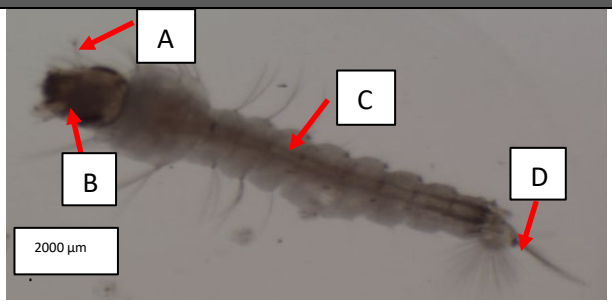
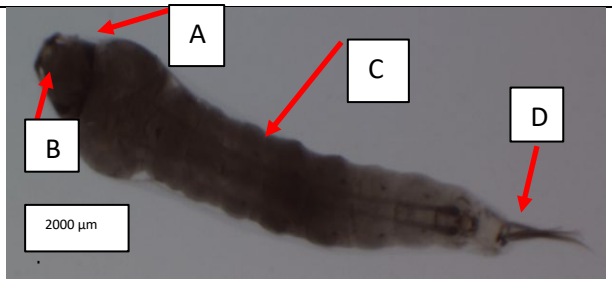
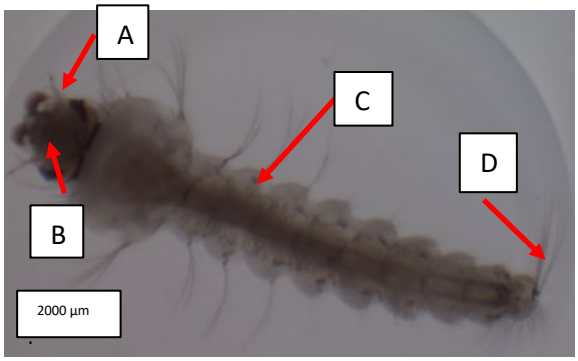
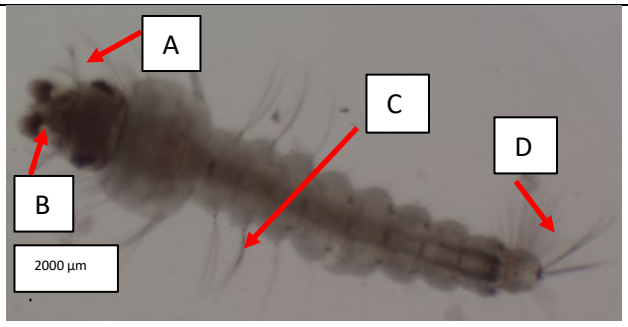


Figure 3.8: The percentage mortality of *An. arabiensis* larvae between 24 and 72 hours after treatment with benzimidazole and imidazole compounds at 0.5 µM compared to DDT.

3.1.6.2 Morphological effects on *Anopheles* larvae

There was no change in the size of the azole-treated larvae and progression to normal growth stages was not halted similar to the untreated control between 24 and 72 hours (Table 3.9). There was also no effect to the development of larvae or progression to the pupae stage while larvae treated with DDT showed morphological changes (such as shrinkage of larvae, antennae and mouth brush) and impairment of movement (Table 3.9). The **QB** compounds-treated larvae also showed no change in size and normal stage progression was not halted.

Table 3.9: Morphological effect of tioconazole and itraconazole (0.5 µM) on *An. arabiensis* larvae in comparison to DDT (0.5 µM).

Treatment	Effect after 72 hours and Morphological changes	*An. arabiensis* (KWAG) larvae
Untreated control	● Alive and no change in mobility. ● No change in size. ● Segments still intact. ● Antennae (A) and mouth brush (B) prominent. ● Abdominal hair (C) and tracheal gills (D) visible.	
DDT (positive control)	● Dead, mobility impaired. ● Shrinkage of larvae. ● Antennae (A) and mouth brush (B) shrunk. ● Abdominal hair (C) and tracheal gills (D) not visible.	
Tioconazole	● Alive and no change in mobility. ● No changes in size. ● Segments still intact. ● Antennae (A) and mouth brush (B) prominent. ● Abdominal hair (C) and tracheal gills (D) still visible.	
Itraconazole	● Alive and no change in mobility ● No changes in size. ● Segments still intact. ● Antennae (A) and mouth brush (B) prominent. ● Abdominal hair (C) and tracheal gills (D) intact.	

3.1.6.3 Ovicidal assay

The ovicidal assay was conducted where KWAG eggs were incubated with **QB**, benzimidazole and imidazole compounds to determine their effect on the ability of the eggs to hatch, their morphological appearance, as well as the development of the hatched larvae. None of the compounds (**QB**, benzimidazole and imidazole) displayed more than 60% inhibition of the eggs from hatching after 72 hours or showed morphological changes on the structure of the eggs, even though eggs only started hatching after 48 hours of treatment with the compounds. After 48 hours, 63.75% of the eggs were inhibited from hatching by **QB6** however, after 72 hours, only 57.50% were inhibited by the same compound in comparison to positive control, formalin which inhibited 100% of the eggs from hatching (Figure 3.9). Ketoconazole, albendazole, clotrimazole and itraconazole showed promising activity after 48 hours, inhibiting more than 50% of the eggs from hatching. After 72 hours, only clotrimazole and itraconazole inhibited more than 40% of the eggs (43.75%) (Figure 3.10). These compounds also had no effect on the hatched larvae and their movement as observed with the 3rd instar larvae.

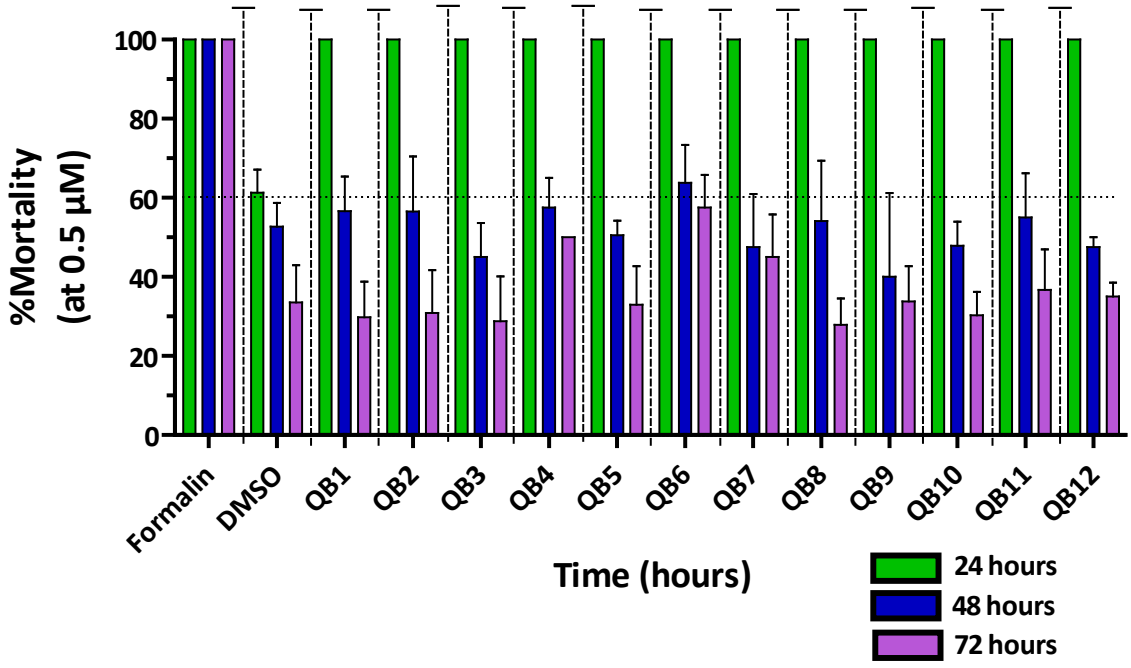


Figure 3.9: The percentage mortality of *An. arabiensis* eggs between 24 and 72 hours after treatment with **QB** compounds at 0.5 µM compared to formalin.

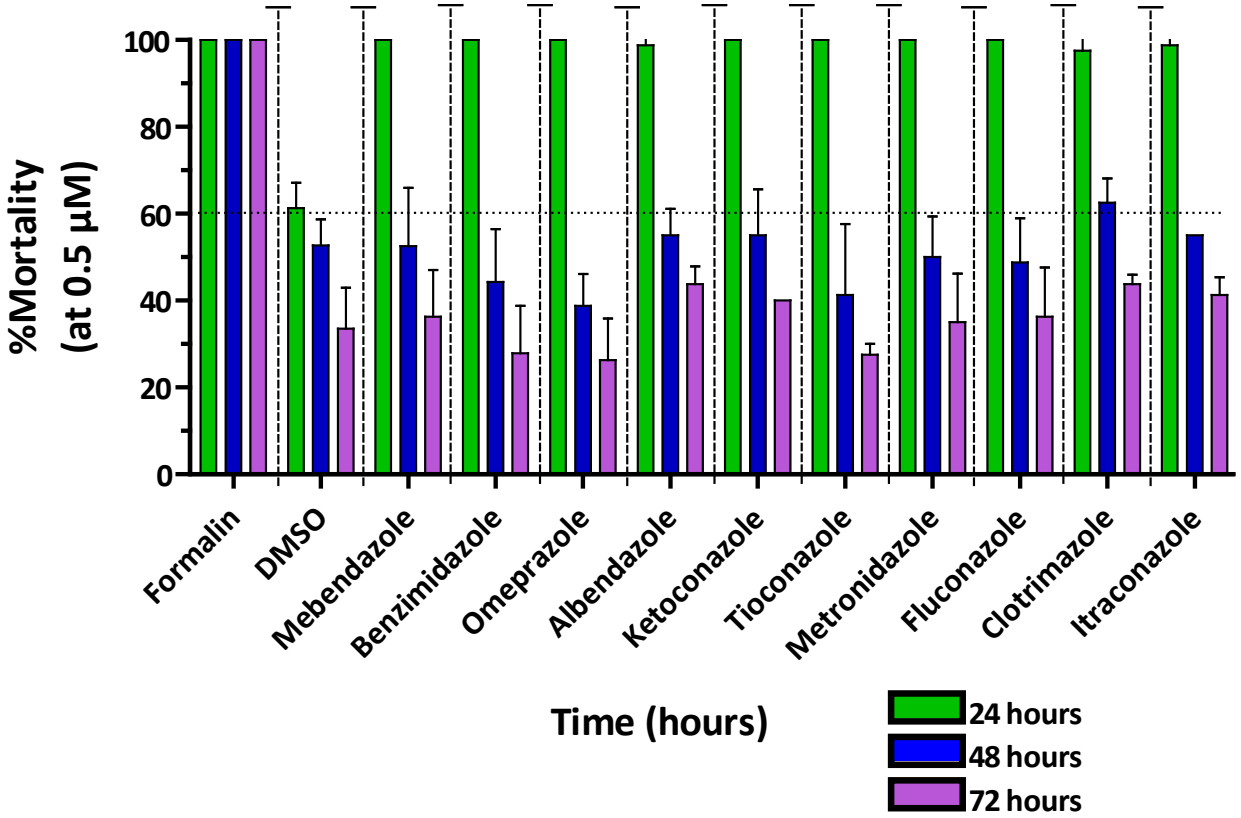


Figure 3.10: The percentage mortality of *An. arabiensis* eggs between 24 and 72 hours after treatment with benzimidazole and imidazole compounds at 0.5 µM compared to formalin.

3.2 6-Oxo-1,6-dihydropyrimidine compounds

3.2.1 *In vitro* effects on the malarial parasite

The antimalarial activity of the **P** compounds was investigated using pLDH assay on the NF54 *Plasmodium* strain.

3.2.1.1 *In vitro* antimalarial activity

The parasite viability of the **P** compounds ranged between 56.78 ± 6.66% and 84.68 ± 3.07% in comparison to quinine (20.51 ± 4.50%) at 50 µM, with compound **P7** as the most active compound (Table 3.10). Unfortunately, as per the criteria set for further investigations the activity of these

compounds was not high enough (60% inhibition or more) to proceed with further evaluations. As a result of the low activity against the malaria parasite, the antioxidant, iron chelation and combination studies were not performed on the **P** compounds.

Table 3.10: The *in vitro* antimalarial activity of **P** compounds after screening at 50 µM.

Compound / Control	Parasite viability (% viability ± S.D.)	Compound / Control	Parasite viability (% viability ± S.D.)
P1	74.85 ± 6.66	**P6**	78.97 ± 25.44
P2	74.46 ± 7.26	**P7**	56.78 ± 7.79
P3	71.56 ± 4.72	**P8**	74.69 ± 14.31
P4	72.58 ± 2.00	**P9**	84.68 ± 3.07
P5	77.04 ± 13.61	**P10**	73.64 ± 7.53
Quinine	20.51 ± 4.50		

3.2.2 Preliminary toxicity profile

To establish the toxicity profile of the **P** compounds, their effects on RBCs, HEK-293, K562 and SH-SY5Y cells were investigated.

