UNIVERSITY OF KWAZULU-NATAL

EVALUATION OF THE EFFICACY OF MASLINIC ACID ON MALARIA PARASITES IN *PLASMODIUM BERGHEI*-INFECTED MALE SPRAGUE-DAWLEY RATS: EFFECTS ON BLOOD GLUCOSE AND RENAL FLUID AND ELECTROLYTE HANDLING

Tshidi Thaane

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By

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

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Discipline of Human Physiology

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TO HIM WHO IS ABLE TO KEEP US FROM FALLING AND BRING US FAULTLESS TO HIS GLORIOUS PRESENCE, TO THE LORD GOD OUR SAVIOUR, BE GLORY, MIGHT AND AUTHORITY FROM ALL AGES PAST, NOW AND FOREVER MORE. AMEN.

I would also like to thank the following:

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Student: Tshidi Thaane **Signature**  **Date** 12/03/2015

Supervisor: Professor C.T. Musabayane **Signature**  **Date** 10/03/2015

LIST OF ABBREVIATIONS

ACT	Artemisinin combination therapy
ANOVA	One analysis of variance
API	Annual parasite index
AVP	Arginine vasopressin
BHT	Butylated hydroxytoluene
BRU	Biomedical Research Unit
b. wt	Body weight
Ca^{+}	Calcium
Cl^{-}	Chloride
CHQ	Chloroquine
DMSO	Dimethyl sulphoxide
ELISA	Enzyme-linked immunosorbant assay
GFR	Glomerular filtration rate
h	Hour
H_2SO_4	Sulphuric acid
IRS	Indoor residual house spraying
IC	Infected control
K^{+}	Potassium
LTD	Limited
Kg	Kilogram

µ	Micro
MA	Maslinic acid
µg	Microgram
µL	Microlitre
Mg	Milligram
mmHg	Millimetres of mercury
mmol	Millimole
MDA	Malondialdehyde
N	Normality
NaOH	Sodium hydroxide
NIC	Non-infected control
NO	Nitric oxide
OD	Optical density of the standards
pRBC	Parasitized red blood cells
pmol	Picomole
p.o.	per os
RBC	Red blood cell
SD	Sprague-Dawley
SEM	Standard error of mean
SOD	Superoxide dismutase

SP	Sulphadoxine/pyrimethamine
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TNF-α	Tumor necrosis factor-alpha
UK	United Kingdom
UKZN	University of KwaZulu-Natal
USA	United States of America

ABSTRACT

Introduction

Malaria remains a major socio-economic burden in Africa despite the numerous global efforts to control and manage the disease through prevention and drug intervention. Clinical studies have shown that malaria infection cases reach a total of 300-500 million every year of which 1 million occur in infants and children. Malaria infection often presents with metabolic complications which include impairment in glucose homeostasis, cardiovascular and kidney functions whose underlying causes can partly be ascribed to *Plasmodium* infection and/or drugs used manage malaria. Malaria cases have increased rapidly due to *Plasmodium* resistance to conventional treatment. The World Health Organisation (WHO) recommended the use of artemisinin combination therapy (ACT) as the first line of defense against malaria. However ACTs are expensive and not accessible to African countries which are the most affected by the disease. Chloroquine (CHQ), therefore, remains the mainstay therapy in some parts of Africa despite the developed *P. falciparum* resistance. *Plasmodium* resistance to CHQ may be due to the inconvenient dosing schedule and bitter taste of the drug which often causes patient non-compliance. The partial use of the drug has been suggested to be a major cause of the rapid development of *P. falciparum* resistance. Furthermore, the high plasma CHQ concentrations following oral administration lead to accumulated deposition of the drug in various organ systems eliciting adverse effects and organ damage. Reports suggest that CHQ accumulates in the kidney (Mc Chesney *et al.*, 1967), heart (Baguet and Fabre, 1999) and adrenal glands (Gustafsson *et al.*, 1987) to alter the physiological functions of these organs (Rodrigues *et al.*, 1994; Nord *et al.*, 2004). Studies have reported that maslinic acid (MA) possesses anti-parasitic, antioxidant properties and averts kidney dysfunction in diabetic rats (Mkhwanazi *et al.*, 2014). The present study evaluated the effects of MA on malaria parasites, glucose homeostasis, renal fluid and electrolyte handling of *P. berghei*-infected male Sprague-Dawley (SD) rats in an effort to further investigate the pharmacological properties of the triterpene. We envisaged that the anti-oxidant effects of MA may ameliorate the malaria and/treatment induced impairment of glucose homeostasis and renal function.

Materials and methods

The extraction of MA was conducted at the University of KwaZulu-Natal (UKZN) Pietermaritzburg chemistry laboratory under the supervision of Prof Van Heerden. MA was extracted using a validated protocol in our laboratory.

The effects of MA and CHQ on malaria parasites, blood glucose concentrations, renal fluid and electrolyte handling were investigated in male SD rats (90-120 g). Studies were carried out on non-infected and *P. berghei*-infected animals for a period of 3 weeks divided into pre-treatment (days 0-7), treatment (days 8-12) and post treatment (days 13-21) periods. During treatment period separate groups of non-infected and *P. berghei*-infected animals were administered MA (40, 80 and 160 mg/kg, p.o.) or CHQ (30 mg/kg, p.o.) twice daily at 9h00 and 17h00 for 5 consecutive days. The selected doses were based on our preliminary findings. % parasitaemia was monitored daily in the *P. berghei*-infected control and *P. berghei*-infected animals treated with MA or CHQ. All groups of animals were individually housed in Makrolon polycarbonate metabolic cages to monitor 24 h food consumption, water intake, % body weight, blood glucose concentrations and urine volume output. These parameters were monitored daily during the treatment period and every third day during the post-treatment period. Overnight urine samples were collected daily during the treatment period and every third day during the post-treatment period for the measurement of Sodium (Na^+), potassium (K^+)and chloride (Cl^-). All groups of animals were anaesthetized in an anaesthetic chamber with 100 mg/kg of isofor inhalation anaesthetic for 3 min for terminal studies. % parasitaemia in the untreated *P. berghei*-infected animals peaked at day 12 of the study. These animals were therefore sacrificed on day 12 as per ethics guidelines. Separate groups of animals (n=6) were sacrificed during pre-treatment days 0 and 7, treatment period days 1, 5 and post-treatment period day 14 to assess the effects of MA and CHQ on biochemical parameters. Blood was collected by cardiac puncture for plasma insulin, arginine vasopressin (AVP) and aldosterone analyses. The collected liver and kidney were used for the measurement of malondialdehyde (MDA), superoxide dismutase (SOD) and GPx concentrations.

