EVALUATION OF THE EFFICACY OF *SYZYGIUM AROMATICUM*-DERIVED OLEANOLIC ACID ON MALARIA PARASITES IN *PLASMODIUM BERGHEI*-INFECTED MALE SPRAGUE-DAWLEY RATS AND EFFECTS ON BLOOD GLUCOSE AND RENAL ELECTROLYTE HANDLING

Bonisiwe Precious Mbatha

2014
EVALUATION OF THE EFFICACY OF SYZYGIUM AROMATICUM DERIVED OLEANOLIC ACID ON MALARIA PARASITES IN PLASMODIUM BERGHEI-INFECTED MALE SPRAGUE-DAWLEY RATS AND EFFECTS ON BLOOD GLUCOSE AND RENAL ELECTROLYTE HANDLING

By
Bonisiwe P. Mbatha (210551793)

Submitted in fulfilment of the requirements for the degree of Master of Medical Science in Human Physiology in the Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, Faculty of Health Sciences

Supervisor: Professor C.T. Musabayane

Discipline of Human Physiology
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
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To my son Mnqobi, I dedicate this to you. I want you to know that education is the key to success.
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I, Bonisiwe Mbatha, hereby declare that the dissertation entitled "Evaluation of the efficacy of Syzygium aromatum-derived oleanolic acid on malaria parasites in Plasmodium berghei-infected male Sprague-Dawley rats and effects on blood glucose and renal electrolyte handling" is a result of my own investigation and research and that this work has not been submitted in part or in full for any other degree or to any other university. Where use was made of the work of others, the work used is duly acknowledged in the text.

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Date: 14/04/2005
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<td>OA</td>
<td>Oleanolic acid</td>
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Abstract

Introduction

Malaria continues to be one of the major causes of morbidity and mortality worldwide even though the disease is treatable. This is as a result of malaria parasites developing resistance to current antimalarial drugs (WHO 2001a). Malaria treatment has been changed in the previous years due to the emergence of resistant strains. WHO currently recommends artemisinin combination therapies (ACT’s) as first line treatment for uncomplicated malaria (WHO 2001b). Following this recommendation many countries changed their treatment policies into using ACT’s as first line treatment for malaria. Although this treatment is effective, the emergence of resistance to artemisinin has been reported in countries like Cambodia and Vietnam (WHO 2013). This poses a threat to halt the effort to eradicate malaria since there is currently no treatment that provides the same level of efficacy as the ACT’s. Another shortfall of the ACT’s is that they are quite expensive and therefore unaffordable to the poorest which are the most burdened by malaria. Also the demand and supply is not balanced. Hence there is an imperative need to develop more effective, affordable and easily accessible antimalarial drugs. Oleanolic acid (OA) is a pentacyclic triterpene derived from the *Syzygium aromaticum* cloves amongst other sources. Various therapeutic effects of OA have been reported including anti-inflammatory, anti-diabetic, and hepatoprotective effects but the antimalarial effects of this triterpene have not been reported (Gao *et al.*, 2009; Ngubane *et al.*, 2011; Lee *et al.*, 2013). Maslinic acid (MA), a triterpene also derived from *S. aromaticum*, has been shown to possess antimalarial effects *in vitro* (Moneriz *et al.*, 2011). These results suggest that OA may also possess antimalarial properties. OA has also been reported to possess anti-oxidant effects, and can therefore alleviate the oxidative stress that is manifested during the malaria infection. Hence this study investigated the effects of OA on the malaria parasites.

Some of the antimalarial drugs such as quinine (QN) and chloroquine (CHQ) have been shown to possess hypoglycaemic effects (Musabayane *et al.*, 2010; Elbadawi *et al.*, 2011). The hypoglycaemic effects of CHQ are mediated in part via an increase in insulin secretion (Musabayane *et al.*, 2010). Hence the effects of OA on blood glucose were also investigated in this study. Studies have shown that orally administered CHQ is also deposited in organs
such as the kidneys therefore altering the functions of these organs (Musabayane et al., 1994). Acute renal failure, pulmonary oedema and metabolic acidosis have been observed in patients with malaria. A study conducted in our laboratory reported that OA increases GFR in streptozotocin (STZ) induced diabetic rats (Mapanga et al., 2009). Another study reported that OA improved kidney function by increasing metabolic function of the kidney cell lines (Madlala et al., 2012). These results indicate that OA may be useful in alleviating renal function disturbances exerted by the malaria infection as well as other antimalarial drugs. Hence we evaluated the effects of OA on kidney function. The mechanisms for the development of kidney dysfunction are mediated, at least in part, via abnormal electrolyte handling by the kidney due to oxidative stress. Therefore, we investigated the effects of OA on oxidative stress in the kidney and liver.

**Materials and methods**

The studies were carried out over a period of 3 weeks and were divided into pre-treatment (days 0-7), treatment (days 8-12) and post treatment (days 13-21) periods. The non-infected control group was monitored for 21 days. In the infected control group, malaria was induced by a single intraperitoneal injection of *P. berghei* infected red blood cells. These rats were also monitored and sacrificed at day 14 for ethical reasons. To evaluate the effects of OA on malaria parasites, blood glucose homeostasis and renal function, separate groups of non-infected and *P. berghei*-infected male Sprague-Dawley rats (90g-120g) were used. These rats were treated with either OA (40, 80 and 160 mg/kg, p.o) or CHQ (30 mg/kg, p.o). The animals were housed individually in Makroon polycarbonate metabolic cages. Percentage parasitaemia, mean body weight changes, food and water intake, blood glucose concentrations, haematocrit, oxidative stress, 24 hour urine volume voided, Na⁺, K⁺, Cl⁻ and creatinine levels were monitored every third day during the pre-treatment and post-treatment periods for all the groups. During the treatment period all these parameters were monitored daily. To assess the effects of OA on AVP, aldosterone and insulin concentrations, separate groups of non-infected and *P. berghei*-infected rats were sacrificed at 0, 12 and 24 hours for acute studies as well as on day 14 and 21 for chronic studies. Blood was collected through cardiac puncture and organs such as heart, liver and kidneys were collected.
Results

There was a continuous increase in percentage parasitaemia of the \textit{P. berghei}-infected control throughout the study and as such the animals were sacrificed at day 14. OA was able to clear the malaria parasites, with the most potent dose (160 mg/kg, p.o.) eliminating the parasites after 6 days of treatment. CHQ (30 mg/kg, p.o) cleared malaria parasites after 5 days of treatment. There was a continuous decrease in blood glucose concentrations of the \textit{P. berghei}-infected control until the rats were sacrificed at day 14. Rats treated with OA showed an increase in glucose concentrations during treatment period when compared to the infected control as well as CHQ-treated rats. CHQ-treated rats displayed a significant decrease in blood glucose concentrations during treatment period when compared to the non-infected control as well as OA-treated rats. There was an improvement in blood glucose levels of these animals during the post treatment period, but glucose levels still remained significantly low when compared to the non-infected control as well as OA-treated animals. There was no significant change in plasma insulin concentrations of rats treated with OA when compared to the non-treated control. There was an increase in plasma insulin concentration of rats treated with CHQ when compared to the non-infected control as well as the OA treated groups. OA was also able to increase haematocrit levels during treatment and continued to maintain these levels at normal ranges throughout the post-treatment period. CHQ also increased haematocrit levels in \textit{P. berghei}-infected rats during treatment period. OA increased urinary \text{Na}^+ output as well as GFR when compared to the non-infected control. OA also decreased urinary \text{K}^+ output significantly during the treatment period when compared to the infected control. CHQ increased urinary \text{Na}^+ output in both non-infected and \textit{P. berghei}-infected rats during the treatment period. OA-treated animals exhibited significantly low malondialdehyde (MDA, a marker of lipid peroxidation) and increased activity of the anti-oxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) in renal and hepatic tissues. However, an increase in MDA levels was observed in CHQ-treated animals as well as a decrease in activities of SOD and GPx.
Discussion

*S. aromaticum* derived-OA demonstrated antimalarial properties by completely clearing the malaria parasites in *P. berghei*-infected rats. OA was able to alleviate malarial hypoglycaemia by improving blood glucose levels in *P. berghei*-infected rats during the treatment period. These anti-hypoglycemic effects may in part be due to the eradication of the malaria parasites from the red blood cells (RBC’s) hence no utilisation of the blood glucose by the parasites. The hypoglycaemia observed in CHQ treated rats may be attributed to the increase in insulin secretion that has been shown in rats orally treated with CHQ. We speculate that an increase in haematocrit levels observed in *P. berghei*-infected rats treated with either OA or CHQ is due to both these drug’s ability to clear the malaria parasites from the systemic circulation hence preventing the lysis of RBC’s. OA improved renal function by increasing Na\(^+\) output and GFR as well as decreasing K\(^+\) output. The results suggest that effects of OA on the kidney function are mediated, in part, via increased improved oxidative status.

Conclusions

The results of the current study demonstrate the ability of OA to clear malaria parasites, maintain glucose homeostasis as well as improve kidney function in *P. berghei*-infected rats. We conclude that this triterpene can play a crucial role in the formulation of more effective antimalarial drugs.

Recommendations

This study has demonstrated the ability of orally administered OA to clear malaria parasites. Future studies should therefore investigate the effects of the transdermally delivered OA via the pectin hydrogel patch. The mechanisms responsible for antimalarial as well as hypoglycaemic effects of OA should also be investigated.
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CHAPTER I
Introduction/ Literature review

1.1 Introduction/ Literature review

Malaria is one of the health challenges in the globe with 300-500 million people infected and 1.1 million people dying annually from the disease (WHO 2012a). Africa is the most affected continent with about 90% of all reported deaths occurring in this region (Hay et al., 2004). Malaria is caused by protozoan parasites of the genus *Plasmodium* and the major vector for this disease in Africa is *Anopheles gambiae* (Sinka et al., 2010). *P. falciparum, P. vivax, P. ovale* and *P. knowlesi* are four *Plasmodium* species that cause malaria in humans (WHO 2013a). *P. falciparum* is the most prevalent of the four species accounting for approximately 90% of all the reported deaths (WHO 2008b). Despite the fact that malaria is treatable, the disease still remains the major cause of morbidity and mortality worldwide. Malaria management includes prevention and treatment of the disease. Method of prevention of malaria include the use of insecticides to prevent the transmission of the disease through mosquito bites. However, resistance to insecticides has been reported in many countries where malaria is endemic (WHO 2013a). Malaria preventive chemotherapy is available especially to pregnant women and children who are the most vulnerable to the disease. The WHO has recommended the use of sulphadoxine-pyremithamine as preventive chemotherapy for both pregnant women and infants (WHO 2013d). Current antimalarial drugs exert their effects by targeting different stages of the *Plasmodium* life cycle. However, the development and spread of drug resistant strains has hindered the efforts to eradicate malaria. WHO currently recommends the use of artemisinin combination therapies (ACTs) as the first line treatment for uncomplicated malaria (WHO 2001b). Following the recommendation, many countries have changed their treatment policies and are using ACT’s as their first line treatment for malaria. Although this treatment is effective, resistance to artemisinin has been reported in countries like Cambodia and Thailand (WHO 2013c). This poses a huge threat in the fight against malaria since there is no current treatment that provides the level of efficacy similar to the ACT’s. Another shortfall for the ACT’s is that they are quite expensive and therefore unaffordable to the poor. There is therefore an imperative need to establish more effective and affordable antimalarial drugs.
Oleanolic acid (OA) is a pentacyclic triterpene derived from *S. aromaticum*. Various therapeutic effects of OA including anti-diabetic, anti-inflammatory, anti-oxidant as well as anti-bacterial effects have been reported (Szakiel *et al.*, 2008; Ngubane *et al.*, 2011; Lee *et al.*, 2013). However anti-malarial effects of this triterpene have not been reported. Maslinic acid (MA), also a triterpene derived from *S. aromaticum* has been shown to have antimalarial effects *in vivo* and hence OA may also possess antimalarial properties (Moneriz *et al.*, 2011). Hence this study evaluated the ability of *S. aromaticum*-derived OA to clear the *Plasmodium* parasites as well as effects of this triterpene on glucose homeostasis and renal function. These pathophysiological effects of malaria manifest following the injection of the *Plasmodium* parasites into the human systemic circulation by an infected Anopheles mosquito. The life cycle of the *Plasmodium* parasites is fully described in the next section.