3.2.2.1 Haemolytic effects

All **P** compounds and quinine displayed negligible haemolysis against the RBCs at 50 µM ranging from 0.08 ± 0.05 % to 4.62 ± 7.46% in comparison to Triton X-100™ (positive control), which displayed 100% haemolysis.

3.2.2.2 Cytotoxic effects on mammalian cells

3.2.2.2.1 *In vitro* cytotoxic activity (MTT Assay)

All the **P** compounds showed minimal toxicity on HEK-293 cells, with % cell viability ranging between 62.66 ± 5.93% and 93.66 ± 12.36% in comparison to camptothecin (77.61 ± 19.18%). **P9** showed the

most activity while **P7** exhibited the least activity. The % cell viability of **P** compounds against K562 ranged between 52.18 ± 11.50% and 77.91 ± 13.59%, and **P6** exhibited the most activity while **P2** exhibited the least activity in comparison to camptothecin (32.81 ± 13.40%). Against the SH-SY5Y cell line, **P10** was the most active compound and **P9** the least active compound with % cell viability ranging between 52.59 ± 8.78% and 92.58 ± 7.37% in comparison to camptothecin (21.36 ± 10.32%) (Table 3.11).

Table 3.11: The cytotoxicity profile of **P** compounds at 50 µM on HEK-293, K562 and SH-SY5Y cell lines.

Compound / Control	HEK-293	K562	SH-SY5Y
	% Cell viability ± S.D.		
P1	84.00 ± 9.63	69.24 ± 9.55	52.81 ± 8.74
P2	77.29 ± 7.63	77.91 ± 13.59	84.19 ± 14.84
P3	62.79 ± 13.81	54.42 ± 9.11	67.98 ± 12.19
P4	89.38 ± 7.71	63.92 ± 12.91	53.22 ± 9.69
P5	89.60 ± 13.12	61.71 ± 14.91	60.40 ± 10.10
P6	85.74 ± 12.95	52.18 ± 11.50	60.72 ± 12.49
P7	93.66 ± 12.36	63.06 ± 11.93	77.60 ± 9.51
P8	87.26 ± 11.99	60.01 ± 8.54	64.13 ± 8.43
P9	62.66 ± 5.93	59.20 ± 6.35	92.58 ± 7.37
P10	82.57 ± 8.00	62.07 ± 6.44	52.59 ± 8.78
Camptothecin	77.61 ± 19.18	32.81 ± 13.40	21.36 ± 10.32

3.2.3 *Artemia* lethality

To further establish the toxicity profile of **P** compounds, the effects on *A. franciscana* nauplii was evaluated at 50 µM.

3.2.3.1. Effects on *Artemia* nauplii viability

The **P** compounds showed variable toxicity against *Artemia* nauplii at 50 µM, and **P3** showed the most toxicity at all three-time intervals (24, 48 and 72 hours – 70.83 ± 24.87%; 91.17 ± 8.39% and

98.67 ± 2.31%, respectively) of treatment, which similar to the positive control, potassium dichromate, showed an increase in toxicity over time (0.00 ± 0.00%; 56.09 ± 6.44% and 97.41 ± 1.09%) (Figure 3.11). **P2** and **P8** also showed some level of toxicity (44.17 ± 11.62% and 51.30 ± 21.63%, respectively) after 72 hours, while **P6**, **P7** and **P9** showed less toxicity, all possessing percentage mortality below 15% after 24, 48 and 72 hours (Figure 3.11). There were no morphological effects seen with all the **P** compounds at 50 µM between 24 and 72 hours in comparison to potassium dichromate. Further investigations such as determining the LD_{50} could not be done with **P3** as it precipitated at high concentrations.

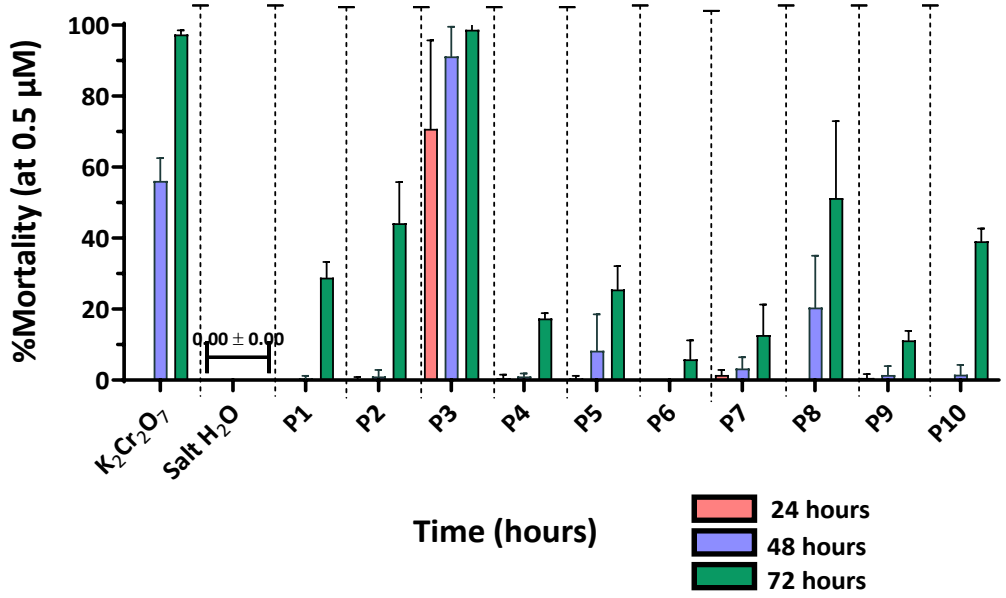


Figure 3.11: The percentage mortality of *A. franciscana* nauplii after 24, 48 and 72 hours treatment with **P** compounds at 50 µM compared to potassium dichromate.

3.2.4 Pharmacokinetic and mechanistic properties

The Lipinski's rule of five was used to evaluate the drug-likeness of **P** compounds, influenced by molecular weight, the number of hydrogen bond donors, the number of hydrogen bond acceptors and the log P value. Ionisation and absorption as a function of pH was also used to further evaluate

whether **P** compounds accumulated in the RBCs (pH 7.4) or in the food vacuole of the parasite (pH 5).

3.2.4.1 Drug-likeness - Permeability and solubility predictions

Only **P6** and **P8** did not comply with the Lipinski's rule of five, both having more than five hydrogen bond acceptors amongst the **P** compounds. These predictions proved **P1**, **P2**, **P3**, **P4**, **P5**, **P7**, **P9** and **P10** to be drug-like, with good solubility (Table 3.12).

Table 3.12: Permeability and solubility predictions of **P** compounds using the Lipinski's rule of five.

Compound / Control	MW (g/mol) <500	Hydrogen bond donors <10	Hydrogen bond acceptors <5	Log P <5	Rule of five violations
P1	392.43	2	5	2.73	0
P2	426.88	2	5	3.33	0
P3	444.87	2	5	3.48	0
P4	420.49	2	5	3.76	0
P5	410.42	2	5	2.87	0
P6	450.47	2	6	3.38	1
P7	442.49	2	5	3.72	0
P8	435.46	3	6	1.58	1
P9	471.33	2	5	3.50	0
P10	406.46	2	5	3.24	0
Quinine	324.42	1	4	2.51	0

3.2.4.2 Predicted ionisation and absorption as a function of pH

Quinine accumulates in the vacuole of the parasites and thereby becomes protonated and trapped in the acidic environment of the vacuole. The **P** compounds displayed similar properties to quinine due to being protonated at pH 5, with 91.76% GIT absorption across all compounds and furthermore, 95.75% of the compounds diffuses into the parasite food vacuole where they accumulate as a small % ionised in the RBCs (Table 3.13).

Table 3.13: Predicted ionisation and absorption of **P** compounds in comparison to quinine (All values predicted using ChemAxon).

Compound / Control	pKa	% ionization @pH 7.4	% ionization @pH 5	% GIT Absorption
P1	6.35	8.24	95.75	91.76
P2	6.35	8.24	95.75	91.76
P3	6.35	8.24	95.75	91.76
P4	6.35	8.24	95.75	91.76
P5	6.35	8.24	95.75	91.76
P6	6.35	8.24	95.75	91.76
P7	6.35	8.24	95.75	91.76
P8	6.35; -0.44	8.24	95.75	91.76
P9	6.35	8.24	95.75	91.76
P10	6.35	8.24	95.75	91.76
Quinine	13.89; 9.05	2.21	90.57	97.79

3.2.5 Effects on the *Anopheles* mosquito

The effect of **P** compounds on the *Anopheles* mosquito was investigated against third instar *An. arabiensis* larvae and eggs at 0.5 µM to determine additional possible target sites of the compounds.

3.2.5.1 Larvicidal effects

3.2.5.1.1 Effects on *Anopheles* larvae

None of the **P** compounds exhibited significant activity when screened at 0.5 µM against third instar *An. arabiensis* larvae (KWAG) between 24 and 48 hours, with percentage mortality ranging between 45 ± 6.88% and 55 ± 7.91% in comparison to DDT (100 ± 0.00%) post 72 hours exposure with the test compounds, three compounds, **P1**, **P2** and **P10** showed the most activity (Figure 3.12). Overall, the **P** compound-treated larvae became moribund over time and insignificant pupae formed. None of the **P** compounds showed morphological changes on *An. arabiensis* larvae at 0.5 µM in comparison to DDT.

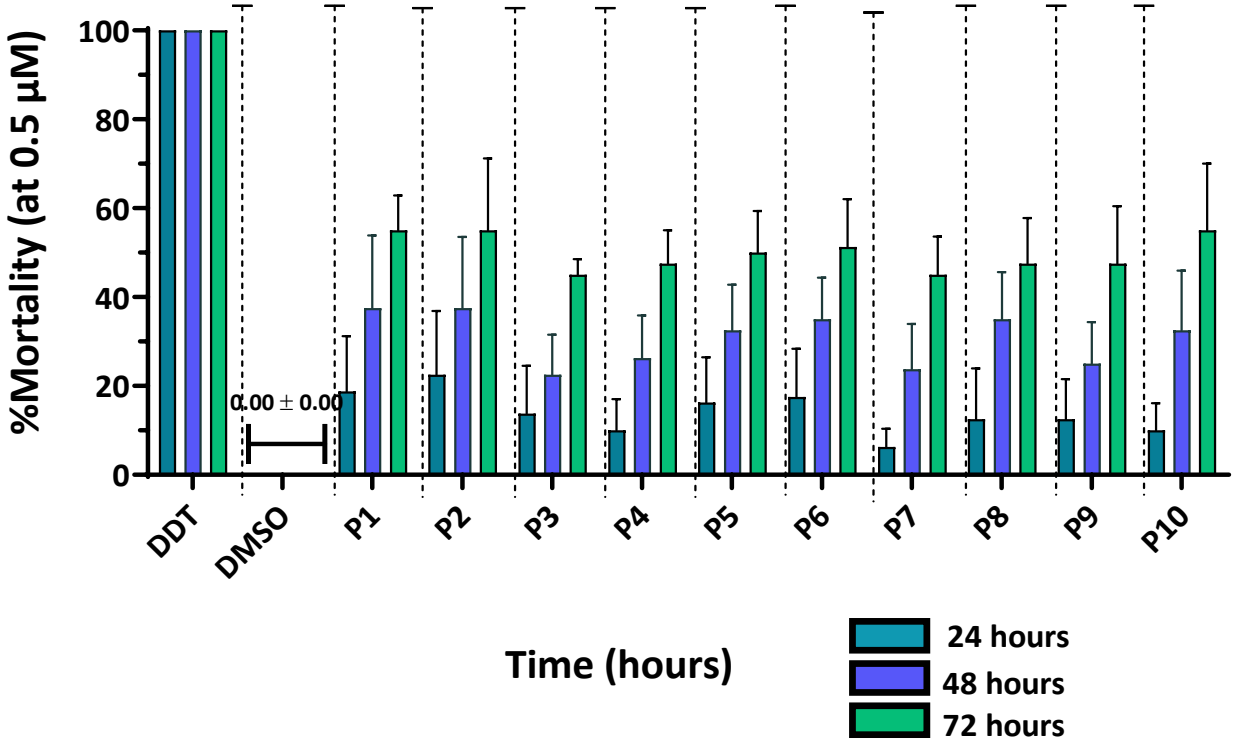


Figure 3.12: The percentage mortality of *An. arabiensis* larvae after 24, 48 and 72 hours treatment with **P** compounds at 0.5 µM compared to DDT.

3.2.5.2 Ovicidal assay

The ovicidal assay was conducted by incubating KWAG eggs with the **P** compounds to determine their ability to interfere with the ability of the eggs to hatch, their morphological appearance, as well as the development of the hatched larvae. **P1**, **P2**, **P3**, **P4** and **P7** showed a delay in the development of the eggs, accounted for by inhibition of more than 60% of the eggs after 24 hours. After 48 hours, only **P3** showed promising activity by inhibiting 74.09 ± 9.67% of the eggs from hatching, however its activity was reduced over 72 hours, with 59.33 ± 7.35% of the eggs being inhibited from hatching after 72 hours (Figure 3.13). None of the compounds had an effect on the hatched larvae and their movement as observed with the 3rd instar larvae.