Results

Percentage (%) parasitaemia in the untreated *P. berghei*-infected control was 55 ± 8 % by day 12 of the experimental period. The *P. berghei*-infected control animals were therefore sacrificed on day 12 of the experimental period and as such all the subsequent results showing the untreated *P. berghei*-infected control animals have no post treatment period (days 13 - 21). The lower dose of MA (40 mg/kg, p.o.) significantly reduced the malaria parasite in comparison with the *P. bergei*-infected control at corresponding time periods. However, this dose did not eliminate the malaria parasite by the end of the 21 days experimental period. The higher doses of MA (80 and 160 mg/kg, p.o.) cleared the parasite from systemic circulation by day 9 following treatment. The effects of MA (80 and 160 mg/kg, p.o.) on % parasitaemia were statistically significant compared to MA (40 mg/kg, p.o.). However, the effects of MA (80 and 160 mg/kg, p.o.) on % parasitaemia were not dose dependent. CHQ (30 mg/kg, p.o.) eliminated the malaria parasites by day 5 following treatment.

Blood glucose concentrations, food consumption, water intake and % body weight changes in the non-infected control animals were not altered throughout the experimental period. The untreated *P. berghei*-infected control animals exhibited a significant reduction in the above mentioned parameters when compared with the non-infected control at corresponding time periods. Plasma insulin concentrations in *P. berghei*-infected animals remained at values comparable with the non-infected control. MA (40, 80 and 160 mg/kg, p.o.) had no significant effects on blood glucose concentrations, food consumption, water intake and % body weight changes of the non-infected animals when compared with the non-infected control. Treatment with MA significantly increased the above mentioned parameters in *P. berghei*-infected. Following treatment with MA plasma insulin concentrations in the non-infected and *P. berghei*-infected animals remained unchanged at values comparable with the non-infected control. When compared with the non-infected control, CHQ (30 mg/kg, p.o.) significantly increased plasma insulin concentrations with a concomitant decrease in blood glucose concentrations in non-infected and *P. berghei*-infected animals. Food and water intake as well as % body weight changes were significantly reduced in these animals.

Plasma AVP and aldosterone concentrations in the non-infected animals served as baseline values. When compared with the non-infected control, untreated *P. berghei*-infected animals exhibited increased plasma AVP concentrations while aldosterone concentrations remained

unchanged. However urinary sodium (Na^+) and urine volume outputs of the untreated *P. berghei*-infected were significantly reduced. Treatment with MA did not alter plasma AVP concentrations and urine volume outputs in the non-infected animals however plasma AVP concentrations in the untreated *P. berghei*-infected animals were significantly increased when compared with the non-infected control. The urine volume outputs in the *P. berghei*-infected animals treated with MA were restored to values comparable with the non-infected control. Plasma aldosterone concentrations of the non-infected and *P. berghei*-infected animals remained unchanged following the administration of MA. However urinary Na^+ outputs were significantly increased in these animals by comparison with the non-infected control. Urinary K^+ and Cl^- outputs in the non-infected and *P. berghei*-infected animals remained unchanged following treatment with MA. CHQ administration significantly increased Na^+ outputs in the non-infected and *P. berghei*-infected animals on day 1 of the treatment period. However urinary Na^+ outputs were significantly reduced in these animals from day 2 of the treatment period until post-treatment day 14. Urinary K^+ outputs were significantly increased during the treatment period while Cl^- outputs remained unchanged throughout the study.

Liver and kidney MDA, SOD and GPx concentrations of the non-infected animals served as baseline. MDA concentrations of untreated *P. berghei*-infected and CHQ treated groups were significantly increased with diminished activity of SOD and GPx when compared with the non-infected control. Interestingly, MDA concentrations following treatment with MA were comparable with those of the non-infected control. MA significantly ($p<0.05$) increased the activity of SOD and GPx in the liver and kidney of *P. berghei*-infected animals to values comparable with the non-infected control.

Discussion

MA eliminated malaria parasites in *P. berghei*-infected animals. Our findings were in agreement with previous studies which reported that MA possesses anti-plasmodial activity *in vitro*. Furthermore De Pablos and colleagues (2010) reported that MA possesses on anti-parasitic effects against the tachyzoites of *Toxoplasma gondii*.

MA maintained blood glucose concentrations of non-infected animals at physiological levels. This could be due to the fact that MA did not alter food intake in these animals. Our results indicate that MA does not influence insulin secretion. This could therefore be one of the mechanisms by which MA maintains glucose homeostasis. MA significantly increased blood

glucose concentrations in *P. berghei*-infected animals to values comparable with the non-infected control. This could be attributed to increased food intake by the *P. berghei*-infected animals. Furthermore, MA administration showed no significant effect on plasma insulin concentrations in these animals. This is another mechanism by which MA could have maintained the blood glucose concentrations at physiological levels. The ability of MA to maintain physiological blood glucose concentrations of indicates that MA may avert the adverse effects on glucose homeostasis that are often observed following infection with malaria and/treatment with CHQ. Indeed treatment with MA showed a significant reduction in blood glucose with concomitant increases in plasma insulin concentrations in non-infected and *P. berghei*-infected animals.