1.2 *Plasmodium* life cycle

*Plasmodium* parasites have a complex life cycle that involves 2 hosts viz, a female mosquito and human (Figure 1). *Plasmodium* parasites are injected into the human blood stream in sporozoite forms via female *Anopheles* mosquito bite (Sidjanski and Vanderberg 1997). From there the parasites are carried by the circulatory system to the liver where they invade hepatic cells (Rennenberg *et al.*, 2010). In the hepatic cells the parasites undergo asexual reproduction and develop to merozoites. The merozoites infect erythrocytes and mature to trophozoites, rapture and release merozoites into the bloodstream. Some of these merozoites go on to infect new erythrocytes while some differentiate into male and female gametocytes which are ingested by the next feeding mosquito. The gametocytes that are ingested by a mosquito undergo sexual replication and produce zygotes. These zygotes differentiate into ookinetes which undergo meiosis and cross the gut wall of the mosquito. The ookinetes undergo endomitosis eventually producing thousands of daughter sporozoites (Clark and Schofield 2000; Mackintosh *et al.*, 2004). The sporozoites migrate from the mid gut to the mosquito’s salivary glands where the life cycle starts all over again when the mosquito bites a human. Current anti-malarial drugs target different stages of the *Plasmodium* life cycle (Bagnaresi 2008). During the erythrocytic stage of the *Plasmodium* life cycle, the parasites acquire amino acids by ingesting the host’s haemoglobin and free haem is produced (Goldberg *et al.*, 1990). This free haem is toxic to the parasites and is thus polymerised to hemozoin. Quinolene-containing antimalarials like CHQ have been shown to prevent the haem polymerisation thereby causing parasite death (Basilico *et al.*, 1998). CHQ is also a
schizonticide but also acts against the formation of gametocytes. Primaquine eradicates pre-erythrocytic liver latent tissue forms of *P. vivax* and *P. ovale* and is also active against gametocyte formation. Anti-folates and ACT’s are also active against the schizont stage malaria parasites. Although these drugs have been effective, resistance has been reported (WHO 2013b). Furthermore, these drugs elicit adverse effects which could be the contributing factor to patient non-compliance which leads to the emergence of resistant strains. Hence studies are being carried out to develop more effective antimalarial drugs. The aim of this study isto investigate the ability of OA to eradicate the malaria parasites as well as to alleviate the complications that arise as a result of the malaria infection. Pathophysiological effects of the malaria infection include hypoglycaemia, anaemia and renal failure.
Figure 1: Illustration of the *Plasmodium* life cycle and the antimalarial drug targets. Majority of antimalarial drugs are schizonticides. Some of these drugs act against more than one stage of the malaria parasite life cycle. Chloroquine, one of the frequently used antimalarial drugs, exerts its plasmocidal effects by inhibiting haematin detoxification during the erythrocytic stage of the life cycle. Primaquine eradicates pre-erythrocytic liver latent tissue forms of *P. vivax* and *P. ovale* and is also active against gametocyte formation.
1.3  Malaria complications

1.3.1  Anaemia

Anaemia is one of the complications normally observed during severe malaria infection. Among the pathophysiological factors that contribute to malarial anaemia is the destruction of red blood cells by the *Plasmodium* parasites, clearance of non-infected red blood cells and suppression of erythropoiesis (Casals-Pascual *et al.*, 2006; Totino *et al.*, 2010). Also the immune system plays a role by removing infected red blood cells through phagocytosis (Ayi *et al.*, 2005). During the *Plasmodium* life cycle, the malaria parasites invade red blood cells, multiply and mature inside these cells, disrupts the cells and go on to infect new RBC’s. This leads to the reduction in the number of red blood cells and hence the development of anaemia. Another factor that contributes to malarial anaemia is the destruction of uninfected red blood cells by phagocytosis (Totino *et al.*, 2010). Malaria has also been reported to suppress erythropoiesis (Casals-Pascual *et al.*, 2006). Studies have shown that haemoglobin residues produced after degradation of haemoglobin by the parasite inhibit erythropoiesis (Casals-Pascual *et al.*, 2006). The anti-inflammatory cytokine TNFα which is produced after the infection is also implicated in dyserythropoiesis (Clark and Chaudri 2008). This imbalance between the destruction and production of red blood cells plays a major role in malarial anaemia. In the current study, haematocrit levels were measured in order to investigate the ability of (OA) to alleviate this malaria complication. Measuring haematocrit was a marker for monitoring the concentration of red blood cells throughout the study. Another factor that also contributes to malarial anaemia apart from the destruction of RBC’s is hepcidin.

1.3.1.1  Hepcidin

Hepcidin, a peptide hormone produced mainly by hepatocytes plays an important role in regulation of iron homeostasis. Inflammation is one of the factors that have been reported to up regulate hepcidin production (Nicolas *et al.*, 2002). Studies have also reported that during the acute malaria infection anti-inflammatory cytokines interleukin 6 (IL-6) and interleukin 10 (IL-10) induce the production of hepcidin (Huang *et al.*, 2014). Hepcidin has been reported to reduce dietary iron absorption in the small intestine and inhibit iron release from macrophages by by internalising and degrading ferroportin, the iron exporter (Ganz 2003). Studies have reported upregulation of hepcidin in mice infected with pathogenic bacteria.
(Peyssonaux et al., 2006). The increased hepcidin levels lead to intracellular iron sequestration thereby reducing the availability of iron to these pathogens (Paradkar et al., 2008). Hence hepcidin is believed to play a protective role during the malaria infection by limiting the availability of iron which is essential for the parasite’s survival (Wang et al., 2011). However this sequestration of iron contributes to the anaemia that is observed during the malaria infection. In this study the effects of malaria infection as well as that of OA and CHQ on haematocrit levels were investigated. This was done in order to establish whether OA is able to alleviate anaemia that is usually observed during the malaria infection.

1.3.2 Hypoglycaemia

Hypoglycaemia is one of the pathophysiological effects the malaria infection (Aziem et al., 2011). During the erythrocytic stage of the *Plasmodium* parasites, the parasites utilise the host’s glucose as the source of energy. Studies have shown an increase in glucose utilisation in the *Plasmodium*-infected RBC’s when compared to the non-infected control (Onyesom et al., 2013). This utilisation of glucose by the *Plasmodium* parasites contributes to the hypoglycaemia observed in most cases of severe malaria. Antimalarial drugs like QN and CHQ have also been shown to possess hypoglycaemic effects. Studies conducted in our laboratory reported that orally administered CHQ exerted hypoglycaemic effects via an increase in insulin secretion (Musabayane et al., 2010a). QN has also been shown to decrease blood glucose through increased insulin secretion (Elbadawi et al., 2011). Therefore these drugs also contribute to severe hypoglycaemia observed during malaria infection when used as treatment. A study conducted in our laboratory showed that OA has no effects on insulin secretion (Musabayane et al., 2010b). These results suggest that OA may not exert hypoglycaemic effects when used for malaria treatment and can therefore be an ideal alternative antimalarial. Hence, in this study blood glucose levels were monitored to investigate the effects of the OA on glucose homeostasis. Glucose concentrations were also monitored in *P.berghei*-infected and non-infected rats to distinguish between the effects of the *Plasmodium* parasites and that of OA on glucose homeostasis.
1.3.3 Impaired kidney function

Acute renal failure has been observed in patients with severe *P. falciparum* malaria (Thanachartwet *et al.*, 2013). In a recent study hyperkalaemia, metabolic acidosis and pulmonary oedema were observed in patients with severe malaria (Thanachartwet *et al.*, 2013). This hyperkalaemia may be attributed to the haemolysis of RBC’s by the malaria parasites thereby releasing potassium to the systemic circulation. Pulmonary oedema and metabolic acidosis further suggest that malaria infection interferes with kidney function. A study conducted in our laboratory reported that OA increases GFR in streptozotocin (STZ) induced diabetic rats (Mapanga *et al.*, 2009). Another study reported that OA improved metabolic function of the kidney cell lines (Madlala *et al.*, 2012). These results suggest that OA may play a vital role in alleviating renal function disturbances exerted by the malaria infection. Hence renal electrolyte levels were monitored to evaluate the effects of OA on kidney function. Current antimalarial drugs are used to treat malaria infection as well as to alleviate complications associated with the disease. The next sections discuss antimalarial drugs, their modes of action, benefits as well as their shortfalls.

1.4 Malaria chemotherapy

1.4.1 Quinine and related drugs

Quinine, a schizonticide derived from cinchoma was reportedly the first chemical compound successfully used to treat malaria (Achan *et al.*, 2011). QN acts against asexual erythrocytic forms of *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. The side effects of QN referred to as cinchonism, include auditory symptoms, gastro-intestinal disturbances, vasodilation, headaches, nausea, blurred vision, vomiting, abdominal pains and thrombocytopenia (Hálfdanarson *et al.*, 2002). QN has also been shown to exert hypoglycaemic effects through increasing insulin secretion (Elbadawi *et al.*, 2011). In addition to these side effects, resistance to QN has also been reported (Pradines *et al.*, 2010). In addition to the resistant strains against the drug, the hypoglycaemic effects also cast a shadow against the use of QN since the malaria parasites also replenish the host’s blood glucose stores. This may lead to severe hypoglycaemia in patients receiving QN treatment. There is therefore a need to uncover alternative drugs with minimal side effects. Hence the current study investigated the ability of OA to clear the malaria parasites in an effort to establish alternative malaria treatment which will elicit minimal side effects.
1.4.2 Chloroquine (CHQ)

CHQ, a QN derivative was one of the most effective and affordable drugs used in treatment and prophylaxis of malaria until the emergence of CHQ-resistant *falciparum* malaria in the 1950’s in Asian countries and in the 1970’s in African countries (Breman *et al.*, 2001). CHQ is a rapid acting schizonticide against *P. falciparum* with gametocytocidal activity against asexual erythrocytic forms of *P. malariae*, *P. ovale* and *P. vivax*. CHQ inhibits malaria parasite’s digestive pathway for haemoglobin by interfering with the release of toxic haem produced when the malaria parasites digest haemoglobin to obtain amino acids which are vital for the parasite’s survival in the red blood cells (Ginsburg *et al.*, 1998). The prevention of haem detoxification is lethal to the *Plasmodium* parasites. CHQ also targets the oxidative pathway in the erythrocytic stage and therefore affects multiple processes in the *Plasmodium* life cycle (Radfar *et al.*, 2008). Following the reports of resistant strains against CHQ, many countries changed their first line treatment to the anti-folates and later to ACT’s (Tjitra *et al.*, 2002). But there are countries that are still using CHQ as their first line treatment against malaria. Inadequate dosing and incomplete courses of therapy due to non-compliance are also believed to be contributing factors to the emergence and spread of resistant malaria strains. CHQ’s bitter taste is thought to be the contributing factor in patient’s non-compliance which leads to mutation of malaria parasites. CHQ is slowly eliminated from the body and hence accumulates in organs such as kidneys and liver and alters physiological functions of these organs (Musabayane *et al.*, 1994). Orally administered CHQ has also been shown to accumulate in the spleen following chronic administration (Desai *et al.*, 2010). Orally administered CHQ has also been shown to cause hypoglycaemia through increasing insulin secretion (Musabayane *et al.*, 2010a). Hence this study investigated antimalarial effects of OA as well as the effects on blood glucose levels and renal electrolyte handling in an effort to explore OA as an alternative antimalarial agent that also stabilizes glucose levels and improves kidney function. Other QN derived drugs include primaquine and mefloquine which are also schizonticides. Mefloquine is also used for malaria prophylaxis and is also active against asexual stages of *P. falciparum* and *P. vivax*. Primaquine eradicates pre-erythrocytic liver latent tissue forms of *P. vivax* and *P. ovale* which cause malaria relapses (Galappathy *et al.*, 2013). However the limitation to the use of primaquine has been the drug’s haemolytic toxicity (WHO 2012b). There is therefore a need to conduct more research that would lead to the formulation of more effective and less toxic antimalarial drugs.
1.4.3 Anti-folates

1.4.3.1 Sulphadoxine/pyrimethamine (SP) combination

When CHQ-resistant malaria strains emerged, many countries changed their first line anti-malarial treatment from CHQ to anti-folates (Plowe et al., 2004). Pyramethamine and sulphadoxine inhibit dihydrofolate reductase and dihydroperoate synthase respectively. These enzymes activate folate metabolism, an essential pathway for the survival of the malaria parasites (Wang et al., 1999). Hence this action leads to parasite death. This combination is the most widely used option for uncomplicated *P. falciparum* malaria. However, resistance to SP combination has also been reported (WHO 2001a).

1.4.3.2 Proguanil

Malorone is a fixed dose combination of atovaquine and proguanil which is highly effective for treatment and prophylaxis for multi-drug resistant *falciparum* malaria (Khositnithikul et al., 2008). Proguanil is an anti-folate which exerts antimalarial effects by interfering with the folate metabolism. This drug inhibits the enzyme dihydrofolate reductase. Following the rise of the resistant strains to anti-folates many countries have changed their policies and are using the artemisinin combination therapies as their first line treatment for malaria. As a result of the reported resistance to artemisinin there is an urgent need to develop more effective drugs. In this study the effects of OA on the malaria parasites were investigated.