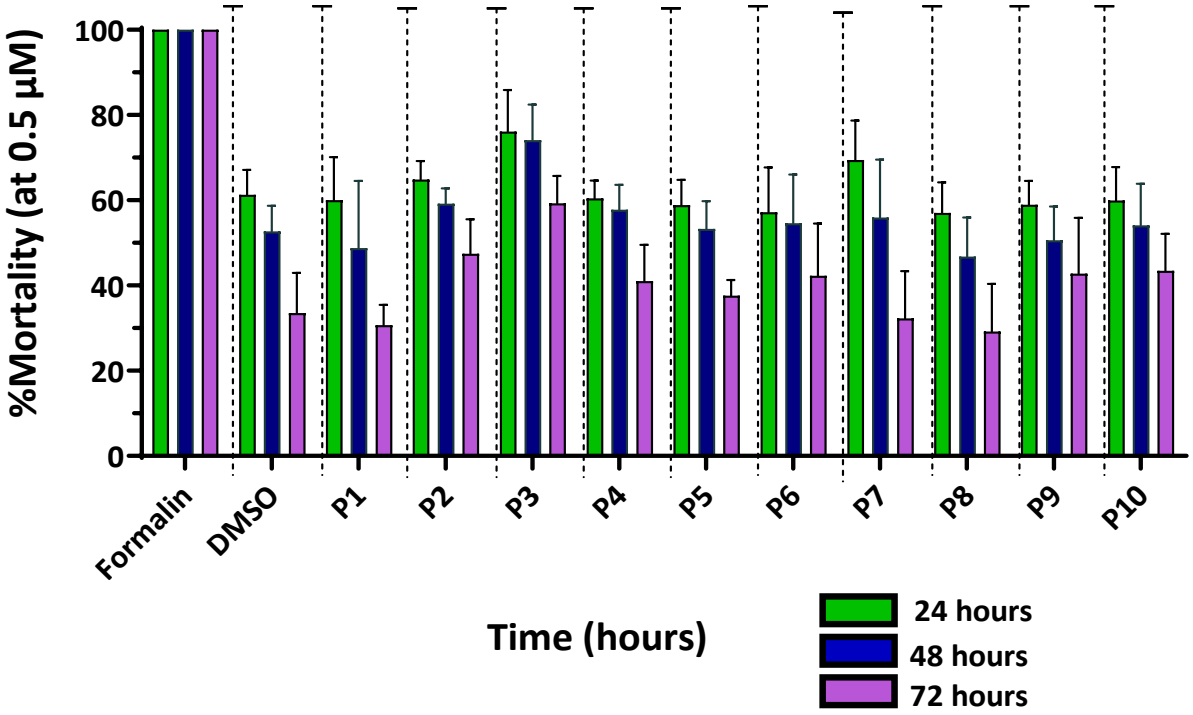


Figure 3.13: The percentage mortality of *An. arabiensis* eggs after 24, 48 and 72 hours treatment with **P** compounds at 0.5 µM compared to formalin.

3.3 Quinoline hydrazone compounds

3.3.1 Effects on the malarial parasite

The antimalarial activity of the **QC** compounds was investigated using pLDH assay on the NF54 *Plasmodium* strain.

3.3.1.1 *In vitro* antimalarial activity

The **QC** compounds showed minimal antimalarial activity, with percentage parasite viability ranging between 77.41 ± 26.40% and 102.12 ± 10.05% in comparison to quinine (20.51 ± 4.50%; Table 3.14). Unfortunately, the activity of these compounds was not high enough to allow for further evaluations. The antioxidant, iron chelation and combination studies were not done on the **QC** compounds due to low antimalarial activity.

Table 3.14: The *in vitro* antimalarial activity of **QC** compounds after screening at 50 µM.

Compound / Control	Parasite viability (% viability ± S.D.)	Compound / Control	Parasite viability (% viability ± S.D.)
QC1	87.96 ± 2.44	**QC7**	93.54 ± 16.08
QC2	77.41 ± 26.40	**QC8**	96.23 ± 17.13
QC3	80.39 ± 9.90	**QC9**	81.33 ± 26.54
QC4	77.87 ± 9.41	**QC10**	97.54 ± 9.55
QC5	102.12 ± 10.05	**QC11**	82.63 ± 28.13
QC6	96.78 ± 8.47	**Quinine**	20.51 ± 4.50

3.3.2 Preliminary toxicity profile

To establish the toxicity profile of the **QC** compounds, their effects on RBCs, HEK-293, K562 and SH-SY5Y cells was investigated.

3.3.2.1 Haemolytic effects

All **QC** compounds and quinine displayed negligible haemolysis against the RBCs at 50 µM ranging from 0.08 ± 0.05% to 2.44 ± 3.72% in comparison to Triton X-100™ (positive control), which displayed 100% haemolysis.

3.3.2.2 *C*ytotoxic effects on mammalian cells

3.3.2.2.1 *In vitro* cytotoxic activity (MTT Assay)

The toxicity profile of the **QC** compounds was evaluated on HEK-293, K562, SH-SY5Y cells using the MTT assay at 100 µM in comparison to the positive control, camptothecin.

The **QC** compounds showed minimal activity on HEK-293 cell line, with % cell growth ranging between 62.03 ± 2.30 % (**QC5**) to 92.47 ± 4.88 % (**QC9**) in comparison to camptothecin (77.61 ± 19.18 %). Their activity against K562 cell line ranged between 60.10 ± 2.46 % (**QC4**) and 81.95 ± 2.26 % (**QC5**) in comparison to camptothecin (32.81 ± 13.40 %). They showed promising activity against SH-SY5Y, with % cell growth ranging between 33.59 ± 2.57 % (**QC4**) and 75.60 ± 5.37 % (**QC5**) in

comparison to camptothecin (21.36 ± 10.32 %). **QC4** was the most active compound against both K562 and SH-SY5Y cell lines, 60.10 ± 2.46% and 33.59 ± 2.57%, respectively (Table 3.15).

Table 3.15: The cytotoxicity profile of **QC** compounds at 100 µM on HEK-293, K562 and SH-SY5Y cell lines.

Compound / Control	HEK-293	K562	SH-SY5Y
	% Cell viability ± S.D.		
QC1	70.43 ± 6.06	66.97 ± 2.91	49.99 ± 1.08
QC2	86.77 ± 4.94	67.54 ± 3.23	47.97 ± 4.10
QC3	88.12 ± 2.54	67.98 ± 1.74	50.27 ± 1.13
QC4	76.55 ± 1.20	60.10 ± 2.46	33.59 ± 2.57
QC5	62.03 ± 2.30	81.95 ± 2.26	75.60 ± 5.37
QC6	78.60 ± 2.16	81.20 ± 3.96	54.45 ± 2.75
QC7	74.70 ± 3.40	69.68 ± 3.95	49.33 ± 1.71
QC8	74.43 ± 3.32	68.93 ± 3.64	50.30 ± 2.57
QC9	92.47 ± 4.88	80.09 ± 2.34	47.46 ± 2.20
QC10	88.26 ± 9.22	73.04 ± 1.13	53.15 ± 1.89
QC11	79.93 ± 4.56	70.68 ± 0.96	49.15 ± 0.59
Camptothecan	77.61 ± 19.18	32.81 ± 13.40	21.36 ± 10.32

3.3.3 *Artemia* lethality

To further establish the toxicity profile of **QC** compounds, the effects on *A. franciscana* nauplii was evaluated at 50 µM.

3.3.3.1. Effects on *Artemia* nauplii viability

The **QC** compounds exhibited minimal activity on *Artemia* nauplii, with percentage mortality ranging from 0.00% to 48.68% at 50 µM after 72 hours. All compounds exhibited % mortality rate below 20% between 24 and 48 hours, and **QC4** was the most active compound (48.68 ± 7.33%) in comparison to potassium dichromate (97.41 ± 1.09%) after 72 hours (Figure 3.14). There were no morphological

effects seen with all the **QC** compounds at 50 µM after 72 hours in comparison to potassium dichromate.

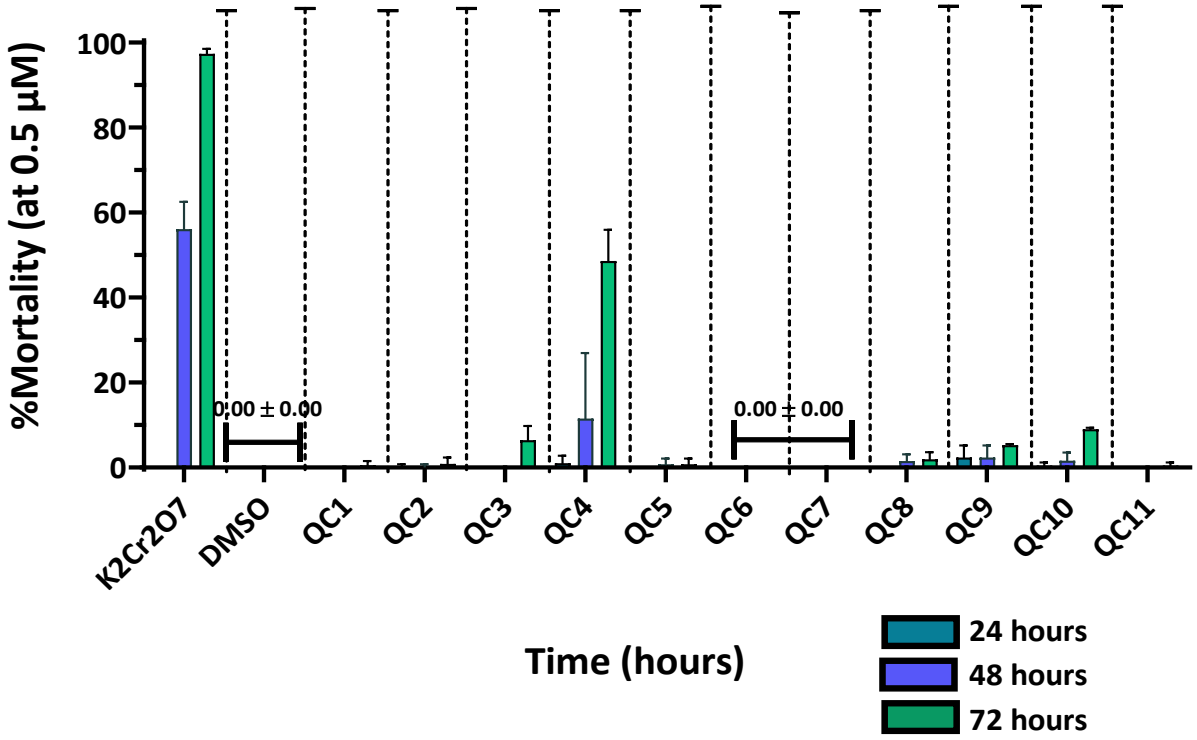


Figure 3.14: The percentage mortality of *A. franciscana* nauplii after 24, 48 and 72 hours treatment with **QC** compounds at 50 µM compared potassium dichromate.

3.3.4 Pharmacokinetic and mechanistic properties

The Lipinski's rule of five was used to evaluate the drug-likeness of **QC** compounds, influenced by molecular weight, the number of hydrogen bond donors, the number of hydrogen bond acceptors and the log P value. Ionisation and absorption as a function of pH was also used to further evaluate whether **QC** compounds accumulated in the RBCs (pH 7.4) or in the food vacuole of the parasite (pH 5).

3.3.4.1 Drug-likeness - Permeability and solubility predictions

Six out of eleven **QC** compounds complied with the Lipinski's rule of five, having molecular weight less than 500 g/mol, less than 10 hydrogen donors, less than 5 hydrogen bonds and the log P ranged

between 0 and 5. These predictions proved **QC1**, **QC3**, **QC5**, **QC7**, **QC8** and **QC11** compounds to be drug-like when compared to quinine with good solubility. **QC2**, **QC4**, **QC6**, **QC9** and **QC10** had a molecular weight above 500 g/mol and therefore did not comply with the Lipinski's rule of five (Table 3.16).

Table 3.16: Permeability and solubility predictions of **QC** compounds using the Lipinski's rule of five.

Compound /Control	MW (g/mol) <500	Hydrogen bond donors <10	Hydrogen bond acceptors <5	Log P <5	Rule of five violations
QC1	491.55	2	5	4.53	0
QC2	519.56	2	6	3.58	2
QC3	443.51	2	5	2.77	0
QC4	527.58	2	5	5.01	2
QC5	495.51	2	5	4.16	0
QC6	529.96	2	5	4.77	1
QC7	491.55	2	5	4.53	0
QC8	491.55	2	5	4.53	0
QC9	519.56	2	6	3.58	2
QC10	505.58	2	5	5.05	2
QC11	495.51	2	5	4.16	0
Quinine	324.42	1	4	2.51	0

3.3.4.2 Predicted ionisation and absorption as a function of pH

All **QC** compounds had a GIT absorption of 2.35% in comparison to quinine (97.79%) and only 14.23% of it was ionised and trapped to contribute to their effect as the majority % is ionised and trapped in the RBCs in comparison to quinine (Table 3.17). Quinine diffuses through the membranes of the parasitised RBCs in its unprotonated form, accumulates in the acidic digestive vacuole where it becomes protonated and as a result trapped.