Renal fluid and electrolyte handling in the non-infected animals remained unchanged throughout the study. *P. berghei*-infected control animals showed a significant decrease in urinary Na^+ excreation and urine volume outputs when compared with the non-infected control. This could be attributed to inappropriate plasma AVP concentrations following malaria infection. Treatment with MA significantly increased urinary Na^+ outputs of the non-infected and *P. berghei*-infected rats. These findings correlate with previous studies in our laboratory which showed that MA increases urinary Na^+ outputs in streptozotocin-induced diabetic animals. The increased urinary Na^+ output can be attributed in part to increased plasma AVP concentrations. Results from the present study show that chronic administration of CHQ causes reduced urinary Na^+ outputs and urine volume outputs in non-infected and *P. berghei*-infected animals. Indeed previous studies in our laboratory reported that CHQ causes renal Na^+ retention via increased plasma aldosterone concentration and reduced glomerular filtration rate (GFR). Urinary K^+ outputs of the *P. berghei*-infected control were significantly increased in comparison with non-infected animals. The *P. berghei*-induced hyperkalaemia is thought to be due to the increased release of K^+ from RBCs due to cell lysis. Indeed, the present study recorded a concomitant reduction in haematocrit levels of the *P. berghei*-infected control. The reduced haematocrit values suggested that there was a significant reduction of RBC's due to malaria parasite-induced hemolysis of infected and non-infected cells. Urinary K^+ output of the *P. berghei*-infected animals decreased following MA administration however this decrease was not of statistical significance. The slight decrease in urinary K^+ output could be due to parasite reduction by MA which led to decreased cell lysis with concomitant improvement of haematocrit values.

Malaria infection increased MDA concentrations and diminished the activity of SOD and GPx in the liver and kidney of *P. berghei*-infected animals. The malaria parasite induced oxidative stress has been attributed to degradation of haemoglobin by the *Plasmodium* parasite as well as increased utilisation of antioxidants to counteract the malaria parasite induced oxidative stress. CHQ has been found to decrease the availability of reduced glutathione to pathways involved in detoxification and reacts with ferriprotoporphyrin IX which produces highly reactive radicals that generate oxidative stress in the host. Indeed finding from the current study show increased MDA concentrations and decreased SOD and GPx in the liver and kidney of non-infected and *P. berghei*-infected animals. Interestingly treatment with MA restored MDA concentrations in the liver and kidney of *P. berghei*-infected animals to normal values. The reduction of MDA levels could be due to increased antioxidant activity. Indeed our results show that MA increases the activity of SOD and GPx. Findings from the current study are in agreement with a previous study in our laboratory which reported that MA improves renal function of diabetic animals by increasing antioxidant activity in the liver, kidney and heart. The antioxidant activity of MA has been suggested to be due to the ability of MA to inhibit NO and reducing susceptibility of plasma to lipid peroxidation. Furthermore MA has been found to possess peroxyl radical scavenging activity and metal chelating effects. In summary, findings from the present study confirm the previously reported anti-plasmodial activity of MA on malaria infected cell lines. Furthermore our results show that MA sustains blood glucose concentrations at physiological levels with a concomitant improvement of renal function of *P. berghei*-infected animals. Findings from the current study not only validate our endogenous knowledge systems but provide scientific evidence that contributes to current knowledge about the therapeutic effects of plant-derived MA in malaria. This study introduces the first *in vivo* evidence of anti-hypoglycaemic and antioxidant effects of MA on malaria and/treatment-induced adverse effects.

Conclusions

S. aromaticum-derived MA possesses anti-plasmodial activity with concomitant improvement of glucose homeostasis and renal fluid and electrolytes handling which are associated with malaria infection and/treatment. Our findings suggest that MA has potential to serve as an alternative anti-malaria compound in malaria management.

Recommendations for future studies

In the present study we have reported decreased haematocrit levels in *P. berghei*-infected rats. Measurements of hepcidin, a recently discovered peptide hormone that plays a major role in iron regulation could provide a mechanism by which malaria anaemia. This could provide information that would aid in the development of therapeutic strategies in the future. In addition to the present study, the anti-plasmodial activity and therapeutic effects of MA have been demonstrated in a number of *in vitro* and *in vivo* studies. Therefore, further studies are required to elucidate the mechanisms of action by which MA improves blood glucose homeostasis and renal function which are associated with malaria infection and/treatment.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION/LITERATURE REVIEW

1.0 Background

Malaria is a tropical disease caused by the protozoan of the genus *Plasmodium*. The mosquito-borne disease accounts for most deaths in sub-Saharan Africa. Although numerous global efforts to control and manage malaria through good surveillance and high intervention coverage are in place, the disease remains a major health and socio-economic crisis in many low-income countries. Malaria infection causes an array of pathophysiological manifestation such as hypoglycaemia, impaired renal function and oxidative stress. Early treatment using effective antimalarial drugs remains the major intervention strategy. However, *Plasmodium* resistance to previously effective anti-malaria drugs has hindered global efforts to control the disease demonstrating a need for new or alternative therapeutic agents. The WHO has recommended use of ACTs as the first line of treatment. However ACTs have proven to be expensive and inaccessible to people in developing countries which are the most affected by malaria. Orally administered CHQ remains the mainstay therapy in some parts of Africa despite the developed *Plasmodium* resistance. The challenge with oral CHQ is the inconvenient dosing schedule of 4 tablets at presentation, 2 tablets 6-8 h later and 2 tablets for the next two consecutive days. Additionally, the bitter taste of CHQ elicits non-compliance in majority of patients resulting in incomplete treatment regimes. The partial use of the CHQ has been suggested to be a major cause of the rapid development of *Plasmodium* resistance to this drug. The high plasma concentrations of CHQ following oral administration result in accumulated deposition of the drug in various organs systems eliciting adverse effects such as impaired glucose homeostasis and renal function as well as cardiovascular disorders. Alternative methods such as the use of plant derived compounds which may target novel biochemical pathways or cell functions of the malaria parasite with minimal adverse effect to the host have taken prominence. Historic and current data show that rural communities use medicinal plants to treat various ailments including malaria. *S. aromaticum*-derived MA has been found to possess antioxidant and anti-parasitic properties. To further investigate the pharmacological properties of MA we isolated the triterpene from the *S. aromaticum* flower buds in an effort to investigate the antimalarial property, effects on physiological parameters and to validate our indigenous knowledge systems.