1.4.4 Artemisinin combination therapies (ACT’s)

Due to the emergence and spread of malaria parasites resistance to some monotherapies including CHQ, WHO recommended the use of combination therapies with artemisinin derivatives (WHO 2001b). Hence, combination therapies are currently the most used drugs in the treatment of malaria worldwide. The shortfall for ACT’s is that they are relatively expensive and therefore inaccessible in malaria endemic countries. The high demand and low supply of these drugs also makes them uneasily accessible.
1.4.4.1 Artemether/lumifantrine (AL)

AL combination is the most widely used ACT combination for treatment of uncomplicated malaria in Africa. AL has good tolerability with few adverse events (Achan et al., 2009). Artemether and lumifantrine are both blood schizonticides with different modes of action. Artemether is an artemisinin derivative that affects multiple stages in the *Plasmodium* life cycle including interference with parasite transport proteins and disruption of parasite mitochondrial function (White et al., 1999). Lumefantrine prevents the detoxification of haem such that toxic haem and free radicals induce parasite death (Kokwaro et al., 2007). These modes of action for the two drugs ensure clearance of the malaria parasites as well as prevent relapse.

1.4.4.2 Dihydroartemisinin/piperaquine (DP)

DP combination is relatively inexpensive and has been shown to be effective in both treating malaria and preventing re-infection.

1.4.2.2.1 Artemisinin resistance

Although ACT’s are currently the most used and effective antimalarials, resistance to artemisinin has been reported in malaria endemic countries like Cambodia and Thailand (WHO 2013b). The emergence of artemisinin resistance raises a major concern since there is currently no treatment that provides the same therapeutic level as the ACT’s. Hence medicinal plant research has become more important and has shown great potential to be a useful tool in formulation of new, effective and affordable drugs.

1.5 Medicinal plants

Medicinal plants have demonstrated a great potential of providing effective drugs for treatment and prevention of malaria with antimalarial drugs QN and artemisinin being derived from plants. As a result more researchers are focusing on medicinal plant research in an effort to uncover novel antimalarial drugs. Due to limited availability or high cost of some antimalarial drugs in tropical countries, the majority of the population in these countries depend on traditional medicinal remedies (Van Andel and Carvalheiro 2013). South Africa is
one of those countries where majority of the population is dependent on traditional remedies especially in the rural areas. Therefore, there is also a need to validate the efficacy of these traditional remedies as this could lead to the discovery of novel and effective drugs.

A number of studies have been conducted to investigate if remedies used by traditional healers have antimalarial activity (Nethengwe et al., 2012; Onguène et al., 2013; Thiengsusuk et al., 2013). Prozesky and his colleagues screened 14 ethnobotanically described antimalarial plants (Prozesky et al., 2001). This in vitro study showed that these plant extracts were not very effective and some were cytotoxic (Prozesky et al., 2001). When Van Zyl and Viljoen screened 34 aloe extracts, the methanol extracts showed variable antimalarial activity and they showed limited toxicity to kidney epithelium cells (Van Zyl and Viljoen 2002). These results provide evidence that medicinal plants could provide alternative novel antimalarial drugs.

In a recent study, medicinal plants used by Zulu traditional healers which include Withania somnifera, Elaeodendron transvalense, Hypoxis hemerocallidea, Vernonia were investigated for their antimalarial activity (Nethengwe et al., 2012). Some of these plants showed high anti-plasmodial activity and some of them possessed antipyretic effects (Nethengwe et al., 2012). Pyrexia is a complication that arises as a result of the malaria infection. Hence these results further indicate that medicinal plants may be the source of new drugs that could eradicate malaria as well as alleviate the symptoms that arise during the malaria infection. Some of these medicinal plants also had anti-oxidant effects (Nethengwe et al., 2012). These results provide evidence that not only can these medicinal plants clear the malaria parasite, but they could also combat oxidative stress that arises as a result of the malaria infection. In this study antimalarial effects of OA which is derived from S.aromaticum were investigated, as well as the effects of this triterpene on blood glucose and renal electrolyte handling.
1.6 Oleanolic acid (OA)

OA is a triterpene that occurs naturally in certain foods like resins and cranberries (Zhang et al., 2013) and also in numerous medicinal plants. In the current study we used S. aromaticum derived OA. S. aromaticum is an evergreen tree that grows up to 8-30 m and is native to Indonesia but also grows in other tropical countries (Bhowmik et al., 2012). OA is a pentacyclic triterpene extracted from the dried flower buds or cloves of the S. aromaticum tree (Figure 2). These flower buds are commonly used as spice and also have various health benefits. S. aromaticum contains other compounds like monoterquine, sequisterpene and triterpenes (Mahmoud et al., 2007).

1.6.1 Therapeutic effects of OA

Various therapeutic effects of OA have been reported including anti-inflammatory (Lee et al., 2013), anti-diabetic (Ngubane et al., 2011) and hepatoprotective effects (Jeong 1999). OA has been reported to exert anti-inflammatory effects through inhibiting the production of tumor necrosis factor-α (TNF-α) (Lee et al., 2013). Various studies have reported that OA has anti-tumor, anti-cancer and chemo protective effects (Feng et al., 2009). Anti-bacterial effects of OA have also been reported, where OA was shown have antibacterial effects against Staphylococcus Aureus, Escherichia Coli and Pseudomonas aeruginosa (Pandey and Singh 2011). (Khathi et al., 2013) reported that OA can be used as a supplement for treating postprandial hyperglycaemia. Furthermore, OA has been shown to work synergistically with insulin, a blood glucose lowering agent (Musabayane et al., 2010b). The same study also showed that OA does not increase insulin secretion, suggesting another possible mechanism for the triterpene’s anti-diabetic effects (Musabayane et al., 2010b). Research has also shown that OA exerts anti-hyperglycaemic effects by increasing glycogen concentration and activities of hexokinase and glucokinase (Ngubane et al., 2011). These results suggest that OA may not contribute to the malarial hypoglycaemia when used for treating the disease. Studies have also shown that maslinic acid (MA) which is also a triterpene derived from S. aromaticum exerts anti-oxidant effects by increasing the activity of anti-oxidant enzymes; superoxide dismutase and glutathione peroxidase in hepatic, cardiac and renal tissues (Mkhwanazi et al., 2014). MA has also been shown to possess antimalarial properties in vitro (Moneriz et al., 2011). These results suggest that OA may also possess antimalarial as well as anti-oxidant effects. Hence this study evaluated antimalarial activity of OA, as well the effects of this triterpene on blood glucose levels and renal electrolyte handling.
Figure 2: OA is extracted from the aromatic flower buds/fruit of the *Syzygium aromaticum* tree (A). The fruits are reddish in colour when they are fully grown and are often referred to as cloves. When they are dried they are brown and appear closed (B). Pictures adapted from (Kamatou *et al.*, 2012; Madlala *et al.*, 2012).
1.7 Aims of the study

The aim of the study was to evaluate the therapeutic effects of *S. aromaticum* derived oleanolic acid on malaria parasites in *Plasmodium berghei*-infected male Sprague-Dawley rats, effects on blood glucose and renal electrolyte handling. The secondary aim was to evaluate the effects of OA on glucose homeostasis and renal function as well as to distinguish between the effects of malaria and that of OA on these parameters.
CHAPTER 2

Materials and methods

2 Materials

2.1 Drugs and chemicals

Drugs and chemicals were sourced as indicated:

Chloroquine diphosphate (C\textsubscript{18}H\textsubscript{26}CIN\textsubscript{3}2H\textsubscript{3}PO\textsubscript{4}), dimethyl sulphoxide (DMSO), Giemsa stain, May-Grunwald solution, acetonitrile (C\textsubscript{2}H\textsubscript{3}N), acetic acid (C\textsubscript{2}H\textsubscript{4}O\textsubscript{2}) ethyl acetate (C\textsubscript{4}H\textsubscript{8}O\textsubscript{2}) thiobarbituric acid (TBA) and butylated hydroxyl toluene (BHT) (Sigma-Aldrich Chemical Company, St Louis, Missouri, USA);

Potassium hydroxide (KOH), sodium sulphate (Na\textsubscript{2}SO\textsubscript{4}), sodium hydroxide (NaOH), sodium carbonate (NaCO\textsubscript{3}), potassium dihydrogen phosphate (KH\textsubscript{2}PO\textsubscript{4}), methanol (CH\textsubscript{3}OH) butanol (C\textsubscript{4}H\textsubscript{10}O) hydrochloric acid (HCl) and 95% ethanol (C\textsubscript{2}H\textsubscript{5}OH) (Merck Chemicals (PTY) LTD, Johannesburg, South Africa); diethyl ether (C\textsubscript{4}H\textsubscript{10}O) and phosphoric acid (H\textsubscript{3}PO\textsubscript{4}) (NT Laboratory Supplies (PTY) LTD, Johannesburg, South Africa);

Sulphuric acid (H\textsubscript{2}SO\textsubscript{4}) (BDH Chemicals LTD, Poole, Dorset, England);

Isofor inhalation anaesthetic (Safeline Pharmaceuticals (PTY) LTD, Weltevreden Park, Roodepoort, South Africa);

The ultrasensitive rat insulin ELISA kit - (DRG Instruments, New Jersey, USA);

Vasopressin ELISA kit, aldosterone ELISA kit (Abcam, Cambridge, Massachusetts, USA) and

Biovision SOD assay kit and Biovision GPx Assay Kit (BioVision research products, Mountain View, CA).

All chemical reagents were of analytical grade.
2.2 Animals

Male Sprague-Dawley rats (90-120 g) bred and housed in the Biomedical Research Unit of the University of KwaZulu-Natal, Westville campus were used in the study. The animals were maintained under standard laboratory conditions of constant temperature (22±2 °C), CO₂ content of <5000 p.p.m., relative humidity of 55±5%, and illumination (12 h light/dark cycles) and the noise levels of <65 decibels. The animals had free access to standard rat chow (Meadows Feeds, Pietermaritzburg, South Africa) and water.

2.3 Ethical considerations

Ethical clearance was obtained from the University of KwaZulu-Natal’s Ethics committee (References 090/13/Animal and 038/14/Animal, see Appendices I and II).

2.4 Experimental design

Effects of OA on malaria parasites, blood glucose and renal electrolyte handling were observed in separate groups on non-infected and P. berghei-infected male Sprague-Dawley rats (Figure 3).
Figure 3: Flow diagram representation of the experimental design summary.

Sprague-Dawley rats (90-120g)

Malaria infected
- Non-treated
- OA
- CHQ

Non-infected
- Non-treated
- OA
- CHQ

Parasitaemia, Blood glucose, Haematocrit, Electrolytes, Antioxidants and Hormones

TERMINAL STUDIES
Blood, heart, liver and kidneys were collected.
2.5 Methods

2.5.1 Extraction of OA

The extraction of OA was carried out in Chemistry laboratory at UKZN Pietermaritzburg campus. OA was isolated from *Syzygium aromaticum* [(Linnaeus) Merrill & Perry] [Myrtaceae] (cavies) flower buds using a standard protocol that has been validated in our laboratory with minor changes (Mapanga *et al.*, 2009; Madlala *et al.*, 2012; Mkhwanazi *et al.*, 2014) (Figure 4). Briefly, air-dried *S. aromaticum* flower buds (500 g) were sequentially extracted twice at 24 h intervals at room temperature with 1 L dichloromethane (DCM), and ethyl acetate (720 mL) on each occasion. Removal of the solvent from the extract under reduced pressure at 55±1°C using a rotary evaporator yielded dichloromethane solubles (DCMS, 63 g) and ethyl acetate solubles (EAS, 85 g). The EAS containing mixtures of oleanolic/ursolic acid was purified by silica gel 60 column chromatography with a hexane: ethyl acetate 9:1, 8:2 solvent system increasing polarity. OA was yielded. The structure of OA was confirmed by spectroscopic analysis using 1D and 2D, $^1$H and $^{13}$C Nuclear Magnetic Resonance (NMR) techniques.

![Figure 4](image.png)

**Figure 4:** The chemical structure of OA as determined through $^1$H and $^{13}$C NMR spectroscopy.
2.5.2  Induction of malaria

A CHQ susceptible strain of *P. berghei* was used for the induction of malaria. Malaria was induced by intraperitoneally injecting male Sprague-Dawley rats with parasitized erythrocytes (10^5 parasitized red blood cells) (Gumede *et al.*, 2003). The *P. berghei* parasites were donated by Professor Peter Smith (University of Cape Town, Division of Clinical Pharmacology, South Africa). Successful malaria induction was confirmed by microscopic examination of Giemsa stained thin smears of the tail blood. Percentage parasitaemia ranging from 15 - 20% was considered as a stable malaria state before commencing any experimental procedures.