Table 3.17: Predicted ionisation and absorption of **QC** compounds in comparison to quinine (All values predicted using ChemAxon).

Compound / Control	pKa	% ionization @pH 7.4	% ionization @pH 5	% GIT Absorption
QC1	11.55; 5.78	97.65	14.23	2.35
QC2	11.55; 5.78	97.65	14.23	2.35
QC3	11.56; 5.78	97.65	14.22	2.35
QC4	11.53; 5.78	97.65	14.22	2.35
QC5	11.20; 5.78	97.64	14.23	2.36
QC6	11.55; 5.78	97.65	14.23	2.35
QC7	11.56; 5.78	97.65	14.23	2.35
QC8	11.56; 5.78	97.65	14.23	2.35
QC9	11.54; 5.78	97.65	14.23	2.35
QC10	11.56; 5.78	97.65	14.23	2.35
QC11	11.56; 5.78	97.65	14.23	2.35
Quinine	13.89; 9.05	2.21	90.57	97.79

3.3.5 Effects on the *Anopheles* mosquito

The effect of **QC** compounds on the *Anopheles* mosquito was investigated against third instar *An. arabiensis* larvae and eggs at 0.5 µM to determine additional possible target sites of the compounds.

3.3.5.1 Larvicidal effects

3.3.5.1.1 Effects on *Anopheles* larvae

None of the **QC** compounds exhibited activity when screened at 0.5 µM against third instar *An. arabiensis* larvae (KWAG) with activity ranging between 15 ± 8.29 % and 38.75 ± 12.44 % at 24 and 48 hours. **QC1**, **QC6** and **QC7** exhibited the highest mortality (48.75 ± 8.93 %; 48.75 ± 14.31 % and 31 %, respectively); while **QC3** showed the least mortality (40.00 ± 8.66 %) in comparison to DDT (100.00 ± 0.00%) after 72 hours (Figure 3.15). Overall, the **QC** compound-treated larvae became

moribund over time and insignificant pupae formed. None of the **QC** compounds showed morphological changes on *An. arabiensis* larvae at 0.5 µM in comparison to DDT.

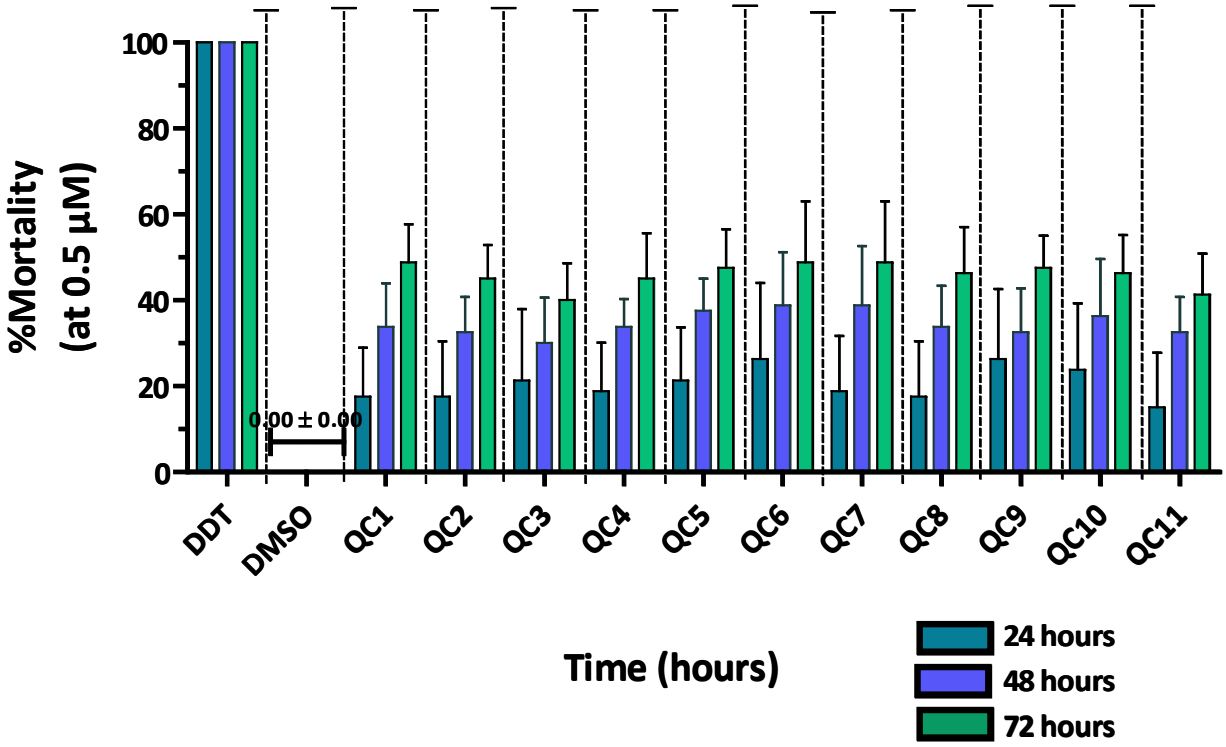


Figure 3.15: The percentage mortality of *An. arabiensis* larvae after 24, 48 and 72 hours treatment with **QC** compounds at 0.5 µM compared to DDT.

3.3.5.2 Ovicidal assay

The ovicidal assay was conducted by incubating KWAG eggs with the **QC** compounds to determine their effect on the ability of the eggs to hatch, their morphological appearance, as well as the development of the hatched larvae. None of the **QC** compounds inhibited more than 60% of *An. arabiensis* eggs from hatching. More than 30% of the eggs hatched after 24 hours, and further increased to more than 40% after 48 hours. Percentage mortality ranged between 25.42 ± 10.90 % and 49.03 ± 11.83 %, and **QC11** exhibited the highest % inhibition in comparison to formalin (100 ± 0.00 %) after 72 hours of treatment (Figure 3.16). None of the **QC** compounds showed morphological changes on *An. arabiensis* eggs at 0.5 µM.

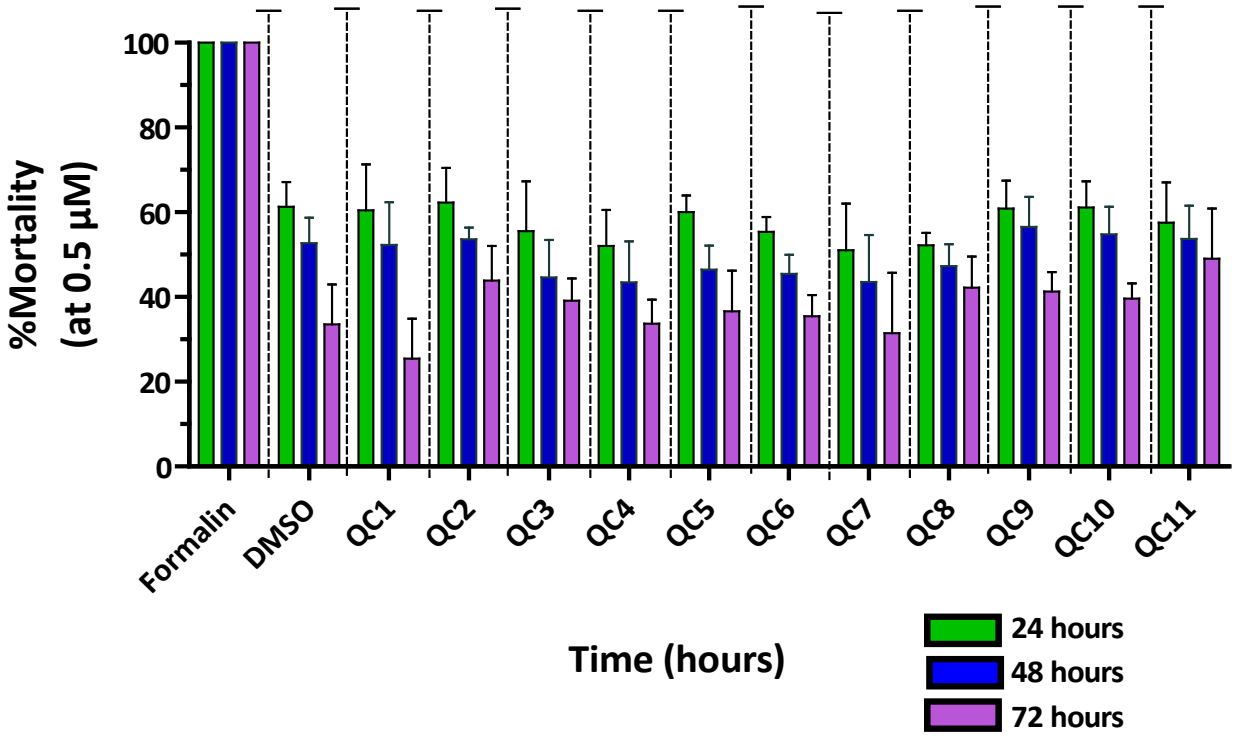


Figure 3.16: The percentage mortality of *An. arabiensis* eggs after 24, 48 and 72 hours treatment with **QC** compounds at 0.5 µM compared to formalin.

Chapter 4: Discussion

The development of resistance to currently used antimalarial treatments necessitates new drugs that are cheaper, easily accessible, with a good safety profile and possess alternative mechanisms of action. This is to allow among other things their use in combination therapy to complement the efficacy and mechanism of action of those antimalarial drugs that remain effective. Quinoline-based compounds are normally limited by resistance, and this has led to their use in combination with other antimalarials (Nqoro *et al.,* 2017) or conjugation with other active moieties (Ansari *et al*., 2017). The possible antimalarial activity of azoles has been investigated over the years as one of the strategies to combat resistance and relieve pressure on the currently used artemisinin derivatives. This led to the development of the pyrimidine-base **P** derivatives, quinolone-base **QC** derivatives and **QB** derivatives that consisted of a quinoline and benzimidazole nucleus, respectively. The latter backbone has attracted great interest in medical research and the ubiquitous functionality of the benzimidazoles and imidazoles nucleus been explored for antimalarial activity. This study further affirms the repurposing of drugs already in use, whilst novel compounds are being developed and investigated.

Unfortunately the WHO goal to eradicate malaria within the next 5 years has not been realized, nor has there been significant progress in terms of developing novel antimalarials to give momentum to this goal. Among others, effective management of malaria programmes, disengaged communities, poorly trained and incentivized staff contribute to operational obstacles in malaria eradication (Feachem *et al.,* 2019). These are some of the fundamental issues that governments should focus on to achieve the eradication goal. Antimalarial pipelines are also unfortunately saturated due to financial challenges, biological complexities of the pathogen and the time factor in drug discovery (Okombo and Chibale, 2017). This has redirected attention to drug repurposing. Repurposing of known and approved drugs for other diseases offers advantages such as reduction in developmental costs and time due to known toxicity, safety and pharmacokinetics evaluations (Agrawal, 2015). As such, it is of importance and worth to further investigate the use of azoles as potential antimalarial and anticancer drugs.

Antimicrobial drugs are some of the drugs that were repurposed for use against malaria, and benzimidazoles and imidazoles were also to be a part of this list (Ramakrishnan *et al.,* 2017). Repurposing of drugs in malaria is not new (Figure 4.1) and the first drugs to be explored in this regard were sulphur-based antibacterial drugs (Nzila *et al.,* 2011).

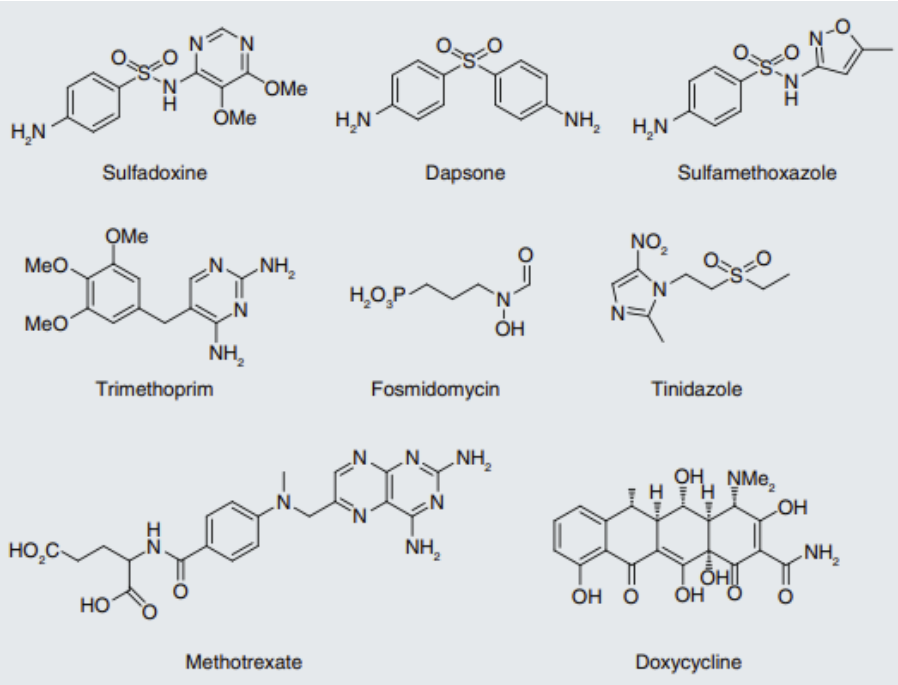


Figure 4.1: Drugs that have been repurposed for treating malaria (Nzila *et al.,* 2011).