1.1 Introduction/ Literature review

Clinical studies have shown that malaria infection cases reach a total of 300-500 million every year (WHO, 2006). Of which 1 million occur in infants and children (Maina *et al.*, 2010). The mosquito-borne disease accounts for 90 % of deaths in sub-Saharan Africa (WHO, 2006; Dale and Knight, 2008; Sadiq *et al.*, 2009). *Plasmodium vivax, P.ovale, P. malariae, P. falciparum* and *P. knowlesi* are the five *Plasmodium* species that are known to cause malaria in humans. *P. vivax* and *P. ovale* only infect young red blood cells, *P. malariae* infects only aging cells while *P. falciparum* invades erythrocytes at any age and is responsible for most of the infections and deaths that occur in sub-Saharan Africa (Tuteja, 2007). The ability of *P. falciparum* to invade erythrocytes of any age explains the heavy parasitaemia generally observed in *P. falciparum* infection. *Plasmodium* infections lead to haemodynamic, immunologic, and metabolic pathogenic features which are initiated by pRBC's (Barsoum, 2000). The *Plasmodium* life cycle has been extensively studied in an effort to manage malaria. The malaria parasite life cycle involves a complex series of interactions between the mammalian-host and the mosquito-vector (Wellems and Plowe, 2001). The cycle begins when the malaria parasite is inoculated into the mammalian host blood stream through the skin by a female *Anopheles* (Figure 1). The injected sporozoites are transported to the liver where they mature asexually into meroziotes. The merozoites are released from the hepatocytes to infect the erythrocytes where they are either released to infect non-infected erythrocytes or produce tropozites (erythrocytic phase). The tropozoites further differentiate into female and male gametocytes. The malaria life cycle continues when the next *Anopheles* mosquito has a blood meal from an infected host. Within the gut of the mosquito the gametocytes develop into male and female reproductive gametes (Wirth, 2002). Once the gametes fuse, they develop into zygotes which in turn develop into ookinetes. The ookinetes cross the wall of the gut and form oocyts filled with sporozoites. The sporozoites are released when the oocyst bursts and move to the mosquito's salivary glands. This mosquito is now ready to infect another mammalian host starting the life cycle once again.