2.5.3  Parasitaemia monitoring

Percentage parasitaemia was monitored every third day during pre-treatment period and every day during treatment and post treatment periods at 09h00 am. Briefly, a small prick at the tip of the rat tail was made followed by placing a small drop of blood on a microscope slide. The blood was smoothly and uniformly smeared using a second microscope slide. The blood film on the slide was air-dried, fixed in 90 % methanol for 30 seconds and then stained in Giemsa stain for 20 minutes. Finally, the stained slide was air-dried and viewed under a microscope (Olympus Cooperation, Tokyo, Japan) with a x50–x100 oil immersion objective (Olympus cooperation, Tokyo, Japan). Parasite density was calculated as a percentage of infected RBCs. This was achieved by counting the total number of red blood cells and the parasitized red blood cells in 5 microscope fields.

\[
\text{% parasitaemia} = \frac{\text{Total parasitized RBC count}}{\text{Total RBC count}} \times 100
\]
2.5.4 Effects of OA on parasitaemia

The studies were carried out over a period of 3 weeks and were divided into pre-treatment (0-7 days), treatment (8-12 days) and post treatment (13-21 days). The non-infected control group was monitored throughout 21 day period. A positive control, infected with *P. berghei* but not treated was also monitored but was sacrificed on day 14 for ethical reasons. Separate groups of non-infected and *P. berghei*-infected male Sprague-Dawley rats were treated with either OA (40, 80 and 160 mg/ kg, p.o ) or CHQ (30 mg/kg, p.o). The selected doses were based on previous studies that were conducted in our laboratory.

The rats were treated orally using a ball-tipped, 18-gauge gavage needle (Kyron Laboratories (Pty) LTD, Benrose, South Africa) attached to a 1 ml syringe. Treatment was given twice daily at 09h00 and at 17h00 for 5 consecutive days. Percentage parasitaemia and blood glucose levels were monitored daily at 09h00. To evaluate the effects of OA on physiological parameters a group of non-infected animals were treated with 80 mg/kg of OA. These animals were monitored for 21 days as well.

2.5.5 Haematocrit levels

Haematocrit levels were monitored in all groups of animals at 09h00 on days 0, 6, 9 14 and 21. Briefly, blood was collected into heparinised capillary tubes using the tail prick method. The sealed capillary tubes were then centrifuged for 3 minutes at 906 x G with a Microhaematocrit centrifuge (346 MSE Centrifuge Manufacturer, London, UK). Haematocrit was determined by calculating the percentage of the packed cell volume to blood volume.

\[
\text{Heamatocrit} = \frac{\text{Packed cell volume}}{\text{Total blood volume}} \times 100
\]
2.5.6 Effects of on blood glucose and physico-metabolic parameters

To evaluate the effects of the malaria parasites, OA and CHQ on blood glucose, blood glucose concentrations were measured in all the groups of animals using blood glucose testing strips (OneTouch select glucometer, Lifescan, Mosta, Malta, United Kingdom). This was done every third day during pre-treatment and post treatment periods and every day during treatment period. Body weights, urine output, food and water intake were measured in control and treated animals at 09h00 every 3rd day during the pre-treatment and post treatment periods, and daily during the treatment period. Body weights, water and food intake were measured in control and treated animals at 09h00 every 3rd day during the pre-treatment and post treatment periods, and daily during the treatment period. The 24h00 urine output, urinary electrolytes (Na⁺, K⁺, Cl⁻), urea and creatinine were also measured.

2.5.7 Terminal studies

To evaluate the effects of OA and CHQ on some biochemical parameters, groups (n=6) of control and groups of non-infected and *P. berghei*-infected rats treated with either OA or CHQ were sacrificed at day 0, 9, 12 and 21 (Total = 24/ group). The animals were anaesthetized by placing them in an anaesthetic chamber with 100 mg/kg of isofor inhalation anaesthetic for 3 minutes. Blood samples were collected by cardiac puncture into pre cooled heparinised tubes and centrifuged for 15 minutes at 959 x G at 4 °C (Eppendorf International, Hamburg, Germany). The plasma samples were stored in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) at -80 °C until assayed. The collected plasma samples were used for arginine vasopressin (AVP), aldosterone, insulin and electrolytes (Na⁺, K⁺, Cl⁻, urea and creatinine) assays. The kidneys, heart, liver, spleen and pancreas were also collected and stored in a Bio Ultra freezer at -80 °C until further use.
2.6 Laboratory analysis

2.6.1 Insulin assay

To determine the effects of OA on insulin secretion, plasma insulin concentrations were measured using the ultra-sensitive rat insulin ELISA kit (DRG diagnostics EIA-2943 GmbH, Marburg, Germany). For acute studies, insulin concentrations were measured on the non-infected as well as *P. berghei*-infected rats treated with OA (80 mg) and CHQ (30 mg) as well the respective controls at 12 and 24 hour intervals. For chronic studies, plasma insulin concentrations were measured on the same groups of animals at day 5 and 14 post treatment.

The insulin assay kit consisted of a 96 well plate coated with mouse monoclonal anti-insulin, enzyme conjugate, standards, enzyme conjugate buffer, substrate 3,3',5,5'-tetramethylbenzidine (TMB), wash buffer and a stop solution. This assay is a solid phase two-site immunoassay based on the direct sandwich technique in which two monoclonal antibodies are directed towards separate antigenic determinants on the insulin molecule. During the incubation period insulin in the sample reacts with peroxidase-conjugate anti-insulin antibodies bound to the microtitration well. The unbound enzyme labelled antibody was removed by the washing step, leaving the bound conjugate to react with TMB. The reaction was stopped by adding sulphuric acid to give a colorimetric endpoint which is read spectrophotometrically on a microplate reader (BMG Labtech GmbH, Ortenberg, Germany) at 450nm. Each determination was done in duplicate for standards and test samples. A 50µl of each insulin standard was added to anti-insulin wells. Plasma samples (50µl) were added to the remaining anti-insulin wells. This was followed by the addition of the enzyme conjugate to standard and plasma samples wells. The plates were incubated at room temperature on a plate shaker (Heidolph, Schwabach, Germany) for 2 hours. This was followed by multiple wash using a wash buffer (350µl). After the final wash, the plates were inverted against absorbent paper to remove all the liquid. The substrate, TMB was then added to all wells and incubated for 30 minutes. The reaction was stopped by adding 50 µl of stop solution to all wells and mixing for 5 minutes. Absorbance was measured using SpectrostarNanomicroplate reader (BMG Labtech GmbH, Ortenberg, Germany). A standard curve was constructed from the absorbance values of the known concentrations of the standards using the spline regression curve. Insulin concentrations of the unknown samples were extrapolated from the standard curve. The lower and upper limits of detection were 1.74 pmol/L and 960 pmol/L, respectively. The intra-assay analytical coefficient of variation
ranged from 4.4 to 5.5% and the inter-assay coefficient variation from 4.7 to 8.9%. Each determination was done in duplicate for standards (0, 3.40, 8.50, 25.50, 68.10 and 170.27 pmol/L) and test samples. A 50 µL of each insulin standard was added to anti-insulin wells.

2.6.2 Arginine vasopressin (AVP) assay

2.6.2.1 AVP extraction

Plasma samples were subjected to an extraction process prior to use. Briefly, 100 µL of plasma sample and 200 µL of ice cold acetone were added into the eppendorf tubes. The mixture was vortex and centrifuged at 12000 x G for 20 minutes. After centrifugation, the supernatant was collected into a glass tube. A volume of 500 µL of ice cold petroleum ether was added to the supernatant. The mixture was centrifuged at 10000 x G for 10 minutes to obtain the ether and aqueous layer. The remaining aqueous layer was transferred into new glass test tubes and dried under gas for 48 hours. After the drying, white crystals were obtained and were reconstituted in assay buffer. The samples were used immediately.

A standard enzymatic method was used to determine plasma AVP concentrations. The assays were performed on an Arg8-Vasopressin ELISA Kit, using reagents purchased from the manufacturer (Abcam, Cambridge, Massachusetts, USA). The lower and upper limits of detection were 4 pmol/L - 923pmol/L, respectively. The intra assay analytical coefficient of variation ranged from 5.9 to 10.6% and the inter-assay coefficient variation from 6.0 to 8.5%. The kit components included a 96 well plate coated goat antibodies, vasopressin conjugate, vasopressin antibody, assay buffer, wash buffer concentrate, vasopressin standards, p-nitrophenyl phosphatesubstrate (pNpp) and a stop solution. The Arg8-Vasopressin ELISA Kit is a competitive immunoassay for the quantitative determination of vasopressin in samples. The assay uses a polyclonal antibody-vasopressin conjugate to bind covalently in a competitive manner with vasopressin in unknown samples. During the incubation period AVP in the sample reacts with phosphatase-conjugate anti-vasopressin antibodies and anti-vasopressin antibodies bound to the micro-titration well. The washing step removes unbound enzyme labelled antibody, leaving the bound conjugate which reacts with pNpp. This reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically on a microplate reader (BMG Labtech GmbH, Ortenberg, Germany) at 405 nm.
Plasma samples were subjected to an extraction process prior to use. Briefly, 100 µL of plasma sample and 200 µL of ice cold acetone were added into the eppendorf tubes. The mixture was vortex and centrifuged at 12000 x G for 20 minutes. After centrifugation, the supernatant was collected into a glass tube. A volume of 500 µL of ice cold petroleum ether was added to the supernatant. The mixture was centrifuged at 10000 x G for 10 minutes to obtain the ether and aqueous layer. The remaining aqueous layer was transferred into new glass test tubes and dried under gas for 48 hours. After the drying, white crystals were obtained and were reconstituted in assay buffer. The samples were used immediately.

Each determination was performed in duplicate for both standards and the test samples. The assay procedure was as follows: A volume of 100 µL of vasopressin standards (4, 10, 23, 59, 148, 369 and 923 pmol/L) was added into anti-vasopressin wells. Samples (100 µL) were then added to the remaining wells followed by 50 µL vasopressin conjugate into all standard and sample wells. The plates were incubated at 4ºC for 24 hours. Following incubation, the reaction volume was emptied. 400µL of wash buffer was added to all wells and aspirated. The process was repeated 3 times. After the final wash, the plates were inverted firmly against absorbent paper to remove all the liquid. 200µL of substrate pNpp was added to all wells and incubated at 37 ºC for 1 hour. The reaction was stopped by adding 50µL of stop solution to all wells and mixing on the shaker for 5 minutes. The absorbance was read at 405 nm using a Spectrostar Nanomicroplate reader (BMG Labtech GmbH, Ortenberg, Germany). A standard curve was constructed from the absorbance values of the known concentrations of the standards using the spline regression curve in Graph Pad Instat software (version 5.00). The respective AVP concentrations of the unknown samples were then extrapolated from the standard curve.

2.6.3 Aldosterone assay

The kit components included a 96 well plate coated with a polyclonal rabbit antibody, aldosterone conjugate, wash buffer concentrate, aldosterone standards, and a stop solution. The DEMEDITEC aldosterone ELISA kit is a solid phase enzyme-linked ELISA based on the principle of competitive binding. The microtiter wells were coated with a polyclonal rabbit antibody directed towards an antigenic site of the aldosterone molecule.
Aldosterone in the sample competes with an aldosterone-horseradish peroxide conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. After addition of the substrate solution, the intensity of the colour is inversely proportional to the concentration of aldosterone in the sample.

The assay procedure was as follows, 50 µL of each standard (0.11, 0.22, 0.43, 0.86, 1.73, 3.46 and 6.92 pmol/L), control and samples were dispensed into appropriate wells, 150 µL of the aldosterone conjugate was then added into each well followed by mixing for 10 minutes and then incubation for 60 minutes at room temperature. The contents of the wells were shaken out and rinsed with 300 µL of wash buffer was added to all wells and aspirated. The process was repeated 3 times. After the final wash, the plates were inverted firmly against absorbent paper to remove all the liquid followed by incubation of 30 minutes at room temperature. The reaction was stopped by adding 100 µL of the stop solution to all well. The absorbance was read at 405 nm using a Spectrostar Nanomicroplate reader (BMG Labtech GmbH, Ortenberg, Germany). A standard curve was constructed from the absorbance values of the known concentrations of the standards using the spline regression curve in Graph Pad Instat software (version 5.00). The respective aldosterone concentrations of the unknown samples were then extrapolated from the standard curve.

2.6.4 Evaluation of oxidative stress

To establish the effects of OA on oxidative stress in the liver, heart and kidney of *P. berghei*-infected rats, we compared levels of MDA, a commonly known marker of oxidative stress and of antioxidant defence enzymes SOD and GPx between the non-infected and *P. berghei*-infected male Sprague-Dawley rats.