Both malaria parasite cells and cancer cells divide rapidly, and some drugs can inhibit critical pathways that control cell division (Nzila *et al.,* 2011). Methotrexate, an anticancer drug is an example of such mechanism that has also shown to block the growth of malaria parasites. Methotrexate (5 mg per day over 5 days) in malaria was found to be safe and well tolerated however, the blood levels achieved were not enough to clear malaria infection completely (Chilengi *et al.,* 2011). There are, however, challenges with drug repurposing such as deep understanding of the biological mechanisms from understanding the gaps between what is known about the molecule to the mechanisms of its re-innovation (Agrawal, 2015). Nonetheless, repurposing of known drugs will further assist in understanding possible analogues that can be explored based on the mechanism of action and toxicity of the known drugs. This study further affirms the repurposing of drugs already in use versus novel compounds in malaria.

4.1 Azole compounds

Azoles are known for their antifungal, antibacterial, anticancer, analgesic and antitubercular properties (Verma *et al*., 2013). The interaction between antimalarial and antimicrobial drugs has been known for years especially against the asexual stages of *P. falciparum* (Huy *et al.,* 2002a; Bell, 2005). In cancer, imidazole and benzimidazole compounds are known to have tubulin modulation mechanisms that are different to those of clinically available molecules such as paclitaxel, vinblastine and docetaxel (Torres *et al.,* 2015). Azoles exert a fungistatic effect through dose-dependent inhibition of CYP-dependent 14α-demethylase, which is important for the conversion of lanosterol to ergosterol, with the latter having importance in the stability of the fungal cell membrane (Gavarkar *et al.,* 2013).

4.1.1 Antimalarial activity

Clinical manifestations of malaria are associated with the proliferative stages, and molecules that target these stages are needed. In the current study, **QB**, benzimidazole and imidazole compounds were investigated for possible antimalarial activity on the asexual blood stages of *P. falciparum* NF54 strain and variable inhibitory and toxicity properties were displayed (Table 3.1). Omeprazole (IC_{50}: 38.80 ± 8.48 µM), ketoconazole (IC_{50}: 16.32 ± 3.28µM), tioconazole (IC_{50}: 4.90 ± 0.41 µM), clotrimazole (IC_{50}: 16.67 ± 4.39 µM), itraconazole (IC_{50}: 2.48 ± 0.37 µM) and two novel compounds namely **QB5** (IC_{50}: 77.81 ± 0.71 µM) and **QB8** (IC_{50}: 35.37 ± 0.67 µM) demonstrated potency against *P. falciparum* NF54 parasites (Table 3.1). Overall, the imidazole compounds showed improved activity compared to the **QB** and benzimidazole compounds. Omeprazole, which is known to have activity against trophozoites displayed better activity amongst the benzimidazole compounds and this is consistent with a findings by Skinner-Adams *et al.* (1997) who suggested that omeprazole has activity against the schizonts and ring forms. Itraconazole and tioconazole were the two most active compounds in comparison to quinine (IC_{50}: 0.18 µM) (Figure 3.1/Table 3.1), and itraconazole demonstrated more potency when compared to tioconazole, with an IC_{50} value that was 1.9-fold more potent (Figure 3.1/Table 3.1)

Tripathi *et al. (*2013) evaluated the activity of ketoconazole on chloroquine sensitive and resistant *P. falciparum* strains, *3D7* and *K1* and found it to have promising activity with IC_{50} values of 5.1 µg/mL and 6.98 µg/mL, respectively (Tripathi *et al.,* 2013). The same study also looked at the combined activity of ketoconazole with an artemisinin derivative, artemether, and this combination was found to be concentration dependent, shown by synergistic and additive interactions at different drug ratios on both strains. Their combined effect on *PfK1* also showed an antagonistic interaction at two of the tested ratios. The *in vitro* antimalarial activity of ketoconazole against *3D7* and *K1 P. falciparum* strains was reported to have IC_{50} values of 4.8 nM and 3.9 nM, respectively (Wisedpanichkij *et al.,* 2009). Tioconazole, clotrimazole, econazole and miconazole were some of the azoles found to be active against *PfK1* with IC_{50} values of 0.63 µM; 0.11 µM; 0.32 µM and 0.49 µM, respectively (Kaiser *et al.,* 2015) and this is consistent with the activity of itraconazole (IC_{50}: 9.3 µM), and posaconazole (IC_{50}: 2.6 µM) against the chloroquine-resistant *P. falciparum* clone *W2* (Penna-Coutinho *et al.,* 2011).

In the current study, the promising activity of tioconazole and itraconazole warranted combination with quinine to assess their *in vitro* interaction in killing the parasite. Tioconazole showed an overall antagonistic effect (∑FIC 1.63), while itraconazole an additive effect (∑FIC 1.12) in combination with quinine against *P. falciparum* (Figure 3.4). The additive effect of the latter might be due to quinine acting on the late trophozoite stage of the parasite, while itraconazole acts on the early trophozoite stage of the parasite. This allows for an additive effect without inhibiting or enhancing the effects of the individual compounds. Tioconazole and quinine showed an antagonistic effect in combination studies, and parasites treated with these compounds did not progress into the schizont parasite stage when analyzing their morphological effects, which could imply tioconazole competes with quinine for uptake into the food vacuole or at the target site. It could be postulated that tioconazole competes with quinine for free haem in the parasite food vacuole, resulting in reduced haem concentrations for quinine or tioconazole to bind, a mechanism similar to that of chloroquine (Parhizgar and Tahghighi, 2017).

Both quinine and tioconazole have some inhibitory effect on haemozoin formation, where quinine has been shown to be more effective than tioconazole with IC_{50} values of 240.07 ± 15.55 µM and 150.37 ± 11.93 µM, respectively (Mahlangu, 2020). Quinine binds haem units to form quinine-haem complexes and these complexes cannot polymerise haem to haemozoin like the uncomplexed units.

As a result, the free haem or quinine-haem complexes damage the parasite's membrane and kills it. This shows how the inhibition of haemozoin formation (Figure 4.2) remains a strategy to be explored in malaria treatment.

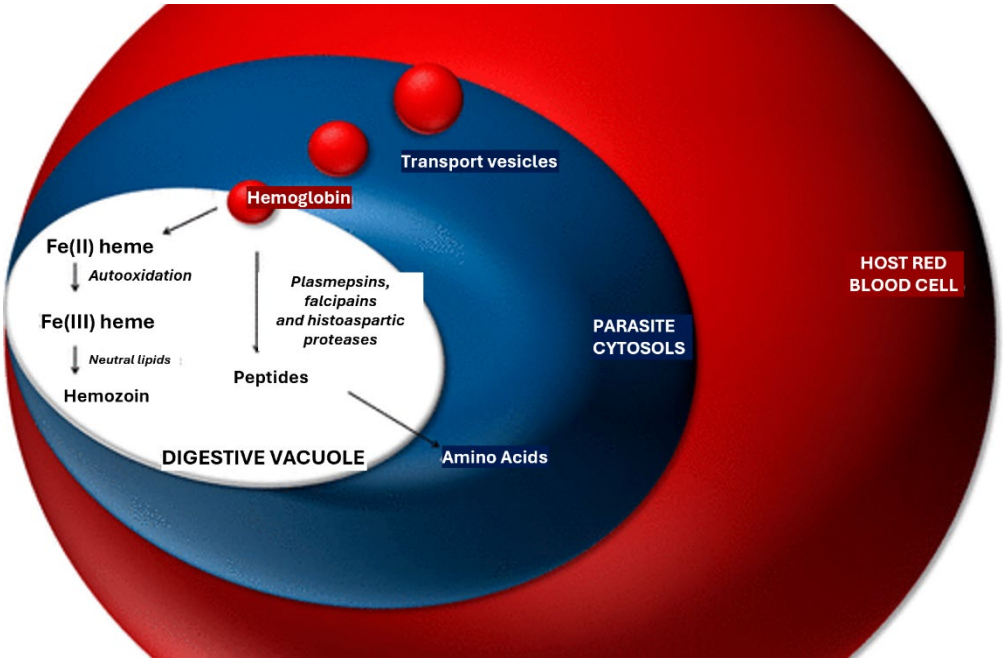


Figure 4.2: Proposed haemozoin formation and degradation pathway in *P. falciparum*. The parasite ingests about 80% of the host's haemoglobin during the trophozoite stage into its digestive vacuole. The haem component of haemoglobin is converted into haemozoin during haemoglobin degradation and globin hydrolysed to amino acids for protein synthesis (Okombo and Chibale, 2017).

The combined effects of omeprazole with quinine have been shown to interact in a synergistic manner *in vitro* (Figure 4.3), while omeprazole with artemisinin displayed an additive interaction. The interaction between omeprazole and artemisinin might be further influenced by *in vivo* pharmacokinetics parameters, and because of this factor, it has been said that this might not be beneficial in the acute phase of the malaria infection due to the rapid parasite clearance (Skinner-Adams and Davis, 1999).

The malarial parasite has developed a protective mechanism against the free haem released during the haemoglobin catabolism through the degradation of the haem that reaches the cytosol of the parasite. In the cytosol, reduced glutathione (GSH) binds to haem via thiol and degrades it (Atamna

and Ginsburg, 1995; Huy *et al.,* 2002b). Despite this protective mechanism, the balance between free haem and detoxification of haem by malaria results in low concentrations of free haem in malaria parasites and red blood cells, allowing the parasite to still multiply (Huy *et al.,* 2002a).

4.1.2 Possible mechanisms of action of azoles

4.1.2.1 Haemozoin formation inhibition

In elucidating the mechanism of action of azoles against *P. falciparum*, of which has been in question for several years, Huy *et al. (*2002b) showed that clotrimazole exhibits high affinity for the haem of the parasite and augments haem-induced haemolysis. It was also reported that the haem-clotrimazole complexes that accumulate in the malarial membrane may lead to its destruction, causing fatal damage to the malaria parasite (Huy *et al.,* 2002b). The same study also investigated the mechanism of action of ketoconazole and miconazole, which both possess the imidazole group in their structures, and these were found to have a mechanism similar to that of clotrimazole by binding to haem with similar affinities to form stable haem-azole complexes. This study showed that clotrimazole possesses a stronger activity for haem-induced haemolysis than ketoconazole, which might imply a stronger activity for clotrimazole. From this current study, the latter proved to be consistent, with antimalarial activity of: clotrimazole (0.00 ± 6.41 µM) and ketoconazole (3.75 ± 1.76 µM) (Table 3.1). The ionisation properties of **QB**, benzimidazole and imidazole compounds however, showed a mechanism of action different to that of quinine, with only a small percentage being trapped in the food vacuole of the parasite.

In addition, clotrimazole was reported to change the morphology and interfere with the development and replication of the parasite (Tiffert *et al.,* 2000). This mechanism was also investigated by Rodrigues *et al.* (2011), where clotrimazole and ketoconazole were found to inhibit β-hematin due to the ability of their structures to form complexes with haem moieties (Rodrigues *et al.,* 2011). The inhibition of β-hematin has been the proposed mechanism of action for azoles against *P. falciparum*, a mechanism similar to that of chloroquine due to their rich electron density and protonatable centers (Sullivan *et al.,* 1996).

4.1.2.2 Oxidative stress and metal homeostasis

Antimalarial agents that also possess antioxidant activity have been suggested to be an advantage as oxidative stress and generation of reactive oxygen species play a vital role in the development of systemic complications in malaria (Pabon *et al.,* 2003). The host's haemoglobin is proposed to be the potential source of free radical production in malaria since the parasite uses haemoglobin as the source of amino acids for nutrition during the erythrocytic stage of malaria. The formed iron-associated (Fe^{2+}-associated) groups then induce intravascular oxidative stress causing changes to erythrocytes and endothelial cells, thus facilitating internalisation of the parasites into the liver and brain of the host (Kumar and Bandyopadhyay, 2005).

The parasite normally develops antioxidant mechanisms through the reduction of its own reactive oxygen species, partly through the synthesis of reduced glutathione and thioredoxin reductase system or transporting the erythrocytic antioxidant enzymes into its cytoplasm (Percário *et al.,* 2012). Due to tremendous oxidative stress caused by the parasites on the host, antimalarials are normally prescribed with an antioxidant, for example ascorbic acid. The effect of antioxidants in erythrocytes is said to be dependent on the absence or presence of glutathione and ascorbic acid has shown a synergistic effect in the presence of glutathione against haem-mediated cell toxicity (Li *et al.,* 2006). In the absence of glutathione, especially in parasitised RBCs as a result of oxidative stress, ascorbic acid reacts with iron or iron-containing molecules to generate hydroxyl radicals or hydrogen peroxide to accentuate haemolytic mechanisms in malaria (Li *et al.,* 2006; Mendiratta *et al.,* 1998).