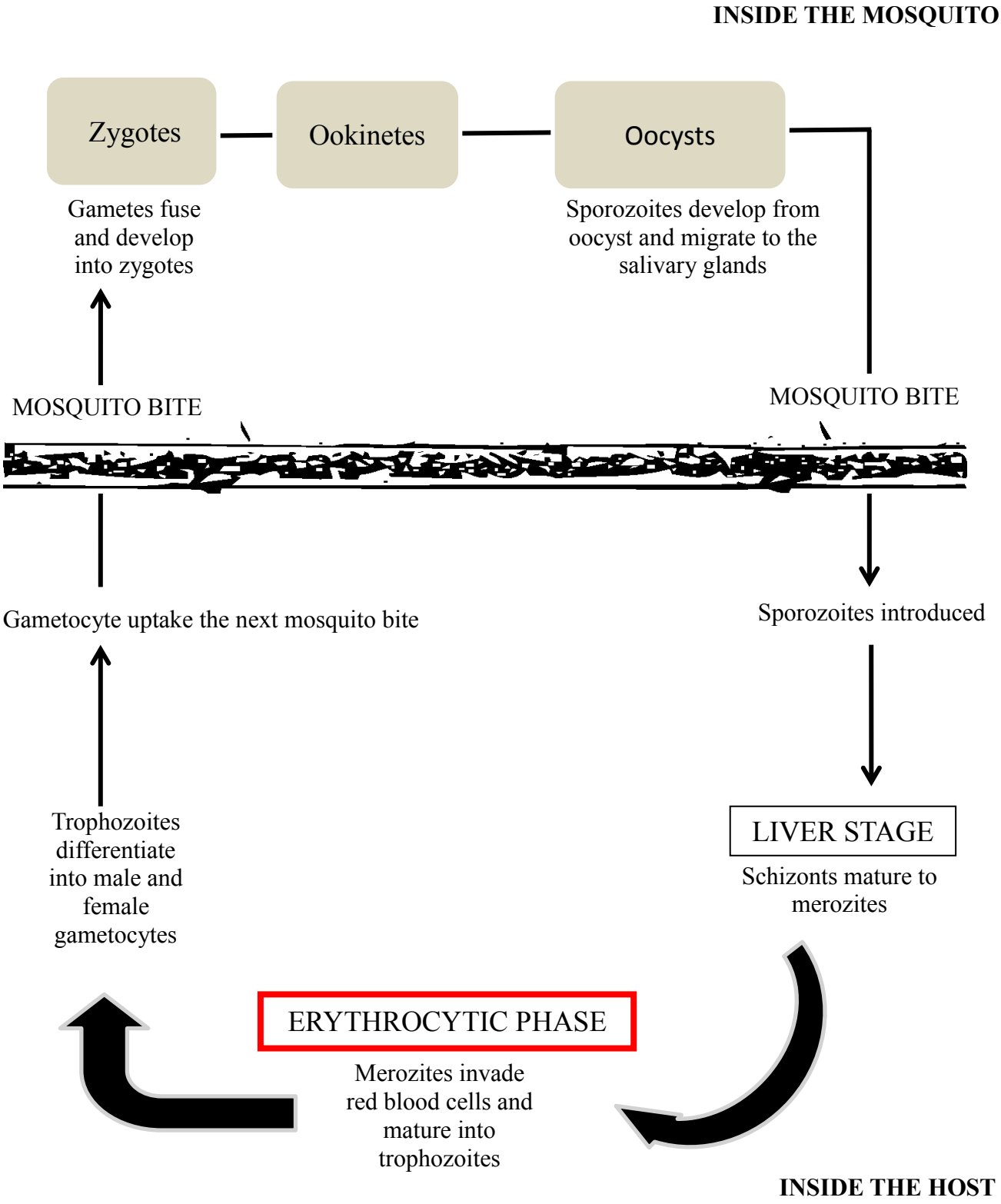

Figure 1: Basic illustration of the *Plasmodium* life cycle. The erythrocytic phase is responsible for clinical manifestations of malaria. CHQ targets the erythrocyte stage and also eliminates gametocytes.

1.2 Oxidative status in malaria

According to literature the oxidative stress that occurs in malaria is due to the degradation of haemoglobin by the *Plasmodium* parasite (Gamboa and Rosenthal, 1996; Ricardo *et al.*, 2012). The haemoglobin degradation separates the oxidant haeme group from the globin chain in which ferrous (Fe^{+2}) ion is oxidized to form ferric (Fe^{+3}) ion, the electrons produced react with molecular oxygen to form oxygen radicals (Atamna and Ginsburg, 1993). Malondialdehyde (MDA), glutathione peroxidase (GPx) and superoxide dismutase (SOD) measured in this study are markers of oxidative stress. The free haem catalyses lipid peroxidation producing the metabolite MDA, an aldehyde which is often used as a measure of oxidative stress (Claudio *et al.*, 2008). Superoxide anions form a great majority of free radicals released during malaria infection, SOD is involved in the clearance of these free radicals. Malaria patients have decreased levels of liver SOD activity than non-infected individuals (Pabon *et al.*, 2003; Dive *et al.*, 2003). Glutathione peroxidase (GPx) reduces lipid peroxides with the oxidation of glutathione. Invasion by the parasite exerts oxidative stress with a concomitant decrease in GPx levels in the liver (George *et al.*, 2012). The relationship between malaria infection and antioxidant levels has been found to be inversely proportional, patients with severe malaria infection are reported to have significantly lower antioxidants than those with mild/moderate malaria (Akpotuzor *et al.*, 2007; Akpotuzor *et al.*, 2012). This is due to increased antioxidant utilization to counteract the effects of free radicals generated during malaria infection. Some studies however have suggested that CHQ which is first line of malaria treatment in some African countries contributes to the oxidative stress observed during malaria infection. CHQ is concentrated in the various organs following oral administration probably causes adverse effects in the liver, kidney and heart (Chaturvedi and Chaturvedi, 2011). CHQ decreases the availability of reduced glutathione to pathways involved in detoxification, induces lipid peroxidation and reacts with ferriprotoporphyrin IX which produces highly reactive radicals that generate oxidative stress in the host (Magwere *et al.*, 1997; Ginsburg *et al.*, 1998). In this study, we measured the levels of MDA, SOD and GPx in the liver and kidney of *P. berghei*-infected rats as well as MA treated animals in an effort to distinguish between the effects of malaria infection to those of treatment. In addition we envisaged that MA which has been shown to possess antioxidant effects in the liver, kidney and heart of diabetic animals could perhaps minimise the oxidative stress observed during malaria infection (Mkhwanazi *et al.*, 2014).

1.3 Malaria symptoms

Hypoglycaemia, impairment of kidney function and anaemia are among an array of pathophysiological manifestations caused by malaria infection or treatment. In this study we investigated the effects of MA on malaria induced hypoglycaemia, impaired renal function and anaemia in *P. berghei*-infected animals. The following section discusses malaria symptoms in detail.

1.3.1 Hypoglycaemia

The aetiology of malaria induced hypoglycaemia is not fully understood and is likely to be multi-factorial (Krishna *et al.*, 1999; Ménard, 2005; Roe and Pasvol, 2009). Studies suggest that the consumption of host glucose to meet the parasite's energy demands precipitates hypoglycaemia through varied mechanisms (Roe and Pasvol, 2009). One such proposed mechanism is through depletion of gluconeogenic substrates such as thiamine. The reduction in thiamine is usually preceded by increased serum transketolase activity in response to increased demand for parasite's glycolytic pathway (Krishna *et al.*, 1999). Thiamine depletion has also been associated with a depression of the host's aerobic glycolysis resulting in increased anaerobic glycolysis and subsequent lactic acid accumulation (Roe and Pasvol, 2009). The rapid proliferation and migration of malaria parasites from one hepatocyte to another during the growth cycle of the parasite has been shown to cause hepatocellular damage (Ménard, 2005). Therefore, in addition to malaria induced depletion of gluconeogenic substrates, liver damage associated with malaria may further precipitate hypoglycaemia. Possible leakage of the intracellular contents of the damaged hepatocytes to the extracellular fluid may occur as evidenced by studies that have reported elevated plasma concentrations of liver enzymes such as aspartate and alanine transaminases as well as alkaline phosphatase indicative of liver damage (Kausar *et al.*, 2010). The liver is a key organ in the maintenance of glucose homeostasis hence any hepatocellular damage may impair glucose homeostasis. In addition the breaking down of the activated insulin-receptor complex occurs in the liver, hepatocellular damage may therefore result in slow insulin receptor recycling thereby precipitating hypoglycaemia MA may minimize liver damage by the parasite and therefore avert the hypoglycaemia observed during malaria infection.