2.6.4.1 MDA

Tissues (50 mg) were homogenized in 500 mL of 0.2% phosphoric acid. The homogenate was centrifuged at 400 x G for 10 min. Thereafter, 400 mL of the homogenate was supplemented with 400 mL 2% phosphoric acid and then separated into two glass tubes, each receiving equal volumes of the solution. Subsequently, 200 mL of 7% phosphoric acid was added into both glass tubes followed by the addition of 400 mL of thiobarbituric acid
(TBA)/butylated hydroxyl toluene(BHT) into one glass tube (sample test) and 400 mL of 3mM hydrochloric acid (HCl) into the second glass tube (blank). To ensure an acidic pH of 1.5, 200 mL of 1M HCl was added to sample and blank test tubes. Both solutions were heated at 100 °C for 15 min, and allowed to cool to room temperature. Butanol (1.5 mL) was added to the cooled solution; the sample was vortexed for 1 min to ensure rigorous mixing and allowed to settle until two phases could be distinguished. The butanol phase (top layer) was transferred to Eppendorf tubes and centrifuged at 13,200 x G for 6 min. The samples were aliquoted into a 96-well microtiter plate in triplicate and the absorbance was read at 532 nm (reference 600 nm) on a BioTek mQuant spectrophotometer (Biotek, Johannesburg, South Africa). The absorbance from these wavelengths was used to calculate the concentration of MDA using Beer’s Law.

2.6.4.2 SOD

SOD activity was measured using the Biovision SOD Assay Kit according to manufacturer’s instructions (BioVision Research Products, Mountain View, CA). In the presence of SOD, tetrazolium salt (WST-1) is reduced into a dye which can be measured photometrically. Rat liver and kidney tissues (50 mg) were homogenized in ice cold 0.1 M Tris/HCl (pH 7.4) containing 0.5% Triton X-100, 5mM b-mercapto ethanol(ME) and 0.1 mgmL_1 phenyl methane sulfonyl fluoride(PMSF) (0.1 mg/kg). Sample solution (20 µL) was added in a sample and blank 2 wells, H2O (20 µL) was added in blank 1 and 3 wells. WST-1 working solution (200 µL) was added in each well. Thereafter, dilution buffer (20 µL) was added in blank 2 and 3 well and SOD enzyme solution (20 µL) was added in sample and blank 1 wells. The plate was then incubated at 37 °C for 20 min followed by reading absorbance at 450 nm. The SOD activity was calculated and expressed as Biovision laboratory protocol. SOD activity was calculated as percentage inhibition using the equation below:

\[
\text{SOD activity (\% inhibition rate)} = \frac{(A_{\text{blank1}} - A_{\text{blank3}}) - (A_{\text{sample}} - A_{\text{blank2}})}{(A_{\text{blank1}} - A_{\text{blank3}})}
\]
2.6.4.3 GPx

Glutathione peroxidase (GPx) is an antioxidant enzyme which reduces liquid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water through the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG). GPx activity was measured in rat liver, kidney and heart tissues using the Biovision GPx Assay Kit according to manufacturers’ instructions (BioVision Research Products, Mountain View, CA). The tissues (50 mg) were homogenized on ice in cold assay buffer (0.2 mL) and subsequently centrifuged at 10,000 g for 15 min at 4°C. The resultant supernatant (100 mL) was loaded into a 96-well plate induplicate. The NADPH standard curve was prepared by diluting the 1mM NADPH standard through a series of concentrations (0, 20, 40, 60, 80, 100 nmol per well). The optical density of the standards (OD) was measured at 340 nm using an Anthos Venytch-200 Spectrophotometer (Biochrom Limited, Cambridge, United Kingdom) and the standard curve was constructed from the values obtained. A reaction mix (90 mL) containing assay buffer, NADPH, glutathione reductase and GSH was added into each sample well, mixed thoroughly and incubated for 15 min at room temperature. The OD was then measured (340 nm) followed by the addition of cumene hydroperoxide (10 mL) and measurement of OD(T1) and another reading following a 5 min incubation in the dark (25°C). GPx activity was calculated using the following equation:

\[
\text{GPx activity} = \frac{(B - B^0) \times \text{Sample dilution}}{(T_2 - T_1) \times V}
\]

2.6.5 Electrolytes measurements

Urinary and plasma Na⁺, K⁺, Cl⁻ and creatinine concentrations were determined by ion activity using the Beckman Coulter (Synchron LX20 Clinical Systems, Fullerton, Carlifornia, USA). Creatinine estimation employed the reaction of creatinine and picric acid at alkaline pH to form a yellow-orange complex, creatinine picrate. For the estimation of urea, the hydrolytic degradation of urea in the presence of the enzyme urease was used. Standard kits and reagents purchased from Beckman Coulter, Dublin, Ireland were used for the analysis. Glomerular filtration rate (GFR) assessed by creatinine clearance (Ccr) was calculated using a
standard formula \[C_{cr} = \text{Urine creatinine concentration} \times \text{Urine flow rate (V)} / \text{Plasma creatinine concentration}\] based on the measurements of the plasma and urinary concentrations of creatinine and urine flow rate.

2.7 Data analysis

All data were expressed as means ± standard error of means (SEM). Data for untreated non-infected and \textit{P. berghei}-infected rats were used as baseline. For chronic studies, the calculation of mean daily fluid voided and urinary amounts of electrolytes excreted and plasma insulin concentrations were used to assess renal function. GFR was evaluated by creatinine clearance as assessed by 24-hour urinary excretion rates of creatinine in relation to plasma concentration. GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, California, USA) was used for all statistical analyses. One way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test was used to establish statistical comparison between various groups. Values of \( p < 0.05 \) were considered statistically significant.
CHAPTER 3
Results

3.1 General

This chapter describes the following results:-

1. Effects of OA on percentage parasitaemia,
2. Effects of OA on glucose homeostasis and
3. Effects of OA on haematocrit levels, anti-oxidant status and renal function.

3.1.1 OA structure elucidation

The purity of the S. aromaticum-derived OA identified by $^1$H NMR and $^{13}$C NMR (1D and 2D) was approximately 98% and the percentage yield varied from 0.79% to 1.72%. The $^1$H NMR and $^{13}$C NMR (1D and 2D) spectroscopic data showing the location of olefonic bonds (carbon 2, 12 and 13) are given as supporting data (Figure 4).
Figure 5: The structure as elucidated by $^1$H- and $^{13}$C-NMR (A) and OA with International Union of Pure Applied Chemistry (IUPAC) numbering (B).
Figure 6: *Syzygium aromaticum*-derived OA $^1\text{H}$ (A) and $^{13}\text{C}$ NMR (B) spectroscopic data. Pure OA was obtained following recrystallization of EAS with methanol and elucidated using $^1\text{H}$ and $^{13}\text{C}$ NMR spectroscopy.
<table>
<thead>
<tr>
<th>Carbon position</th>
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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>183.5</td>
</tr>
<tr>
<td><strong>2</strong></td>
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</tr>
<tr>
<td>3</td>
<td>122.7</td>
</tr>
<tr>
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<td><strong>38.9</strong></td>
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</tr>
<tr>
<td>30</td>
<td>15.5</td>
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</table>

**Table 1**: \(^{13}\text{C}\) Bruker NMR spectra showing the location of important carbon atoms of \( S.\ aromaticum\)-derived OA.
3.2 Short-term studies

The study was divided into pre-treatment (0-7 days), treatment (8-12 days) and post-treatment (13-21 days). The effects of OA (40, 80 and 160 mg/kg, p.o.) and CHQ (30 mg/kg, p.o.) were evaluated in separate groups of non-infected and *P. berghei*-infected male Sprague-Dawley rats treated twice daily at 09h00 and 17h00 during the treatment period.

3.2.1 Effects of OA on parasitaemia

In the infected control group there was a continuous increase in the parasitaemia reaching 38 ± 4 % by day 14 and thus these rats were sacrificed for ethical reasons. Hence there are no post-treatment results for this group.

Groups treated with OA showed a significant decrease in percentage parasitaemia throughout the treatment period for all the three doses compared to the infected control (Figure 6). OA was able clear the malaria parasites completely with the most potent dose clearing the parasites by day 6 following treatment. CHQ (30 mg/kg, p.o.) was also able to significantly reduce parasitaemia during the treatment period, clearing the parasites from the systemic circulation by day 6 following treatment (Figure 6). Both treatments completely cleared the parasites from the systemic circulation during the post-treatment period.
Figure 7: Comparison of the effects of OA (40, 80 and 160 mg/kg, p.o.) on percentage parasitaemia in *P. berghei*-infected male Sprague-Dawley rats and CHQ (30 mg/kg, p.o.) treated rats with untreated infected animals (IC). Values are presented as means ± SEM and vertical bars indicate SEM (n=6 in each group). ★ p<0.05 by comparison with the infected control (IC). α p<0.05 by comparison with the CHQ.
3.2.3 Effects on physico metabolic changes

Body weight changes, food and water intake were measured in separate groups of non-infected and *P. berghei*-infected male Sprague-Dawley rats treated with OA (40, 80 and 160 mg/kg, p.o.) and CHQ (30 mg/kg, p.o.). The animals were treated twice daily at 09h00 and at 17H00 for 5 consecutive days.

3.2.3.1 Body weight, food and water intake

There were no significant changes in food consumption and water intake in non-infected control animals throughout the study, but these animals progressively gained weight (Table 2). Similarly, non-infected rats treated with OA (40, 80 and 160 mg/kg, p.o.) progressively gained weight and exhibited no changes in food and water intake. The above mentioned parameters were significantly decreased in *P. berghei*-infected control animals when compared to the non-infected control (Table 2). OA significantly increased food and water intake as well % body weight changes of *P. berghei*-infected animals (Table 2). Both non-infected and *P. berghei*-infected animals treated with CHQ (30 mg/kg, p.o.) showed a significant (p<0.05) decrease in the above mentioned parameters when compared with non-infected control animals (Table 2).
Table 2: Food consumption, water intake and % body weight changes of non-infected and *P. berghei*-infected animals treated twice daily with OA (80 mg/kg, p.o.) and CHQ (30 mg/kg, p.o.). Values are presented as means ± SEM (n = 6 in each group).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Groups</th>
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<th>Treatment</th>
<th>Post-treatment</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>13 ± 1</td>
<td>14 ± 2</td>
</tr>
<tr>
<td></td>
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<tr>
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</tr>
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<td>11 ± 2☆</td>
<td>10 ± 3☆</td>
</tr>
<tr>
<td></td>
<td>I CHQ 30</td>
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<td>6 ± 1☆</td>
<td>9 ± 1☆</td>
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<td>10 ± 1☆</td>
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<td>7 ± 2</td>
<td>9 ± 1☆</td>
<td>18 ± 1☆</td>
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<td>-7 ± 1</td>
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<td>-5 ± 1☆</td>
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☆ p<0.05 by comparison with the non-infected control (NI) animals
★ p<0.05 by comparison with the infected control (IC) animals.
# p<0.05 by comparison with the CHQ-treated animals
∞ p<0.05 by comparison with the OA-treated animals
3.2.4 Effects on blood glucose

Blood glucose concentrations were monitored throughout the 3-week period in separate groups of non-infected and *P. berghei*-infected male Sprague-Dawley rats treated with either OA (40, 80 and 160 mg/kg, p.o.) and CHQ (30 mg/kg, p.o.). The non-infected control group displayed no significant changes in blood glucose concentrations throughout the study (Figure 8). However, in the untreated infected group, there was a significant (p<0.05) decline in blood glucose concentrations when compared to the non-infected control with the glucose levels reaching 2.92 ± 0.13 mmol/L by day 12. Figure 8 shows a significant increase in blood glucose concentrations of the OA-treated infected groups during the treatment period and euglycaemic levels were maintained throughout the post-treatment period for all the doses. *P. berghei*-infected rats treated with 30 mg of CHQ exhibited significant decreases in blood glucose concentrations, but glucose concentrations slightly increased during the post-treatment period. Similar trends were observed in non-infected rats administered with 30 mg of CHQ. There was no significant change in blood glucose concentrations of non-infected rats treated with OA.

3.2.4.1 Effects on plasma insulin concentrations

To evaluate the acute and chronic effects of OA on plasma insulin concentrations blood was collected at 0, 0.5, 1, 5 and 14 days following treatment from non-infected and *P. berghei*-infected groups treated with either OA (80 mg/kg, p.o.) or CHQ (30 mg/kg, p.o.). There was no dose-dependent significant difference in blood glucose concentration of OA-treated animals hence a median dose was selected for the evaluation on plasma insulin concentrations. There were no significant differences in plasma insulin concentrations of the *P. berghei* infected control compared to non-infected control animals (Table 3). OA did not alter the plasma insulin concentrations in both groups of animals (Table 3). However, CHQ (30 mg/kg, p.o.) administration increase in plasma insulin concentrations of non-infected rats both acute and chronic studies. A similar trend was observed in *P. berghei*-infected rats treated, but CHQ increased plasma insulin concentrations of both the non-infected and the infected animals.
Figure 8: Comparison of blood glucose concentration profiles in non-infected (A and B) and *P. berghei*-infected (C and D) animals treated with either OA (40, 80 and 160 mg) and CHQ (30 mg). Values are presented as mean ± S.E.M, where columns represent means and vertical bars represent SEM (n=6 in each group). ★ p<0.05 by comparison with the infected control (IC). α p<0.05 by comparison with the non-infected control. ♦ p<0.05 by comparison with the CHQ treated group.
Table 3: Comparison of plasma insulin and blood glucose concentrations in non-infected and *P. berghei*-infected rats treated with OA (80 mg/kg, p.o) or CHQ (30 mg/kg, p.o) with respective controls. Data are expressed as means ± SEM (n = 6 in each group).