A similar mechanism to ascorbic acid was investigated with **QB**, imidazole and benzimidazole compounds. Unfortunately, all compounds displayed minimal free radical scavenging activity in comparison to ascorbic acid. These results are consistent with literature for quinoline and benzimidazole derivatives bearing a methyl group and in reference to their antioxidant activity (Karmaker *et al.,* 2017). Wiseman *et al.* (1991) investigated two scenarios where rat liver microsomes were incubated with ferric oxide (Fe^{+3})-ascorbate or ferric oxide (Fe^{+3})-ADP/NADPH along with three azoles (ketoconazole and miconazole). In both systems, ketoconazole was found to be the most effective lipid peroxidation inhibitor (IC_{50} value: 15 µM and 13 µM) for the two systems, respectively. The activity of miconazole was 3.5 fold less active (IC_{50} value: 40 µM) in the (Fe^{+3})-ascorbate system.

In contrast, tamoxifen and 4-hydroxytamoxifen were used as positive controls and their activity with IC_{50} values of 11 µM and 3 µM for the (Fe^{+3})-ascorbate system, respectively, and with IC_{50} values: 18 µM and 3 µM for the (Fe^{+3})-ADP/NADPH system, respectively (Wiseman *et al.,* 1991).

4.1.2.3 Iron chelating mechanisms

A fine balance of the iron homeostasis systems is essential to ensure parasite development and propagation, however by targeting the available iron and withholding it from the parasites adversely affects their life cycle. The latter has been investigated as a potential strategy in controlling malaria. Unfortunately, none of the tested compounds showed notable iron chelating activity when compared to EDTA (Section 3.1.5.4). Thiosemicarbazone analogues (known for their iron-chelating effects) of benzimidazole were found to exhibit excellent antimalarial activity in comparison to chloroquine and quinine against *P. falciparum*, with activity ranging between 0.003 µg/mL and 0.025 µg/mL (Divatia *et al.*, 2019). As such further research is needed if these dual mechanisms of action are to be taken advantage of.

4.1.2.4 Other possible mechanisms of action

Itraconazole acts by inhibiting cytochrome P450 enzymes, particularly 14-α-demethylase, which is an enzyme involved in the progressive removal of methyl groups from lanosterol in the biosynthesis of ergosterol (Lestner and Hope, 2013). Ketoconazole also acts by inhibiting 14-α-demethylase, thereby interrupting the synthesis of ergosterol that forms an important part of the plasma membrane of apicomplexan parasites, including *P. falciparum* (Docampo and Moreno, 2001). It also inhibits the cytochrome 3A4 enzyme, which is responsible for metabolism of most drugs (Horn and Hansten, 2008). Even though there is no evidence to support the presence of a complementary gene sequence with any cytochrome P gene in the *Plasmodium* genome, the possibility of the malaria parasite possessing a cytochrome-like gene uncomplimentary to the protozoan or mammalian one with the a similar functions cannot be ruled out (Wisedpanichkij *et al.,* 2009).Tioconazole has been proposed to be an analogue of nicotinamide adenine dinucleotide (NADH), occupying the same position as NADH in the active site pocket of *P. falciparum* enzyme lactate dehydrogenase (*Pf*LDH) and thereby inhibiting the malaria parasite (Penna-Coutinho *et al.,* 2011).

4.1.3 Vector effects

Since IRS and LLINs target adult mosquitoes, targeting the mosquito at its larval stage has become one of the most important strategies. This is because they are concentrated in smaller areas, they cannot avoid control measures unlike adult mosquitos and they are killed before dispersing to human dwellings making it possible to control malaria transmission (Hanafi-Bojd *et al.,* 2011). Mosquitoes undergo complete metamorphosis and develop in mature stages in standing water. Some anopheline species breed in a wide range of water bodies, while others breed in water storage containers (Tusting *et al.,* 2013). Unlike adult mosquitoes, mosquito larvae are susceptible to vector control measures as they cannot develop behavioural resistance to avoid these measures (Geissbühler *et al.,* 2007). Larval source management intervention is quite old and has been forgotten in malaria control, however, it remains important, and targeting as many larval habitants as possible is important as adult mosquitoes depend on among other things the size and number of possible habitants (Tusting *et al.,* 2013).

Unfortunately, none of the tested azole compounds showed potential as insecticides against *An. arabiensis* larvae and eggs and this shows that the pharmacological properties of the active compounds were directed towards the intra-erythrocytic stage of the malaria parasite rather than that of the vector, displaying lack of ovicidal and larvicidal activity when compared to formalin and DDT (Figure 3.7/3.8/3.9 and 3.10). This lack of activity indicates that no further work is necessary in elucidating their mechanism against the malarial vector.

4.1.4 Cytotoxicity

As much as efficacy is important in the development of new compounds, in the same manner, safety is one of the critical aspects that must be fulfilled. It is for this reason why newly developed compounds must be effective against the malarial parasites without affecting the integrity of the host cell. Negligible haemolysis was observed with all the tested azole compounds and quinine, indicating minimal toxicity (Section 3.1.2.1). This effect demonstrated the activity of the active compounds to be directed to the intra-erythrocytic malaria parasite, which is favorable towards their safety profile and use. In addition, all tested azole compounds, apart from itraconazole,

demonstrated low selectivity toward the malaria parasite or the human epithelial cells with safety indices ranging between 1.00 and 35.39 (Table 3.4). Despite the favourable antimalarial activity, tioconazole was the only azole compound that displayed toxicity against the HEK-293 cells.

According to drug discovery guidelines, new drugs need to be able to be administered orally. As such the pharmacokinetic and chemical profile of the drugs need to be closely monitored. As such, the Lipinski's rule of five was used to predict oral bioavailability of potential antimalarial drugs based on molecular weight, number of hydrogen bond acceptors, number of hydrogen bond donors and the value of the partition coefficient (log *P*). Amongst the **QB**, benzimidazole and imidazole compounds, the surface area of ketoconazole and itraconazole showed a potential challenge with regards to absorption due to high molecular weight. The log *P* of a compound, which measures its hydrophilicity is known to be favourable for values less than five. Tioconazole, clotrimazole and itraconazole showed log *P* values greater than 5 and are therefore less hydrophilic which may cause poor permeation or absorption. To further investigate the toxicity of the test compounds, their toxic hazard characteristics were estimated, and this displayed all azoles to be of high-class toxicity (Section 3.1.5.).

The addition of methyl groups contributes greatly to the reduction of toxicity due to the donation of electrons, while electron withdrawing substituents tend to increase toxicity (Tsakovska *et al.,* 2006). Itraconazole has a methyl constituent bearing structure which may ascribe to its non-toxic properties on HEK-293 cells though it is lipophilic. It was predicted to target amino oxidase A and prostaglandin G/H synthase 1, mainly causing hepatotoxicity and immunotoxicity (Rakhshan *et al.,* 2023). Tioconazole is also lipophilic and highly bound to plasma proteins, with toxicity seen on both the HEK-293 cells and *A. franciscana* nauplii (Table 3.3; Table 3.5/Figure 3.6). Tioconazole is predicted to target aromatase enzyme, nuclear factor (erythroid-derived 2)-like 2/ antioxidant responsive element (nrf2/ARE), heat shock factor response element (HSE) and has minimal activity against phosphoprotein (tumour suppressor) p53 (Zarn et al., 2003). The position of the substituted sidechain on the quinoline might play a role in the activity of the **QB** derivatives as **QB10** and **QB3** also have methyl and bromide side chains, but have less activity compared to **QB5** and **QB8**, respectively (Figure 4.3) The introduction of other groups such as the methyl group can also be of benefit depending on the structure of the compound against *P. falciparum* (Mayoka *et al.,* 2019).

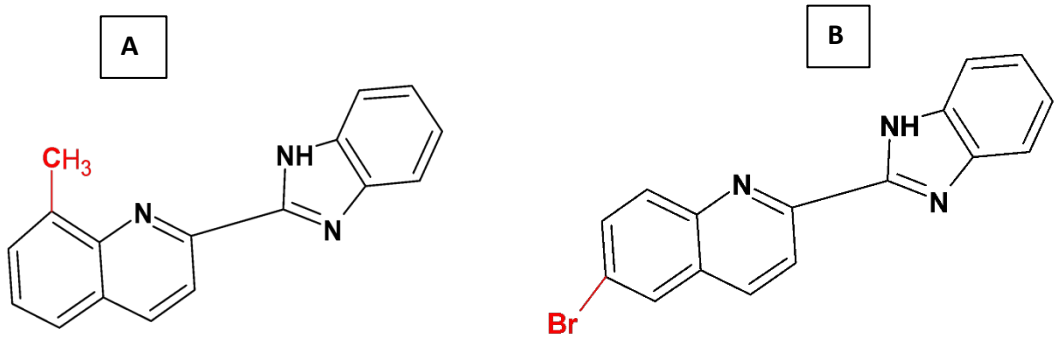


Figure 4.3: The structure of **QB5** (A) and **QB8** (B) showing the respective side chains.

The favorable safety profile of the compounds was further tested with their toxicity against *Artemia* using the *Artemia* lethality assay, which is a simple and inexpensive assay that serves as a preliminary cytotoxicity test of medicines, pesticides and heavy metals prior to further tests on mammalian animal models (Banti and Hadjikakou, 2021). It has also played an important role in determining the cytotoxicity of most drugs as possible anticancer drugs. After 24 hours of treatment with the compounds, **QB5**, **QB9**, **QB11** and tioconazole showed more than 50**%** *A. franciscana* nauplii mortality; while it took 48 hours of treatment for albendazole, tioconazole, clotrimazole and itraconazole to kill the nauplii (Figure 3.6). After 72 hours, over 80% mortality of the *A. franciscana* nauplii was observed with **QB1** (100%), **QB5** (100%), **QB9** (99.10%), **QB11** (100%), mebendazole (100%), ketoconazole (92.63%), tioconazole (100%), albendazole (100%), clotrimazole (100%) and itraconazole (100%). None of the novel **QB** series that showed toxicity against *A. franciscana* nauplii showed toxicity against the HEK-293 and K562 cells. This could imply that their toxicity is directed towards more complex multicellular organisms than the single cells. Azoles were found to be less toxic on epithelial cells in a study where novel ring-A fused azole derivatives of botulin were prepared and their toxicity evaluated (Grishko *et al.,* 2017).

4.1.5 Anticancer activity

Azoles are used as antifungal agents however they have also been found to possess anticancer properties (Bae *et al.,* 2018); where they are proposed to easily bind to target proteins and receptors through different forces and interactions due to their electron-rich containing structure (Ahmad *et al.,* 2018). This promising chemotherapeutic effect was also seen with tioconazole and mebendazole

against K562 cell line (Table 3.3). Proposed mechanisms of action of azoles in cancer may be due to their ability to inhibit transglutaminase 2 (TG 2), which is a cross-linking enzyme responsible for first line protection of infections and tissue stabilization with its activation leading to the development of cancer (Choi *et al.,* 2016).

Mebendazole has shown a high degree of toxicity to triple-negative breast cancer (TNBC) cells *in vitro* as well as possess favorable toxicity profile at high doses in humans over long periods of time and has been approved by health authorities (El-On, 2003). The promising antiproliferative effects of mebendazole against cancer cells is not observed in normal cells, where minimal toxicity has been observed on a fibroblast cell line and human umbilical vein endothelial cells (HUVECs) (Mukhopadhyay *et al.,* 2002), as well as the HEK-293 cells (Table 3.3). Indicating that a wide panel of cells need to be evaluated before any conclusion on the toxicity profile of a compound can be made.

Itraconazole displayed the best safety profile amongst the active compounds against the malarial parasite (safety index: 35.39); and tioconazole and mebendazole were the two most toxic compounds against the leukaemic K562 cell line (IC_{50} 15.01 ± 1.79 µM and 3.98 ± 0.49 µM, respectively) in comparison to cisplatin 71.22 ± 7.63 µM. However, since anticancer drugs are ideally administered in combination, that of tioconazole and cisplatin would not be an advisable combination due to the observed antagonistic interaction. In contrast, the additive interaction seen with the mebendazole-cisplatin combination against the K562 cell line warrants further investigation.

Tioconazole has the ability to reduce tumor viability, sensitize cancer cells to chemotherapeutic drugs and induce their starvation (Liu *et al.,* 2018). It was also suggested that tioconazole could be a potential autophagy modulator by inhibiting autophagy related proteins, as cancer cells use autophagy as a defense system against apoptosis induced by chemotherapy (Liu *et al.,* 2018). The imidazolic nitrogen on tioconazole plays an important role in its activity (Crisóstomo-Lucas *et al.,* 2018). To potentiate the anticancer activity of tioconazole, the addition of metal ions (copper, zinc, cadmium and cobalt) to tioconazole was found to be superior to the unbound form against cervical uterine adenocarcinoma (HeLa) cancer (Crisóstomo-Lucas *et al.,* 2018). This further supports the potential use of tioconazole in cancer and the necessity for further investigations.