1.3.2 Impaired renal function

Malaria parasite-induced kidney dysfunction is ascribed to hyponatraemia and hyperkalaemia. The increased release of potassium (K^+) from red blood cells (RBCs) during haemolysis results in high plasma K^+ concentrations resulting in hyperkalaemia (Etim and Ekaidem, 2011). Parasitized red blood cells stick to one another and tend to adhere to adjacent unparasitized erythrocytes as well as to the capillary endothelium. This in turn results in the formation of intravascular clumps which impede the renal microcirculation (Elsheikha and Sheashaa, 2007; Das, 2008). Indeed, the constriction of renal vascular vessels has been reported in malaria associated renal failure (Sriamornsak, 1998). This constriction of the renal vascular bed often causes a decrease in oxygen supply, the resulting ischaemia causes a reduction in urine volume, GFR and urinary Na^+ excretion (Elsheikha and Sheashaa, 2007; Das, 2008). The pathophysiology of hyponatraemia in severe malaria is incompletely understood, some studies found evidence for "appropriate" vasopressin release due to hypervolaemia whereas other studies found evidence for "inappropriate" vasopressin release (Holst *et al.*, 1994; Hanson *et al.*, 2009). Chronic administration of CHQ has been reported to cause renal Na^+ retention possibly via increased plasma aldosterone concentration and reduced GFR (Musabayane *et al.*, 1994; Musabayane *et al.*, 2000a). However a study by Musabayane *et al*., 1993 reported no significant changes in aldosterone following CHQ administration. Thus the CHQ induced natriuresis is thought to be attributed to CHQ stimulated increase in plasma AVP concentrations (Musabayane *et al.*, 1996) . In addition, the increased Na^+ retention following chronic CHQ administration may also be accredited to the CHQ-induced kidney damage (Musabayane *et al.*, 2000a). In this study we measured plasma AVP and aldosterone concentrations in *P. berghei*-infected and MA treated rats. Since MA has been reported to be a reno-protective compound (Mkhwanazi *et al.*, 2014), we envisaged that the MA antioxidant property may ameliorate the malaria and/treatment-induced kidney damage.

1.3.3 Anaemia

Malaria infection has been associated with anaemia for a long time (Haldar and Mohandas, 2009). Recent studies however have reported the role of hepcidin in malarial anaemia. Hepcidin is a peptide hormone that regulates iron metabolism and is thought to play a central role in the anaemia of chronic inflammation (Drakesmith and Prentice, 2012). Literature reports that this hormone reduces dietary iron absorption in the small intestine and inhibits iron release from macrophages by a mechanism which promotes the internalization and degradation of ferroportin, the sole known iron exporter. This leads to hypoferremia and limited iron availability for erythropoiesis (Howard *et al.*, 2007). Hepcidin levels are regulated by three independent mechanisms, namely inflammation, iron loading and erythropoietic (EPO) activity. Inflammation and iron loading induce hepcidin production while EPO activity suppresses production. Inflammation has a potent effect on iron homeostasis by reducing intestinal iron absorption and sequestering iron in macrophages thereby decreasing serum iron levels. In addition current literature shows substantial evidence that these effects of inflammation are also mediated by hepcidin (Young and Zaritsky, 2009). The presence of the malaria parasite triggers the host's innate immune system to release pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-22. These pro-inflammatory cytokines together with type I interferon induce hepcidin transcription through STAT3 signalling (Howard *et al.*, 2007). This hepcidin then blocks the release of iron from enterocytes, hepatocytes and macrophages leading to hypoferremia and limited iron availability for erythropoiesis (McDevitt *et al.*, 2004) .

The regulation of hepcidin via iron loading seems to be mediated by the bone morphogenetic protein receptor complex at the surface of hepatocytes. This complex includes two proteins that are known to be mutated in various forms of hereditary hemochromatosis, human hemochromatosis protein (HFE) and hemojuvelin (Bennett *et al.*, 2000). Although the exact molecular mechanism is not yet completely understood, this bone morphogenetic protein receptor complex interacts with transferrin receptors 1 and 2, linking the sensing of serum iron with hepcidin production (Young and Zaritsky, 2009). The regulation of hepcidin production by erythropoiesis remains poorly understood. One or more unidentified bone marrow-derived signals generated during increased erythropoiesis decreases hepcidin production. Thus, the increased demand for iron incorporation into haemoglobin is met by increased internal iron absorption and release of stored iron from the reticuloendothelial

system. This signal transduction mechanism seems very robust and is able to keep hepcidin levels low during systemic iron overload as is seen in the iron-loading thalassemias. The exact metabolism of hepcidin remains to be determined. However, N-terminally truncated forms of hepcidin (hepcidin-20 and hepcidin-22) have been identified and are likely to be degradation products that are biologically inactive (Park *et al.*, 2001). Hepcidin-25 may be filtered and reabsorbed in the proximal tubule in a manner similar to 2-microglobulin as indicated by the presence of measurable amounts of hepcidin-25 in urine. Renal excretion of hepcidin has been suggested to play a major role the clearance of this peptide hormone (Swinkels *et al.*, 2008).