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<tr>
<th>Treatment</th>
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<th>Blood glucose concentration (mmol/L)</th>
<th>% Parasitaemia</th>
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<tr>
<td></td>
<td>5</td>
<td>9.60 ± 1.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.10 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>9.34 ± 2.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.71 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/A</td>
</tr>
<tr>
<td>NI CHQ(30 mg/kg)</td>
<td>0.5</td>
<td>18.57 ± 1.00&lt;sup&gt;★&lt;/sup&gt;</td>
<td>2.83 ± 0.51&lt;sup&gt;★&lt;/sup&gt;</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>28.00 ± 2.00&lt;sup&gt;★&lt;/sup&gt;</td>
<td>3.98 ± 0.25&lt;sup&gt;★&lt;/sup&gt;</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>39.00 ± 4.00&lt;sup&gt;★&lt;/sup&gt;</td>
<td>2.03 ± 0.19&lt;sup&gt;★&lt;/sup&gt;</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>10.40 ± 2.00&lt;sup&gt;★&lt;/sup&gt;</td>
<td>5.46 ± 0.07&lt;sup&gt;★&lt;/sup&gt;</td>
<td>N/A</td>
</tr>
<tr>
<td>IC</td>
<td>0</td>
<td>11.00 ± 3.00</td>
<td>5.73 ± 0.28</td>
<td>22.36 ± 2.16</td>
</tr>
<tr>
<td>OA (80 mg/kg)</td>
<td>0.5</td>
<td>10.37 ± 2.00&lt;sup&gt;★&lt;/sup&gt;</td>
<td>5.81 ± 0.2&lt;sup&gt;★&lt;/sup&gt;</td>
<td>21.89 ± 1.62</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10.44 ± 3.02&lt;sup&gt;★&lt;/sup&gt;</td>
<td>5.63 ± 0.2&lt;sup&gt;★&lt;/sup&gt;</td>
<td>19.02 ± 2.03</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>9.38 ± 3.00&lt;sup&gt;★&lt;/sup&gt;</td>
<td>5.81 ± 0.4&lt;sup&gt;★&lt;/sup&gt;</td>
<td>2.89 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>11.02 ± 3.01&lt;sup&gt;★&lt;/sup&gt;</td>
<td>6.30 ± 0.3&lt;sup&gt;★&lt;/sup&gt;</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>I CHQ (30 mg/kg)</td>
<td>0.5</td>
<td>18.57 ± 4.00&lt;sup&gt;★&lt;/sup&gt;</td>
<td>2.83 ± 0.27&lt;sup&gt;★&lt;/sup&gt;</td>
<td>22.05 ± 1.87</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>28.00 ± 7.00&lt;sup&gt;★&lt;/sup&gt;</td>
<td>3.98 ± 0.25&lt;sup&gt;★&lt;/sup&gt;</td>
<td>21.33 ± 1.67</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>29.00 ± 4.00&lt;sup&gt;★&lt;/sup&gt;</td>
<td>2.29 ± 0.13&lt;sup&gt;★&lt;/sup&gt;</td>
<td>1.02 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>16.00 ± 4.00&lt;sup&gt;★&lt;/sup&gt;</td>
<td>4.40 ± 0.28&lt;sup&gt;★&lt;/sup&gt;</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

★ p<0.05 by comparison with the non-infected control (NIC) animals.
# p<0.05 by comparison with the infected control (IC) animals
α p<0.05 by comparison with the CHQ-treated animals
∞ p<0.05 by comparison with the OA-treated animals
3.3 Effects on renal electrolytes handling

To investigate the effects of malaria infection, OA and CHQ on renal function, 24 hour urine samples were collected at 09h00 every third day throughout the study from control and treated non-infected and *P. berghei*-infected rats. Urinary Na\(^+\), K\(^+\), Cl\(^-\) outputs as well as 24 hour volumes were monitored. Plasma Na\(^+\), K\(^+\), Cl\(^-\) concentrations as well GFR were also assessed in these groups of animals.

3.3.1 Non-infected groups

There was no significant change in the Na\(^+\) output of the non-infected control animals throughout the study (Figure 8). All the three doses of OA as well as CHQ increased Na\(^+\) output significantly in the non-infected groups when compared to non-treated group.

There were no changes in urinary K\(^+\) output of the non-infected control throughout the study. The non-infected group treated with OA did not alter urinary K\(^+\) and Cl\(^-\) outputs. However, CHQ increased K\(^+\) output, but did not influence Cl\(^-\) output in the non-infected rats.

There were no significant changes observed in the 24 hour urine output in the non-infected control throughout 21 day period (Figure 8). A similar trend was observed in non-infected rats administered with OA. The non-infected group administered with CHQ however exhibited a decrease in 24 hour urine output during treatment period.

3.3.2 Infected groups

A continuous decrease in urinary Na\(^+\) output was observed in *P. berghei*-infected control throughout the study. Both OA and CHQ increased urinary Na\(^+\) outputs during the treatment period. In the infected control group there was a continuous increase in K\(^+\) output throughout the 14 day period. OA treatment decreased K\(^+\) outputs of *P.berghei*-infected animals during the treatment period. On the contrary, CHQ increased urinary K\(^+\) output in the *P.berghei*-infected rats during treatment period, but Cl\(^-\) outputs were not altered.

The *P. berghei*-infected non-treated group showed a gradual decrease in the 24 hour urine output throughout the 14 day period which was reduced by OA as well as CHQ treatment. OA significantly increased 24 hour urine outputs during the treatment period.
However, CHQ decreased the urine outputs of the *P. berghei*-infected animals. There was a slight increase in the urine outputs of the CHQ-treated group during the post-treatment period.

Table 4 represents the plasma Na\(^+\), K\(^+\), Cl\(^-\) concentrations as well as the GFR of both the non-infected and *P. berghei*-infected groups treated with either OA or CHQ. There were no significant changes observed in plasma Na\(^+\), K\(^+\) and Cl\(^-\) concentration of non-infected control as well as rats treated with CHQ and OA (Table 4). There was a significant increase in GFR of rats treated with OA when compared to the control as well as the CHQ treated group.

### 3.4 Effects OA on haematocrit levels

Haematocrit levels were monitored in separate groups of non-infected and *P. berghei*-infected male Sprague-Dawley rats treated with OA (40, 80 and 160 mg/kg, p.o.) and CHQ (30 mg/kg, p.o.) on days 0, 6, 9, 14 and 21. OA treatment did not alter the haematocrit levels of non-infected rats treated throughout the 21 day period (Figure 10). Administration of non-infected rats with CHQ (30 mg/kg, p.o.) decreased the haematocrit levels. A continuous decrease in haematocrit levels was observed in *P. berghei*-infected rats throughout the 14 day period. OA increased the haematocrit levels in *P. berghei*-infected rats during the treatment period and these levels remained constant throughout the post-treatment period. CHQ also increased haematocrit levels in infected rats during the treatment period when compared to the infected control.
Figure 9: Comparison of the urinary Na⁺, K⁺, Cl⁻ and 24 hour urine outputs of the non-infected animals treated with OA (40, 80 and 160 mg/kg, p.o.) (E-H) and CHQ (30 mg/kg, p.o.) (A-D). Values are presented as mean ± S.E.M, where columns represent means and vertical bars represent SEM (n=6 in each group). *p<0.05 by comparison with the non-infected control (NIC) animals. α p<0.05 by comparison with the CHQ treated animals.
Figure 10: Comparison of the urinary Na⁺, K⁺, Cl⁻ and 24 hour urine outputs of the *P. berghei*-infected animals treated with OA (40, 80 and 160 mg/kg, p.o.) (E-H) and CHQ (30 mg/kg, p.o.) (A-D). Values are presented as mean ± S.E.M, where columns represent means and vertical bars represent SEM (n=6 in each group). ★ p<0.05 by comparison with the *P. berghei*-infected control (IC) animals. α p<0.05 by comparison with the CHQ treated animals.
Table 4: Plasma biochemical parameters of non-infected and *P.berghei* infected animals treated twice daily for 5 days with OA (80 mg/kg, p.o.) and CHQ (30 mg/kg, p.o.). Values are presented as means ± SEM (n=6 in each group).

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Parameter (mmol/L)</th>
<th>NIC</th>
<th>NI OA 80</th>
<th>NI CHQ 30</th>
<th>IC</th>
<th>I OA 80</th>
<th>I CHQ 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment</td>
<td>Na⁺</td>
<td>138 ± 3</td>
<td>137 ± 4</td>
<td>140 ± 2</td>
<td>136 ± 4</td>
<td>138 ± 4</td>
<td>140 ± 5</td>
</tr>
<tr>
<td></td>
<td>K⁺</td>
<td>5.89 ± 0.80</td>
<td>5.62 ± 0.62</td>
<td>4.33 ± 0.82</td>
<td>6.70 ± 0.36</td>
<td>5.62 ± 0.62</td>
<td>6.70 ± 0.36</td>
</tr>
<tr>
<td></td>
<td>Cl⁻</td>
<td>101 ± 8</td>
<td>103 ± 6</td>
<td>102 ± 9</td>
<td>98 ± 8</td>
<td>100 ± 6</td>
<td>99 ± 7</td>
</tr>
<tr>
<td></td>
<td>GFR (ml.min 100 g⁻¹)</td>
<td>0.80 ± 0.02</td>
<td>0.87 ± 0.01</td>
<td>0.90 ± 0.02</td>
<td>0.90 ± 0.01</td>
<td>0.90 ± 0.01</td>
<td>0.90 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Kidney mass (g/100g)</td>
<td>0.80 ± 0.02</td>
<td>0.82 ± 0.02</td>
<td>0.89 ± 0.03</td>
<td>0.89 ± 0.01</td>
<td>0.82 ± 0.02</td>
<td>0.86 ± 0.03</td>
</tr>
<tr>
<td>Treatment</td>
<td>Na⁺</td>
<td>131 ± 2</td>
<td>146. ± 2</td>
<td>134 ± 2</td>
<td>137 ± 4</td>
<td>136.2 ± 4</td>
<td>140 ± 4 *α</td>
</tr>
<tr>
<td></td>
<td>K⁺</td>
<td>5.51 ± 0.30</td>
<td>5.12 ± 0.36</td>
<td>4.63 ± 0.40</td>
<td>4.50 ± 0.58</td>
<td>5.46 ± 0.19*</td>
<td>10.50 ± 1.56 **α</td>
</tr>
<tr>
<td></td>
<td>Cl⁻</td>
<td>104 ± 3.00</td>
<td>124 ± 3</td>
<td>103 ± 3.00</td>
<td>101 ± 3</td>
<td>104.0 ± 3</td>
<td>101 ± 2</td>
</tr>
<tr>
<td></td>
<td>GFR (ml.min 100 g⁻¹)</td>
<td>0.91±0.03</td>
<td>1.84 ± 0.03 *</td>
<td>0.80±0.02</td>
<td>0.83±0.02</td>
<td>1.80 ± 0.02 ***α</td>
<td>0.90 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Kidney mass (g/100g)</td>
<td>0.90 ± 0.04</td>
<td>0.91 ± 0.03</td>
<td>0.89 ± 0.02</td>
<td>1.01 ± 0.40</td>
<td>0.90 ± 0.02</td>
<td>0.93 ± 0.04</td>
</tr>
<tr>
<td>Post-treatment</td>
<td>Na⁺</td>
<td>131 ± 2</td>
<td>144.2 ± 4</td>
<td>131 ± 3</td>
<td>N/A</td>
<td>145.00 ± 2.00</td>
<td>137 ± 4.00</td>
</tr>
<tr>
<td></td>
<td>K⁺</td>
<td>5.51 ± 0.30</td>
<td>5.98 ± 1.00*</td>
<td>3.73 ± 0.30</td>
<td>N/A</td>
<td>6.25 ± 0.20*</td>
<td>10.09 ± 4.06 **α</td>
</tr>
<tr>
<td></td>
<td>Cl⁻</td>
<td>104 ± 3</td>
<td>121 ± 3</td>
<td>101 ± 3</td>
<td>N/A</td>
<td>102.00 ± 2.00</td>
<td>103.00 ± 2.00</td>
</tr>
<tr>
<td></td>
<td>GFR (ml.min 100 g⁻¹)</td>
<td>0.91±0.03</td>
<td>0.41 ± 0.01</td>
<td>1.02±0.03</td>
<td>N/A</td>
<td>0.94 ± 0.01</td>
<td>1.00 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Kidney mass (g/100g)</td>
<td>0.90 ± 0.04</td>
<td>0.84 ± 0.02</td>
<td>0.90 ± 0.01</td>
<td>N/A</td>
<td>0.90 ± 0.02</td>
<td>0.90 ± 0.03</td>
</tr>
</tbody>
</table>