Clotrimazole has also been found to have *in vitro* anticancer properties, particularly directed to the human breast cancer cell line by triggering apoptosis (Majewski *et al.,* 2004), ability to prevent cell migration (Furtado *et al.,* 2012) and ability to suppress metabolic enzymes in lung carcinoma and colon adenocarcinoma cells (Kadavakollu *et al.,* 2014). Clotrimazole is also used in the management of *Tinea pedis*, sickle cell anaemia, beriberi- vitamin B1 deficiency and Chagas disease (Buckner and Urbina, 2012). Itraconazole has been reported to inhibit Hedgehog signaling in non-small cell lung carcinoma (Kim *et al.,* 2010), along with anti-angiogenesis effects in non-small lung cancer (Aftab *et al.,* 2011) and antitumor activity in prostate cancer (Antonarakis *et al.,* 2013). Ketoconazole has the ability to block numerous steps in androgen synthesis by blocking cytochrome P450 CYP17A1 enzyme in castration-resistant prostate cancer patients receiving dutasteride for benign prostatic hyperplasia (Taplin *et al.,* 2009; Vasaitis *et al.,* 2011). Although all the azoles have been found to possess *in vitro* anticancer properties, little clinical research has been undertaken to confirm these activities.

4.2 6-Oxo-1,6 dihydropyrimidine (P compounds)

Pyrimidines are heterocyclic compounds with an important role in medicinal chemistry due to possessing anti-cancer, anti-viral and anti-bacterial activity (Figure 4.4). Essential building blocks of DNA and RNA namely thymine, cytosine and uracil as well as vitamins like thiamine, riboflavin and folic acid include the pyrimidine ring. The fact that the malaria parasite relies only on the *de novo* biosynthesis of pyrimidines in contrast to mammalian cells, which can salvage on both existing pyrimidines and synthesize pyrimidines, makes them vulnerable to inhibition as they lack the salvage enzymes (Singh and Kaur, 2016).

Pyrimidine-based falcipain inhibitors represent some of the derivatives with activity against falcipain; where falcipain-2 (FP2) and falcipain-3 (FP3) are cysteine proteases involved in the hydrolysis of haemoglobin in the acidic (pH 4–6) vacuole of the parasite. These proteases are expressed predominantly in trophozoite and schizont stages in the life cycle of the malaria parasite and therefore serve as important targets (Singh and Kaur, 2016). Furthermore, dihydropyrimidines also display varied activity including antiplatelet aggregation, antihypertensive, antimalarial, antioxidant, antifungal, analgesic and anti-inflammatory activity (Bahekar and Shinde, 2004).

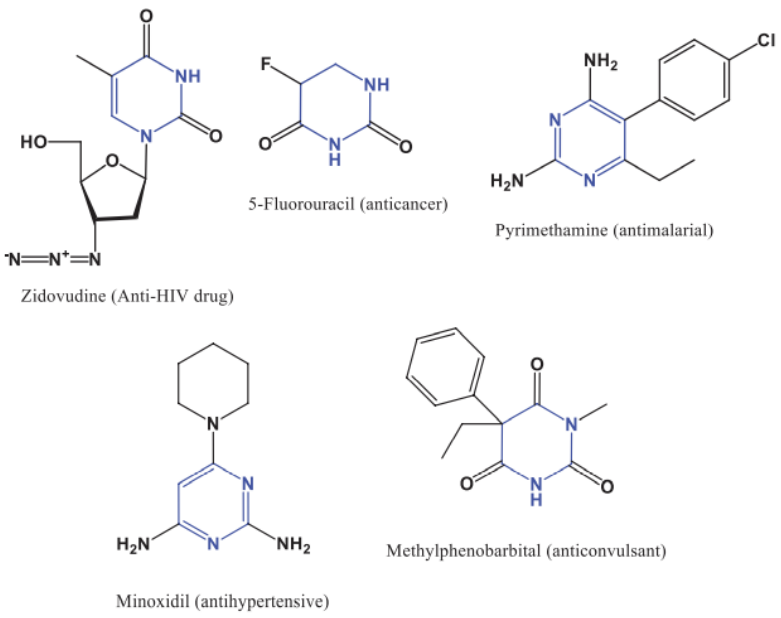


Figure 4.4: Chemical structure of active molecules with the pyrimidine pharmacophore (Padmashali *et al.,* 2019).

4.2.1 Antimalarial activity

The 6-oxo-1,6 dihydropyrimidine (**P**) compounds were evaluated for antimalarial properties and displayed varying activity with compound **P7** having the most activity by inhibiting 43% parasite growth, however, it was not as active when compared to the positive control, quinine (85.5% parasite inhibition). This antiplasmodial inhibition by compound **P7** appeared to be directed against the intraerythrocytic parasite as it did not induce haemolysis, thereby not affecting the host's cell membrane by increasing permeability properties or interfering with metabolism.

Agarwal *et al. (*2006) showed that there is a relationship between the activity and the partition coefficient of a compound, and the synthesized analogs from this study had an increase in activity with a decrease in the partition coefficient value (Table 3.12). In terms of the structure-activity, the substitution of a benzene ring with thiophene ring reduced the partition coefficient value to 2.75 with the MIC of 1 µg/mL. In addition, phenyl substituted compounds showed a two-fold MIC value with a partition coefficient value of 3.07 and substituting the phenyl group with a methoxy group increased the partition coefficient and had no effect on the MIC value (Agarwal *et al.,* 2006). It was concluded from this study that these analogs have potential in malaria chemotherapy, and their

structure could be modified further to increase their activity. In addition, electron withdrawing groups substituted at the *para*-position of aromatic rings are crucial for cytotoxicity of pyrimidine derivatives (Zhang *et al.,* 2019) and this might to a certain extent explain the cytotoxic effect of compound **P3** on *A. franciscana* nauplii by the *para*-substituted chloride (Figure 4.5).

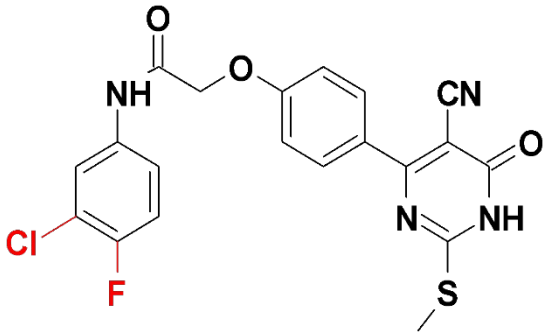


Figure 4.5: The structure of **P3** showing the para-position substituted group.

4.2.2 Vector effects

Dihydropyrimidines have been found to have larvicidal activity and the proposed mechanism of action was attributed to their high lipophilicity, leading to disturbance of the cell membrane and subsequent activation of different signals (Ghosh *et al.,* 2012). In this current study however, none of the **P** compounds exhibited significant activity against third instar *An. arabiensis* larvae, but it has been proven that pyrimidines do have the potential to possess larvicidal and insecticide activity (Rajanarendar *et al.,* 2010).

4.2.3 Cytotoxicity and anticancer activity

Safety is important in the discovery of new drug entities and the challenge is to develop antimalarial drugs which selectively target the malaria parasite without affecting the integrity of the red blood cells of the host. The safety of the **P** compounds was evaluated against the HEK-293, K562 and SH-SY5Y cell lines, as well as *A. franciscana* nauplii. Of the tested **P** derivatives, compound **P7** displayed minimal toxicity on all cell lines, with K562 cell line being the most sensitive to compound **P7** derivative compared to HEK-293 and SH-SY5Y cell lines (Table 3.11). **P3** showed noticeable toxicity

against *A. franciscana* nauplii (98.67% kill after 72 hours) and its effects appeared to be more selective for more complex multicellular organisms than the single cells. The toxicity of this compound on *A. franciscana* nauplii appeared to be concentration dependent with higher concentrations not yielding an LD_{50} value, shown by its precipitation at high concentrations used to determine the LD_{50} value (Figure 3.11).

The *in vitro* anticancer properties of 4-substituted 2-amino pyrido[3,4-*d*]pyrimidine derivates have been evaluated against the leukemia, melanoma, and cancers of lung, colon, kidney, ovary, breast, prostate and the central nervous system from the NCI 60 human cancer cell line panel (Wei and Malhotra, 2012). It was found that some of the derivatives possessed selective growth inhibition against MCF-7 (breast cancer cell line), MDA-MB-468 (breast cancer cell line) and UO-31 (renal cancer cell line) when compared to the other cell lines, suggesting disease-oriented selectivity (Wei and Malhotra, 2012). To further establish the safety profile of **P** compounds, the Lipinski's rule of five was used. All **P** compounds apart from **P6** and **P8** complied with the Lipinski's rule of five and showed a log *P* value less than 5, which is indicative of good absorption.

Fatima *et al.* (2012) investigated the antimalarial activity of compounds with a pyrimidine core against the chloroquine-sensitive and -resistant *P. falciparum* strains, 3D7 and K1, respectively. Most of the compounds displayed potent antimalarial activity with IC_{50} values less than 2 µg/mL, with three compounds potently inhibiting the chloroquine-resistant strain. Furthermore, they possessed favourable selective indices ranging between 20.39 to 1183.89, when compared to the Vero monkey cell lines.

4.3 2-(Quinolin-8-yloxy)acetohydrazone (QC compounds)

Hydrazones have been shown to have antimicrobial, anticancer, antiprotozoal, cardioprotective, larvicidal, anti-inflammatory and antiplatelet activity (Tabanca *et al.,* 2012; Altintop *et al.,* 2019; Dandawate *et al.,* 2012). Quinolines have also been shown to possess antimalarial properties (Golden *et al.,* 2015). The current study evaluated the potential antimalarial and anticancer activity of the **QC** compounds that contained a quinoline nucleus with a hydrazone linkage. The design of

hybrid compounds to target malaria have shown advantages such as reduced drug-drug interactions by eliminating competition for binding to the plasma protein (Nqoro *et al.,* 2017).

4.3.1 Antimalarial activity

Quinoline-based compounds have shown antimalarial activity by interfering with *P. falciparum* haem metabolism (Bawa *et al.,* 2010) and over the years structural modifications have been evaluated towards exploring this mechanism of action even further, especially with increased resistance on the currently used antimalarials. Blood feeding organisms such as *Plasmodium* species have proteases that convert the host's hemoglobin to heme and globin, peptides and amino acids essential for their survival and growth (Figure 4.6). Haem is oxidized to hemin, releasing hydrogen peroxidase. Hemin is detoxified by being converted to haemozoin. Quinoline compounds act by inhibiting the formation of non-toxic haemozoin, leading to increased levels of free heme in the food vacuole, which promotes peroxidase reactions with hydrogen peroxidase to higher oxidative iron forms that may cause damage to the parasite (Herraiz *et al.,* 2019).

There was minimal activity observed with the **QC** compounds against *P. falciparum* NF54 parasites with the highest parasite growth inhibition by **QC2** and **QC4** (22.59% and 22.13%, respectively), which was not comparable to that of the standard antimalarial agent, quinine (Table 3.14). The effect of these compounds was directed towards the malaria parasite as they did not affect the permeability or viability of the host red blood cell as evident by negligible haemolysis. In one particular study, conjugated hydrazones were synthesized and their potential as antimalarial agents through possible haemozoin inhibition and iron chelation activity was evaluated against *P. falciparum* 3D7 and K1 parasite strains (Sarkar *et al.,* 2016). Compound 5f displayed iron chelating (IC_{50}: 1.87 ± 0.24 µM) and antimalarial (IC_{50}: 16.95 ± 1.19 µM) potential, and the aromatic system with a nitrogen- and sulfur-substituted five-membered ring was the proposed mechanism for its activity (Sarkar *et al.,* 2016). This further shows the opportunity for structure-activity possessed by the quinoline bearing molecules.