Literature reports that EPO inhibits hepcidin expression in hepatocarcinoma cell lines and hepatocytes. In addition, upregulation of hepcidin expression was also observed in spleens and livers of mice infected with pathogenic bacteria (Wang *et al.*, 2011). This response lead to intracellular iron sequestration and reduced the availability of iron thus inhibiting the growth of pathogens. Iron deficiency is a common condition in many malaria-endemic regions. Malarial anaemia leads to hypoxia which causes upregulation of EPO production in the kidney and serum EPO levels. These physiological factors could influence the expression of hepcidin during malaria infection. Suppressed erythropoiesis contributes to malarial anaemia as new red cells are not produced to replace those lost by haemolysis and ingestion by macrophages. Normally, hepcidin levels are inhibited during periods of strong bone marrow activity, this increases iron release from stores and absorption from the diet. As a result, more iron is available to supply erythropoiesis. In contrast, raised hepcidin levels during malaria infection would reverse this flow of iron and further starve the erythron when the need for iron is urgent. Work by Howard *et al*., 2007 showed that increased hepcidin is indeed present during the blood stage of malaria infections *in vivo*. Additionally *P. falciparum* parasitized eryrthocytes were found to induce hepcidin expression from co-cultured leucocytes (Howard *et al.*, 2007). If hepcidin does contribute to malarial anaemia, further understanding of the molecular mechanism of *Plasmodium*-induced increase in hepcidin may enable interventions to reduce hepcidin expression during infection. This could provide a new approach to modify the anaemia associated with malaria. Some studies have suggested that the quinolone ring of CHQ and other quinine related drugs that act as hepten and bind with specific RBCs proteins to elicit an immune response leading to the destruction of RBCs, thus aggravating anaemia (Clark and Cowden, 2003). The details on the anaemia

during malaria infection are obscure. In this study therefore, we monitored percentage haematocrit and in an effort to establish the mechanism by which malaria anaemia occurs.

1.4 Malaria management

1.4.1 Vector control

Malaria management strategies make use of environmental, biological and chemical techniques as means of controlling the malaria vector (Walker and Lynch, 2007). Environmental management focuses on avoiding creation of vector breeding areas, changing natural habitats or improving human habitation to reduce the abundance of target vectors with minimal adverse impacts on the environment and social settings (Rozendaal, 1997; Shiff *et al.*, 2011). Environmental management techniques were successfully applied in the early 20th century to ensure that no water of the quality required by the identified local vector is available (Goh, 1983). This technique however was not universally applicable because implementation requires careful consideration of ecological, socio-economic and cultural settings (Ramirez *et al.*, 2009). A wide range of organisms regulate the *Anopheles* populations naturally through predation, parasitism and competition (Lacey and Lacey, 1990; Das and Amalraj, 1997).

Biological techniques therefore make use of natural rivals of the targeted mosquito to achieve effective vector control. A study by Das and Amalraj, 1997 reported successful control of *Anopheles* populations by larvivorous fish, bacterial pathogens *Bacillus thuringiensis israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) (Johnson *et al.*, 1995; Das and Amalraj, 1997). These predators have been reported to attack the larval stages of the mosquito (Das and Amalraj, 1997; Majambere *et al.*, 2007). Although effective and inexpensive, biological larvae control agents are difficult to use and they generally require substantial information about vector ecology, distribution of larval habitats and local environmental conditions (Kumar and Hwang, 2006). Furthermore, these interventions tend to be effective only under certain conditions therefore successful vector control in one location may not be predictive of results elsewhere (Das and Amalraj, 1997).

Chemical methods of malaria vector control have been employed in an effort to eliminate or reduce vector populations. Literature evidence shows successful use of temephos (Trade name Abes) as a malaria vector larvicide (Kumar *et al.*, 1994; Gopaul, 1995). However larval resistance to this widely applied larvicide is a growing concern (Brooke *et al.*, 2001). Indoor

residual house spraying (IRS) has been the most successful chemical control method for adult female mosquitos since 1940 (Hemingway and Ranson, 2000). The rationale for IRS is based on the behaviour of *Anopheles* species that rest on walls before or after biting humans (Govella *et al.*, 2013). Pilot IRS projects showed dramatic reductions in annual parasite index (API). This inspired the WHO to adopt a malaria eradication goal in 1995 (Olliaro and Trigg, 1995). Although the goal of malaria eradication proved elusive in malaria endemic areas the effectiveness of IRS is thought to be well established (Krogstad, 1996). The major limitation of IRS however is the developed *Anopheles* resistance to insecticides probably due to risks associated with exposure to pesticides (Milam *et al.*, 2000). The information in the preceding paragraphs shows that efforts to manage malaria through vector control have been unsuccessful. The increasing problems associated with the developed vector resistance argue for multiple approaches to manage malaria. The current study investigated the plant-derived MA as a potential novel drug for the management of malaria. The following section discusses different classes of drugs that are currently used to treat malaria.

1.4.2 Anti-malaria drugs

According to the WHO, the use of antimalarials ensured a significant reduction of malaria related morbidity and mortality rates in India and sub-Saharan Africa (WHO, 2008). Current anti-malaria drugs can be classified according to their target site on the life cycle of the *Plasmodium* parasites.

Casual prophylaxis drugs

This class of drugs include primaquine, proguanil and pyrimethamine (Le-Bras and Duran, 2003). These drugs have been found to prevent the relapse of malaria infection by eradicating pre-erythrocytic liver latent tissue forms of *P. vivax* and *P.ovale* (Hyde, 2002; Le-Bras and Duran, 2003). This class of anti-malaria drugs however is not without limitations. Primaquine for example is administered daily due to the short half-life. The use of primaquine has therefore been associated with adverse effects such as haematological disorders and gastrointestitial disturbances (Richie and Saul, 2002). Proguanil and pyrimethamine are prophylactic drugs that have been found to be highly effective against the primary tissue forms of *P.falciparum* (Snow et al., 2005).