★p<0.05 by comparison to the non-infected control
α p<0.05 by comparison to the infected control
# p<0.05 by comparison with CHQ
Figure 11: Comparison of % haematocrit in non-infected (A and B) and *P. berghei*-infected (C and D) animals treated with OA (40, 80, and 160 mg/kg, p.o.) and CHQ (30 mg/kg, p.o.) with respective control animals. Values are presented as means ± S.E.M, where columns represent means and vertical bars represent SEM (n=6 in each group). ★p<0.05 by comparison to the non-infected control. αp<0.05 by comparison to the infected control.
3.5 Effects of OA on plasma AVP and aldosterone concentrations

Plasma AVP and aldosterone concentrations were measured on days 5 and 14 in samples collected from non-infected control animals and non-infected and *P. berghei*-infected male Sprague-Dawley rats treated with OA (80 mg/kg, p.o.). By comparison with untreated non-infected rats both plasma AVP and aldosterone concentrations of untreated *P. berghei*-infected animals were elevated (Table 5). OA administration did not alter plasma AVP and aldosterone concentrations, although the triterpene increased the levels of these hormones of *P. berghei*-infected rats.
Table 5: Comparison of plasma AVP and aldosterone concentrations of non-infected and *P. berghei*-infected rats following 5 and 14 days of treatment with OA (80 mg/kg, p.o.) with respective control animals. Data are expressed as means ± SEM (n = 6 in each group).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (days post-treatment)</th>
<th>AVP (pmol/L)</th>
<th>Aldosterone (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIC</td>
<td>0</td>
<td>2.82 ± 0.47</td>
<td>0.78 ± 0.04</td>
</tr>
<tr>
<td>NI OA (80 mg/kg, p.o.)</td>
<td>5</td>
<td>2.90 ± 0.86</td>
<td>0.78 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>2.76 ± 0.64</td>
<td>0.74 ± 0.04</td>
</tr>
<tr>
<td>IC</td>
<td>0</td>
<td>4.98 ± 0.62</td>
<td>1.79 ± 0.08</td>
</tr>
<tr>
<td>OA (80 mg/kg, p.o.)</td>
<td>5</td>
<td>4.61 ± 0.73</td>
<td>0.80 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>4.09 ± 0.39</td>
<td>0.79 ± 0.05</td>
</tr>
</tbody>
</table>

♦ p<0.05 by comparison with the non-infected control (NIC) animals.
★p<0.05 by comparison with the infected control (IC) animals.
3.5 Effects of OA on oxidative status

MDA levels, SOD and GPx activity were measured in kidney and liver tissues of separate groups of non-infected and *P. berghei*-infected rats treated with either OA (80 mg/kg, p.o.) and CHQ (30 mg/kg, p.o.). Non-infected control results represent normal MDA, SOD and GPx concentrations found in these tissues.

OA had no effects on MDA levels and activities of SOD and GPx of non-infected rats in both the liver and kidney tissues (Table 6). However, CHQ increased MDA levels and lowered the activities of antioxidant enzymes. On the other hand *P. berghei* infection significantly increased MDA levels and depressed SOD and GPx activities (Table 6). OA significantly decreased MDA levels and restored the activities of SOD and GPx of *P. berghei*-infected animals to near normalcy.
Table 6: Comparison of MDA, SOD and GPx concentrations of non-infected and *P. berghei*-infected rats treated with either OA (80 mg/kg, p.o.) or CHQ (30 mg/kg, p.o.). Data are expressed as means ± SEM (n = 6 in each group)

<table>
<thead>
<tr>
<th>Parameter measured</th>
<th>Group</th>
<th>Organ</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/g protein)</td>
<td>NIC</td>
<td>1.94 ± 0.67</td>
<td>1.15 ± 0.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NI OA</td>
<td>1.63 ± 0.68</td>
<td>1.42 ± 0.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NI CHQ</td>
<td>5.30 ± 0.38*</td>
<td>5.88 ± 0.25*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IC</td>
<td>4.07 ± 0.91*</td>
<td>6.07 ± 0.62*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I OA</td>
<td>1.88 ± 0.56*</td>
<td>1.21 ± 0.60*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I CHQ</td>
<td>5.92 ± 0.85**</td>
<td>7.75 ± 0.41**</td>
<td></td>
</tr>
<tr>
<td>SOD activity (nmol/min mL/g protein)</td>
<td>NIC</td>
<td>9.27 ± 1.80*</td>
<td>19.64 ± 1.89*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NI OA</td>
<td>8.21 ± 2.02**</td>
<td>18.01 ± 2.59**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NI CHQ</td>
<td>4.57 ± 1.29*</td>
<td>3.49 ± 1.46*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IC</td>
<td>3.43 ± 0.90*</td>
<td>5.31 ± 0.66*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I OA</td>
<td>8.62 ± 1.98**</td>
<td>19.02 ± 3.01**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I CHQ</td>
<td>0.26 ± 0.01**</td>
<td>1.87 ± 0.04**</td>
<td></td>
</tr>
<tr>
<td>GPx activity (nmol/min mL/g protein)</td>
<td>NIC</td>
<td>2.08 ± 0.15*</td>
<td>4.36 ± 0.25*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NI OA</td>
<td>2.21 ± 0.10**</td>
<td>4.40 ± 0.32**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NI CHQ</td>
<td>1.33 ± 0.10*</td>
<td>2.27 ± 0.25*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IC</td>
<td>0.92 ± 0.07*</td>
<td>0.36 ± 0.05*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I OA</td>
<td>2.12 ± 0.17*</td>
<td>4.32 ± 0.22*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I CHQ</td>
<td>0.45 ± 0.06**</td>
<td>0.19 ± 0.02**</td>
<td></td>
</tr>
</tbody>
</table>

♦ p < 0.05 by comparison with the non-infected control (NIC) animals.
★p < 0.05 by comparison with the infected control (IC) animals.
# p < 0.05 by comparison with the CHQ-treated animals.
∞ p < 0.05 by comparison with the OA-treated animals.
CHAPTER 4
Discussion

The main purpose of the study was to evaluate the ability of Syzygium aromaticum derived OA to clear malaria parasites in P. berghei-infected male Sprague-Dawley rats. The secondary aim was to distinguish between the pathophysiological effects of malaria, OA and CHQ on blood glucose and renal electrolytes handling. The results show that OA possesses anti-malarial properties as well as the ability to ameliorate kidney function and also maintain blood glucose in infected animals. The findings are of considerable clinical importance as they show for the first time the ability of OA to clear malaria parasites as well as alleviate the some of the complications associated malaria.

Weanling male Sprague-Dawley rats (90-120g) were used in the current study. This is an ideal age as the rats are highly susceptible to infection by rodent plasmodia, because of the Plasmodium preference for immature red blood cells (Martín-Jaular et al., 2013). The Plasmodium strain used for malaria induction in this study is a rodent-infecting P. berghei strain widely used to study human malaria (Baptista et al., 2010; Sarfo et al., 2011; Nganou-Makamdop et al., 2012). The similarities of the life cycles of P. berghei and the human infecting Plasmodia make the strain an ideal experimental model to study malaria.

The results of the current study have demonstrated antimalarial properties of OA. This was observed when OA cleared the malaria parasites completely and prevented relapse during the post-treatment period. OA was able to ameliorate hypoglycaemia in P. berghei-infected rats. Hypoglycaemia is one of the complications normally observed during the malaria infection (Aziem et al., 2011). Various mechanisms by which malaria infection exerts blood lowering effects have been reported. Plasmodium parasites utilizing the host’s glucose for their own survival has been shown to be one of these mechanisms (Mehta et al., 2005). This mechanism is believed to be the major contributor to the hypoglycaemia that is observed during malaria infection. Studies have also shown that upon invading the RBC’s, Plasmodium parasites utilize the host’s glucose thereby causing hypoglycaemia and also decrease glucose uptake in non-infected RBC’s (Mehta et al., 2006). Hence we speculate that the anti-hypoglycaemic effects of OA were mediated partly via the eradication of the malaria parasites from the systemic circulation.
There may be various mechanisms through which blood glucose was restored to normalcy. For instance an increase in food consumption during the treatment and post-treatment periods (Table 2) may have also contributed to the increase in glucose concentrations. A decrease in food consumption was observed in *P. berghei*-infected rats and this may also have contributed to the low blood glucose levels observed in these animals.

An increase in insulin secretion has also been associated with malarial hypoglycaemia (Elased and Playfair 1994). However in the current study no changes in insulin concentrations were observed in *P. berghei*-infected rats (Table 2). Research has also reported a decrease in gluconeogenesis during malaria infection as a contributing factor in malarial hypoglycaemia (Dekker *et al*., 1997). Liver damage is one of the pathophysiological effects of malaria infection (Guha *et al*., 2006). During an infection, *Plasmodium* parasites invade hepatic cells, mature inside the cells and then rapture these cells. We speculate that this liver damage may inhibit glucose synthesis in the liver hence contributing to malarial hypoglycaemia. There were no significant changes in plasma insulin concentrations of both the non-infected and *P. berghei*-infected rats following treatment with OA. The current data indicate that OA has no effect on insulin secretion. These results are in agreement with previous studies which have shown that OA has no effects on insulin concentrations (Musabayane *et al*., 2010b).

The current study also compared the effects of OA and CHQ treatments on blood glucose in both non-infected and *P. berghei*-infected rats. Although CHQ is able to eradicate malaria parasites, adverse effects of this drug including hypoglycaemia and kidney dysfunction have been reported following CHQ treatment (Musabayane *et al*., 2010a). Studies have implicated CHQ’s ability to increase insulin secretion as one of the mechanisms by which the drug exerts hypoglycaemic effects (Musabayane *et al*., 2010a). Current data show that CHQ significantly decreased blood glucose concentrations of *P. berghei*-infected rats during the treatment period. These blood glucose lowering effects and decreased food intake of CHQ were also observed in non-infected rats treated with CHQ, confirming the drug’s capability to reduce blood glucose is independent of the malaria infection. We speculate that the bitter taste of CHQ may be the cause of a decrease in food consumption in orally treated rats, which consequently reduced the blood glucose concentrations. This data shows that OA does not induce hypoglycaemia and has no effects on insulin secretion and hence is a better alternative for malaria management.
To evaluate the effects of OA on malarial anaemia and to distinguish between the effects of OA and that of CHQ on malarial anaemia, haematocrit (HCT) levels were monitored in separate groups of non-infected and *P. berghei*-infected male Sprague-Dawley rats treated with either OA or CHQ. Following infection with *P. berghei*, continuous decrease in haematocrit levels was observed (Figure 10). Studies have reported various mechanisms that contribute to anaemia during malaria infection. The decrease in HCT levels may be attributed to the uncontrolled destruction of infected RBC’s by the *Plasmodium* parasites during their life cycle. When *Plasmodium* parasites are injected into the systemic circulation by an infected female mosquito they are transported via the systemic circulation to the liver where they invade hepatic cells (Montagna *et al.*, 2014). After they mature in the liver they rupture the hepatocytes and infect the RBC’s. During the erythrocytic stage of the *Plasmodium* life cycle, parasites ingest haemoglobin and toxic haem is released into the food vacuole (Akompong *et al.*, 2000). In an effort to prevent cell death this haem is oxidized by the parasites to hemozoin (Nagaraj *et al.*, 2013). Studies have implicated this haemozoin in erythropoiesis inhibition, thereby decrease in the number of RBC’s which ultimately results in anaemia (Skorokhod *et al.*, 2014). This imbalance between destruction and replacement of RBCs also contributes to malarial anaemia. In addition, the production of reactive oxygen species (ROS) has been reported to cause anaemia (Chirico and Pialoux 2012) and studies have reported the production of ROS in malaria. Hence we believe that malarial induced oxidative stress also plays a role in anemia observed during the malaria infection.

In this study we demonstrated the ability of OA to restore HCT levels no normalcy during the treatment period in *P. berghei*-infected rats (Figure 10). We speculate that this ability of OA to alleviate malarial anaemia is partly attributed to OA being able to eradicate the malaria parasites from the systemic circulation thereby halting the destruction of RBC’s. We also speculate that after the elimination of the malaria parasites the erythropoiesis rate will be normalized since there will be no production of haemozoin by the parasites.