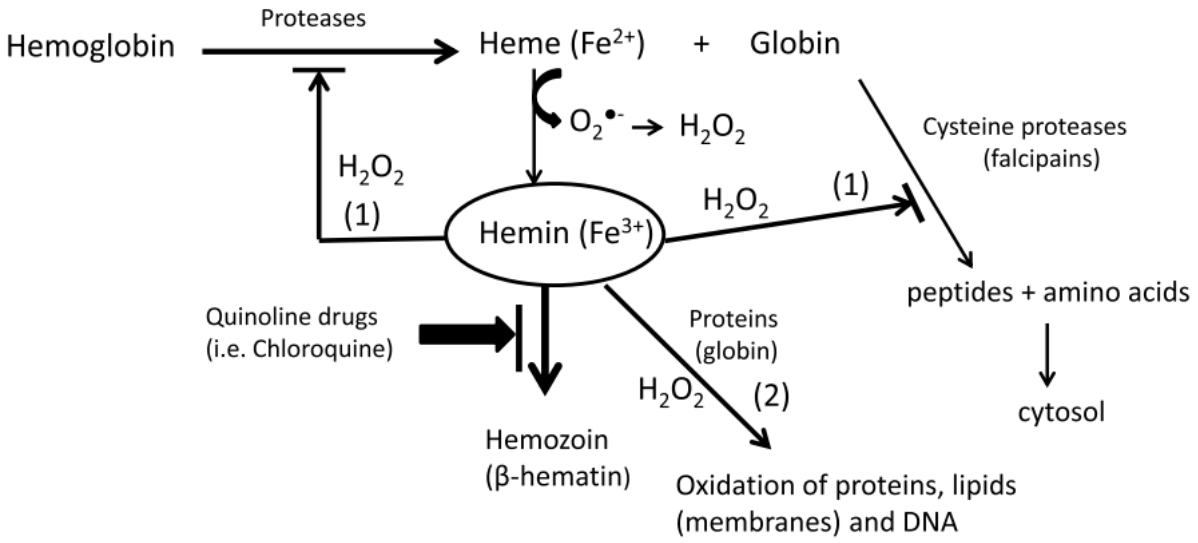


Figure 4.6: Mechanism of action of quinolines inhibiting haemozoin in the digestive vacuole of *P. falciparum* (Herraiz *et al.,* 2019).

Iron chelation has been identified as a potential mechanism to target the malaria parasite, as such all hydrazones screened to date have shown iron-chelating properties, however their antimalarial activity was not as promising as proposed with IC_{50} values ranging between 1.85 µM and >100 µM compared to the deferoxamine (0.72 ± 0.19 µM). Hydrazones with free hydroxyl groups, such as 3,4-hydroxy substituted benzothiazole, have been shown to be more potent in inhibiting malaria growth compared to methoxylated hydrazones against the K1 parasite strain (IC_{50}: 14.17 ± 1.34 µM) inducing minimal haemolysis (Sarkar *et al.,* 2016).

Some quinoline compounds with anticancer properties act by intercalating into the DNA and are currently used as topoisomerase inhibitors, causing permanent damage to the DNA molecule that triggers apoptosis (Jain *et al.,* 2016). There are two classes of DNA topoisomerase and type I acts by breaking one DNA strand for the passage of the second strand while type II breaks both strands of one DNA duplex to allow the passage of the second DNA double strand. Several quinolines target the latter to elicit their activity (Jain *et al.,* 2016). The *para*-substitution of side chains on quinoline derivatives contributes to their activity as anticancer agents (Ahsan *et al.,* 2016). The 2,4-

disubstitution on the quinoline ring also plays a role in its anticancer properties through growth inhibition by apoptosis, modulation of nuclear receptor responsiveness, disruption of cell migration, inhibition of angiogenesis and cell cycle arrest (Lu *et al.,* 2008).

4.3.2 Vector effects

In evaluating the potential of **QC** compounds as larvicides, this study showed that their activity was not directed towards the *An. arabiensis* vector as there was no significant mortality after 72 hours as compared to DDT which resulted in 100% mortality (Table 3.17). Compound **QC1**, **QC6** and **QC7** exhibited the highest mortality overall and none of the **QC** derivatives showed morphological changes on *An. arabiensis* larvae. Hydrazone compounds have been shown to possess larvicidal activity (~92%) against *An. arabiensis* after 48 hours, with comparable activity to the positive control, temephos (99%). The presence of halogens (fluorine or chlorine) on the phenyl ring of the two most active compounds was suggested to be the attribution to their high activity (Nefisath *et al.*, 2021). Based on the lack of activity of the **QC** compounds in comparison to those in the current study, it indicated that no further investigation into these compounds as larvicides is warranted.

4.3.3 Cytotoxicity and anticancer activity

Even with continuous progress in the development of anticancer drugs, there is still a need for novel, diverse and targeted molecules with increased safety. Quinoline derivatives have shown activity against cancer cells and the quinoline scaffold has an important role in drug design in optimization of physicochemical properties (Mohamed and Abuo-Rahm, 2020).

Minimal toxicity was displayed by the **QC** compounds on both the HEK-293 and K562 cells. Overall, the human SH-SY5Y neuroblastoma cell line was the most sensitive to all the **QC** compounds and warrants further investigation (Table 3.15). Several quinoline compounds have been investigated as anticancer agents over the years and camptothecin serves as an example of a quinoline-containing compound with anticancer properties (Ahsan *et al.,* 2016). The Lipinski's rule of five was used to predict the drug-likeness of the **QC** compounds and **QC1**, **QC3**, **QC5**, **QC7**, **QC8** and **QC11** complied with this rule and proved to have good solubility, similar to quinine (Table 3.16). Compounds **QC2**,

QC4, **QC6**, **QC9** and **QC10** violated the Lipinski's rule of five by having molecular weights above 500 g/mol, and **QC2** and **QC9** further had hydrogen bond acceptors more than 5. Due to the minimal antimalarial activity, these compounds do not warrant further investigation.

Chapter 5: Conclusion, Limitations and Recommendations

5.1 Conclusion

The WHO remains committed to the eradication of malaria, however, the development of resistance to the currently used antimalarials and insecticides is still of great concern (WHO, 2023). As such, new compounds need to be investigated. To contribute to this search, this study tested three sets of novel compounds: the **P**, **QC** and **QB** compounds as well as known azoles (benzimidazoles and imidazoles) for their potential activity against the *P. falciparum* NF54 malaria strain and *Anopheles arabiensis* KWAG strain.

None of the tested novel compounds showed significant activity against both *Plasmodium* and *Anopheles*. Unfortunately none of the tested compounds exhibited potential as insecticides against *An. arabiensis* larvae and eggs. However, two of the **QB** compounds, **QB5** and **QB8**, showed activity against malaria and this could be related to the position of the side chains on the quinoline base. Azoles have shown to have potential against malaria in this study, with tioconazole and itraconazole the most active against chloroquine-sensitive NF54 malaria parasite, potentially inhibiting haemozoin formation as a mechanism of action. Itraconazole interacted in an additive manner when combined with quinine, while the tioconazole- quinine combination had an antagonistic interaction. The safety profile of the most active compounds were favourable, as shown by low toxicity against human RBCs and HEK293 cells.

The potential antimalarial activity of azoles gives them an edge in drug repurposing, a strategy that can greatly contribute towards saving time and money in drug development. Literature has reported concerns around the toxicity profile of azoles as oral drugs and through drug repurposing and structure-activity relationship, itraconazoles could be structurally modified or the delivery formulation using nanotechnology could overcome this to improve their activity and toxicity profile.

Research shows that compounds with antioxidant properties are prescribed with antimalarial drugs and this is one of the strategies this study investigated. Unfortunately, none of the compounds showed antioxidant activity through free radical scavenging or iron chelation activity.

5.2 Limitations

The following limitations were identified and need to be taken into consideration for future studies:

- The study was limited to investigating the antimalarial activity on only the *P. falciparum* NF54 malaria strain, and did not explore possible activity against resistant strains, where the need for new antimalarials are needed.
- The haemozoin inhibition as a possible mechanism of action was not determined for the novel compounds due to technical issues in the laboratory. But previous work undertaken in the laboratory should be extended.
- The possible effect of the two active compounds (tioconazole and itraconazole) against the gametocyte stages of the malaria parasite was not determined as inhibition of the sexual stages would decrease the malaria transmission. Time did not allow for gametocyte differentiation of the NF54 strain assay to be established in the laboratory to evaluate the compounds.
- Due to limited compound being supplied, other mechanistic studies could not be determined.
- The toxicity profile of the tested compounds against an extended panel of cell lines was not determined to have comparable data for the selectivity of the compounds against normal and cancerous cells.

5.3 Recommendations

Considering the volume of compounds tested, the number of assays done, further investigations need undertaking.

- When looking at the toxicity profile generated for **P** compounds, only **P7** could be considered for further derivatization to increase its antimalarial activity but maintaining a low toxicity profile.
- Tioconazole, itraconazole and clotrimazole require further studies to investigate their antimalarial activity against resistant strains and to elucidate their mechanism of action in malaria.
- Further studies with the active imidazoles can be done to assess their combined activity with artemisinin derivatives against *P. falciparum*. To further understand combined interactions

of tioconazole and itraconazole with quinine, in-depth studies can be done to capitalize on their interaction and mechanism of action.

- Tioconazole and mebendazole warrant further investigations on their anticancer properties.
- In addressing toxicity, especially of the combined compounds, reformulation is one of the strategies that can be explored and this facilitate better oral absorption of tested compounds.
- Further studies are needed to understand the toxicity of **QB1**, **QB5**, **QB9**, **QB11**, mebendazole, ketoconazole, tioconazole, albendazole, clotrimazole and itraconazole against *A. franciscana* nauplii and in other toxicity models.

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Appendix A: Ethics waiver for obtaining and using fresh blood (M140669)

R14/49 Dr Robyn L van Zyl

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140669

NAME: (Principal Investigator) Dr Robyn L van Zyl

DEPARTMENT: Pharmacy & Pharmacology
Medical School

PROJECT TITLE: The Chemotherapeutic Properties of Novel Synthetic and Natural Compounds (Renewal previously M090532)

DATE CONSIDERED: 29/05/2009 (Initial Approval) 26/06/2014 (Renewal)

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR:

APPROVED BY: Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 26/06/2014

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

DECLARATION OF INVESTIGATORS

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

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

Principal Investigator Signature

Date 21-07-2014

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

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/

November 23, 2017

It has come to my attention that there is some misunderstanding concerning waivers for a few postgraduate students in the Pharmacology Division, Department of Pharmacy and Pharmacology. The waivers below are all that is needed for ethics approval for the listed postgraduate students' research.

Each postgraduate received fresh blood weekly needed to grow malaria parasites from healthy volunteers in an approved project (renewal number M140669; original number M 090532) of Prof R L van Zyl.

Waiver details:

W-CJ-130301-3 Prof R L van Zyl, Mr T I Mahlangu – student no 667999.

W-CJ-150330-2 Prof R L van Zyl, Mrs N Jansen van Vuuren – student no 0748672W.

W-CJ-160129-2 Prof R L van Zyl, Ms P Thabana – student no 609529.

W-CJ-161129-1 Ms F Kathrada – student no 0602240N, Prof R L van Zyl.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2017 -11- 23
JOHANNESBURG

Professor Peter Cleaton-Jones
Chair

Copies: HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkanzi, Lebo Moeng.

Appendix B: Ethics waiver for culturing malaria parasites (20180104)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Research Office

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

CLEARANCE CERTIFICATE **PROTOCOL NUMBER: 20180104**

BRIEF DESCRIPTION OF APPLICATION:

The effecr of novel 6-OXO-1,6 dihydropyrimidyne and 2-(quinolil-8-ylo) acetohydrazone derivarives on the life cycyle of the malaria parasite

APPLICANT: Ms PA Thabana

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)

Faculty of Health Sciences, University of the Witwatersrand
Johannesburg, Republic of South Africa
Tel: +27 (0) 11 717 6531

DECLARATION OF APPLICANT:

To be completed in duplicate and **one copy** returned to the Research Office, Phillip 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

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
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SOUTH AFRICA
Tel: +27 (0)11 717 2454
Email: Kennedy.Erlwanger@wits.ac.za

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

Assoc Prof Kennedy H. Erlwanger
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Appendix D: Human ethics waiver for the use of human cell lines (W-CJ-160129-2)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Ms P Thabana
School: Therapeutic Sciences
Department: Pharmancy and Pharmacology
Medical School
University

E-mail: 609529@students.wits.ac.za

CC: Supervisor: Professor R van Zyl <Robyn.vanZyl@wits.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 28/06/2021

REF: *R14/49*

PROTOCOL NO: W-CBP-210628-01 (This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)

PROJECT TITLE: *The effect of novel 6-oxo-1,6-dihydropyrimadine derivative on the life cycle of the malaria parasite*

Renewal of W-CJ-160129-2

Please find attached the Ethics Waiver Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/ClearScanWaiver.wps

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

28/06/2021

Ref: W-CBP-210628-01

TO WHOM IT MAY CONCERN

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

Investigator: Ms P Thabana
Student No. (if appropriate): 609529
Staff No. (if appropriate):

Supervisor: Professor R van Zyl

School: Therapeutic Sciences
Department: Pharmancy and Pharmacology
Medical School
University

Project title: *The effect of novel 6-oxo-1,6-dihydropyrimadine derivative on the life cycle of the malaria parasite*

Renewal of W-CJ-160129-2

Reason: In vitro laboratory study
No human participants will be involved in the study

CB Penny

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

Research Office Secretariat:
Third Floor, Phillip Tobias Building, corner of St Andrews and York Roads, Parktown, Johannesburg 2193
Postal address: Private Bag 3, Wits 2050
Tel Nos: +27 (0)11 717 1234/1252/2656/2700
Office E-mail: HREC-Medical.ResearchOffice@wits.ac.za
Website:
https://www.wits.ac.za/research/researcher-support/research-ethics/ethics-committees/

Appendix E: Ethics waiver for the use of Artemia eggs and nauplii 07-11-2017-O

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

Assoc Prof Kennedy H. Erlwanger
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