Disturbances in renal function during malaria infection have been reported including acute kidney injury, acute renal failure and glomerulopathy (Mehta *et al.*, 2001; Kute *et al.*, 2013; Wichapoon *et al.*, 2014). Hence the effects of OA on renal electrolytes handling were evaluated in the current study. Following the infection with *P. berghei*, a continuous decrease in urinary Na$^+$ output was observed. This may be attributed to an increase in plasma aldosterone concentrations that was observed in these animals (Table 4). The mechanisms by
which the malaria infection increases aldosterone secretion are not clear and remain to be elucidated. An increase in urinary Na\textsuperscript{+} output was also observed in P. berghei-infected animals treated with OA. These results confirm findings of previous studies conducted in our laboratory that also showed an increase in Na\textsuperscript{+} outputs of rats treated with OA (Mapanga et al., 2009; Madlala et al., 2012; Mkhwanazi et al., 2014). We speculate that an increase in plasma AVP levels observed in these rats may be partly responsible for this natriuresis. There was a significant increase in urinary Na\textsuperscript{+} output of both non-infected and P. berghei-infected rats treated with CHQ during the treatment period. Indeed previous studies have reported increased Na\textsuperscript{+} excretion following treatment with CHQ (Musabayane et al., 1993). Studies have attributed this natriuresis to CHQ stimulating an increase in AVP secretion (Musabayane et al., 1996).

An increase in urinary K\textsuperscript{+} output was observed in untreated P. berghei-infected rats throughout the 14 day period. This increase in K\textsuperscript{+} concentration may be due to the lysis of RBC’s that occurs during the erythrocytic stage of the Plasmodium life cycle (Clark and Cowden 2003). Following treatment with OA there was a significant decrease in urinary K\textsuperscript{+} output of the P. berghei-infected rats. We speculate that this change in K\textsuperscript{+} concentrations may be due to elimination of the malaria parasites from the systemic circulation by OA. CHQ however increased urinary K\textsuperscript{+} output in infected as well non-infected rats during the treatment period. The mechanisms by which CHQ causes this hyperkalaemia are not clear.

There was a continuous decrease in urine output of the P. berghei-infected rats throughout the study period perhaps due to the increased AVP levels that were observed in these animals. This may also be due to a decrease in fluid intake that was observed in the P. berghei-infected rats. Following treatment with OA, there was a significant increase in the urine output due to natriuresis. This may also be attributed to an increased water intake that was observed during the treatment period in these animals.

A significant increase in plasma Na\textsuperscript{+} concentrations was observed in P. berghei-infected rats treated with CHQ during the treatment period which can be attributed to increased plasma aldosterone concentrations observed in these rats. Although, aldosterone levels were not measured in CHQ-treated rats in the current study, CHQ has been shown to increase aldosterone secretion and this may be partly responsible for this increase in plasma Na\textsuperscript{+} concentrations.
CHQ also increased plasma K+ concentration in *P. berghei*-infected rats during the treatment period. This hyperkalaemia may be partly attributed to the lysis of the RBC’s which leads to the release of cellular K+ into the systemic during the malaria infection (Clark and Cowden 2003). There was a significant increase in GFR of non-infected and *P. berghei*-infected rats treated with OA during the treatment period when compared to the non-infected and infected control. OA also improved GFR when compared to CHQ. These results are in agreement with previous studies conducted in our laboratory which showed that OA improves kidney function (Mapanga *et al.*, 2009).

An increase in oxidative stress has been reported in malaria infected patients (Das and Nanda 1999). Research has shown that malaria infection induced the production of the hydroxyl radical (OH·) in the liver which caused oxidative stress and led to the apoptosis of hepatocytes (Guha *et al.*, 2006). The degradation of host’s haemoglobin by the *Plasmodium* parasites is thought to be the major cause of oxidative stress in malaria infection (Beckera *et al.*, 2004). This process is beneficial to the *Plasmodium* parasites because this is how they acquire amino acids necessary for their survival. However toxic haem as well as reactive oxygen species are produced during this process (Beckera *et al.*, 2004). There was a significant increase in MDA levels of untreated *P. berghei*-infected rats and decrease in SOD and GPx activities. These results are in agreement with previous studies that have reported increased oxidative stress during the malaria infection (Beckera *et al.*, 2004). Following treatment with OA, there was a significant decrease in MDA levels which was accompanied by an increase in SOD and GPx activities in both the kidney and liver tissues of the *P. berghei*-infected rats. These results show that OA possesses anti-oxidative effects since this triterpene was able to increase the activities of SOD and GPx which play a significant role in scavenging free radicals during oxidative stress. This data is in agreement with previous studies which have reported the anti-oxidative effects of OA (Gao *et al.*, 2009; Wang *et al.*, 2010). When compared with non-infected control and OA treatment, CHQ elevated MDA levels in both the kidney and liver tissues. Furthermore, a significant decrease in SOD and GPx activities of both non-infected and *P. berghei*-infected rats was recorded following CHQ treatment. CHQ has been shown to increase MDA, a lipid peroxidation marker as well as decrease the activity of the anti-oxidative enzyme SOD (Magwere *et al.*, 1997). These findings indicate that CHQ also induces oxidative stress and they are in agreement with the findings of the current study.
CHAPTER 5

Conclusions

OA possesses antimalarial effects and alleviated malarial induced hypoglycaemia. In comparison to CHQ, OA demonstrated potential of being an ideal alternative treatment for malaria because of this triterpene’s ability to alleviate hypoglycaemia. In addition, OA improved renal function by increasing urinary Na⁺ output and GFR. These findings demonstrate that OA has the potential to alleviate renal disturbances brought about by the malaria infection. OA was also able to ameliorate malaria induced oxidative stress by decreasing MDA levels and increasing the anti-oxidant enzymes activities. Hence we conclude that Syzygium aromaticum-derived OA has anti-malarial, anti-oxidant as well as anti-hypoglycaemic effects. Therefore OA can be further explored as an alternative treatment for malaria.
CHAPTER 6

References


Antigen. American Society for Microbiology 5: 12.


CHAPTER 7
Appendices
Appendix I

27 March 2013

Reference: 090/13/Animal

Miss B Mbatha
School of Laboratory Medicine
and Medical Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Miss Mbatha

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2013 on the following project:

"The effects of some medicinal plant extracts on malaria parasites, blood glucose, and renal electrolyte handling in Plasmodium berghei infected male Sprague-Dawley rats."

Yours sincerely

[Signature]

Professor Theresa HT Goetze
Chairperson: Animal Ethics Sub-committee

Registrar – Prof. J Meyerowitz
Research Office – Dr N Singh
Supervisor, Prof. C Musabayane
Head of School – Prof. W Danis

[Signature]

Founding Campuses:
- Edgewood
- Howard College
- Medical School
- Pietermaritzburg
- Westville
23 December 2013

Reference: 038/14/Animal

Miss B Mbatha
Discipline of Physiology
School of Laboratory Medicine &
Medical Sciences
WESTVILLE Campus

Dear Miss Mbatha

RENEWAL: Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for 2014 on the following project:

"Evaluation of therapeutic efficacy of some medicinal plants on Plasmodium berghei-infected male Sprague-dawley rats and effects on blood glucose and renal electrolyte handling."

Yours sincerely

[Signature]

Professor Theresa MT Coetzee
Chairperson: Animal Research Ethics Committee

Cc: Registrar – Prof. J Meyerowitz
Research Office – Dr N Singh
Supervisor – Prof. C Musabeyane
Head of School – Prof. W Daniels
BRU – Dr S Singh.
Appendix III

Evaluation of efficacy of transdermal delivery of chloroquine on *Plasmodium berghei*-infected male Sprague-Dawley rats; effects on blood glucose and renal electrolyte handling, College of Health Sciences research symposium. 2013, 12-13 September.

**Sibiya, H.,** Mukaratirwa, S., Mbatla, B, Musabayane, C.T.

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Reports indicate that oral administration of chloroquine (CHQ) evokes adverse effects on glucose homeostasis and kidney function in many African children. This is partly due to transiently high plasma CHQ concentration following oral CHQ administration and/or malaria parasites. We have, however, observed that oral administration of amidated pectin chloroquine beads (PC) formulation sustains controlled release of CHQ into the bloodstream (Munjeri *et al*., 1998). We speculated that topical application of an amidated CHQ-pectin patch may also afford sustained, controlled release of CHQ into the bloodstream. Accordingly, the current study investigated whether CHQ delivery via the transdermal route can reduce malaria parasites and ameliorate the adverse effects associated with oral CHQ administration. The pectin-CHQ matrix patch was prepared using a protocol similar to that previously described (Musabayane *et al*., 2003) with slight modifications. Parasitaemia, blood glucose and renal function were monitored in groups of non-infected and *Plasmodium berghei*-infected male Sprague-Dawley rats following oral or transdermal delivery of CHQ. These parameters, were monitored over a 21-day period divided into pre-treatment (days 0-7), treatment (days 8-12) and post-treatment (days 13-21) in separate groups following a once off application of the CHQ patch (767µg/kg) or twice daily administration of CHQ (30 mg/kg, p.o.) during the treatment period. Transdermally delivered CHQ equally reduced *P. berghei* parasites by comparison with twice daily oral CHQ. Oral CHQ treatment was associated with increased urinary Na⁺ outputs and hyperkalaemia. The CHQ matrix patch did not influence these parameters. We conclude that the CHQ patch sustains plasma CHQ concentrations and has the potential avert the adverse effects on glucose homeostasis and renal function associated with oral administration of CHQ.
Appendix IV

Chloroquine profiles in pectin-chloroquine hydrogel patches formulation in male Sprague-Dawley rats and in vitro over a period of time, School of Laboratory Medicine and Medical Sciences research symposium, 2014, 26 May.

Thaane, T., Mbatha, B and Musabayane, CT

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The use of orally-delivered CHQ in malaria treatment is often associated with impaired glucose homeostasis, cardiovascular and kidney functions. The pathophysiological manifestations arise from accumulated deposition of CHQ caused by high initial concentrations required for oral administration. Thus there was need for an alternative formulation. We investigated the transdermal formulation provides sustained controlled drug release thus averting the adverse effects observed with oral CHQ. Against this background we then evaluated the patch stability and effects on electrolyte handling. In vitro; the CHQ concentrations in patches prepared with various amounts of CHQ were assessed via spectrophotometer on days 1, 7, 14, 21 and 28 following patch preparation. In-vivo; the animals were treated with once off application of 2.5g patch, the 21 day study was divided into pre-treatment 0-7, treatment 8-12 and post treatment 13-21. The animals were individually housed in metabolic cages for 24-hour urine samples. Sodium, potassium, urea and creatinine outputs were measured daily during treatment and every third day pre- and post-treatment. Separate groups of animals (n=6) were sacrificed and plasma obtained for CHQ assessment. In-vitro studies revealed that CHQ concentration of various pectin-CHQ patches remained constant for 28 days with the percentage drug incorporation ranging from 44-58%. In-vivo we found that plasma CHQ concentrations remained constant for 21-days and no significant changes were observed in the renal electrolyte handling of the animals following 2.5g CHQ transdermal treatment. The TDDS is become popular due to unique advantages the pectin-CHQ patch could be an alternative to conventional formulation of malaria management.

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Schools of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu Natal, Private Bag X54001, Durban, 4000, South Africa.

Despite the fact that malaria is treatable, the disease still remains the major cause of morbidity and mortality worldwide. The major factor that hinders efforts to eradicate malaria is drug resistance; hence there is a need to formulate more effective drugs. *Syzygium aromaticum*-derived Oleanolic acid (OA) has been shown to possess various therapeutic effects including anti-tumor, anti-cancer and anti-diabetic effects. However, anti-malarial effects of this triterpene have not been investigated. The aim of the study was to evaluate the effects of OA on *Plasmodium berghei*-infected male Sprague-Dawley rats, as well as effects on blood glucose and renal electrolyte handling. *P. berghei*-infected erythrocytes (10⁵ parasitized RBC) were injected intraperitoneally into male Sprague – Dawley rats. To monitor parasitaemia, blood was collected on to microscopic slides via tail prick method and stained using Giemsa stain. Smeared slides were then viewed under the light microscope. Once the parasitaemia reached (10⁵ - 10⁸), rats were treated orally with 40, 80 and 160 mg/kg of OA for 5 days twice daily. Percentage parasitaemia, blood glucose, haematocrit and renal electrolyte levels were measured. All the doses of OA demonstrated anti-malarial activity and were also able to regulate blood glucose and haematocrit levels. These results show that not only does OA possess anti-malarial effects but is also able to alleviate complications that arise as a result of malarial infection.