Schizonticidal drugs

Blood schizonticides act on the asexual forms of the erythrocytic stage of the malaria parasites. Literature evidence shows that these drugs are highly potent against the malaria parasites and can be used to suppress clinical symptoms that result from malaria infection (Sullivan *et al.*, 1996). Quinine was the first drug to be used successfully against malaria. This drug is both a schizonticide and gametocytocide (targets sexual stages of the malaria parasite life cycle). However the complex dosing regimen and poor tolerability of this drug has resulted in *Plasmodium* resistance (Sullivan *et al.*, 1996). CHQ, a diprotic compound derived from quinine possesses both blood schizonticidal and gametocytocidal activities against CHQ sensitive *Plasmodium* parasites (Sullivan *et al.*, 1996; WHO, 2006). CHQ was once a highly successful anti-malaria drug. However the bitter taste of CHQ and inconvenient dosing schedule of 4 tablets at presentation, 2 tablets 6-8 h later and 2 tablets for the next two consecutive days has been suggested elicits non-compliance in majority of patients resulting in incomplete treatment thus precipitating *Plasmodium* resistance (Munjeri *et al.*, 1998; Musabayane *et al.*, 2003). Furthermore, the high plasma CHQ concentrations following oral administration lead to accumulated deposition of CHQ in various organ systems eliciting adverse effects and organ damage. Reports suggest that CHQ accumulates in the kidney (Mc Chesney *et al.*, 1967) and adrenal glands (Gustafsson *et al.*, 1987) to alter the physiological functions of these organs (Rodrigues *et al.*, 1994; Nord *et al.*, 2004). CHQ however remains the main anti-malarial agent in some parts of Africa including South Africa despite the developed *Plasmodium* resistance. In the current study CHQ was used as the standard drug.

Anti-relapse drugs

Also known as secondary tissue schizonticides this class of anti-malaria drugs has pronounced action on the secondary pre-erythrocytic phase of *P.vivax* and *P.malariae* infections in the liver. Primaquine and quinocide have been identified as the most potent anti-relapse drugs in malaria management.

Numerous global efforts are in place to manage malaria through vector control and use of anti-malaria drugs (Breman *et al.*, 2004). However *Plasmodium* infections persist (Dale and Knight, 2008). This is largely due to the high emergence of parasite resistance especially observed for *P. falciparum* (Ogbonna and Unekeb, 2008). The developed resistance has been attributed to complex treatment regimens and adverse effects caused by current malaria management strategies (Bloland, 2003). The use of plant-derived bioactive compounds as a novel approach to malaria management has taken centre stage and is highly advocated by the

WHO (WHO, 2010). Herbal remedies are inexpensive, effective and often have minimal side effects (Gupta and Raina, 1998; Tyler, 1999). In an effort to validate our endogenous knowledge systems the current study investigated the ability of MA to clear malaria parasites in *P. berghei*-infected rats. We envisaged that this medicinal plant extract may avert the adverse effects observed with malaria infection and/treatment.

1.5 Medicinal plants with anti-malaria activity

Historic and current data shows that populations in African and Asian countries rely of medicinal plants as their primary health care system (Winslow and Kroll, 1998; Kamboj, 2000). The use of herbal medicine by these populations has been suggested to be due to cultural preferences, low cost and abundant availability (Gupta and Raina, 1998; Mesfin *et al.*, 2009). With approximately 80 % of the global population depending on medicinal plants, traditional healers are using herbal medicines for the treatment of various ailments including malaria (Kamboj, 2000; Willcox and Bodeker, 2004). However, major limitations of traditional remedies are due to un-established doses and unpredictable efficacy (Chinedu *et al.*, 2014). In addition, the short and long-term toxic effects of these herbal treatments are not known (Firenzuoli and Gori, 2007). Therefore, the practice of traditional medicine is not completely recognised by modern medicine (Chang and Story, 2005). The search for plant derived anti-plasmodial compounds which are not toxic to the host has therefore taken prominence in the medical research field (Wanyoike, 2003; Zofou *et al.*, 2011; Zofou, 2011). Current literature suggests that the development of new anti-malaria drugs from highly active natural products is crucial to overcome the developed *Plasmodium* resistance to available anti-malaria drugs (Pohlit *et al.*, 2013). Ogbonna and Unekeb, 2008 reported that aterminisin which is isolated from the Chinese herbal medicine *Artemisia annua* decreased mortality and morbidity associated with malaria in different parts of the world (Ogbonna and Unekeb, 2008). The WHO therefore recommended use of ACT's as the first line of malaria treatment (WHO, 2010).

The artemisinin based regimen possess schizonticidal and gametocytocidal properties which inhibit parasite transmission and possibly reduce the development of *Plasmodium* resistance (Nosten and White, 2007). The mechanism of action of ACTs is unclear, however studies speculate that, like quinolines, ACTs block the conversion of heme to hemozoin (Hyde, 2002). Although highly effective, artemisinins are recommended for use in combination with the more long-lasting antimalarials such as quinolines due to their short half-life *in vivo* in

order to ensure complete elimination of residual parasites (Hyde, 2002; Rahmatullah *et al.*, 2011). The imbalance between demand and supply, comparatively high cost, dosing complexity and lack of clinical experience caused high failure rates which lead to *Plasmodium* resistance to ACT's (Bloland, 2003). The developed resistance to available anti-malaria drugs has hindered global efforts to control the disease demonstrating an urgent need for alternative or new anti-malaria drugs (Hyde, 2002). Table 1 presents some medicinal plants that have been found to possess anti-malarial activity. The current study investigated the *S. aromaticum*-derived MA as a novel anti-plasmodial compound.

Table 1: A list of medicinal plants that have been found to possess antimalarial activity

Family	Name of plant	Parts used	Reference
Amaranthaceae	*Amaranthus spinosus*	Root	(Moshi *et al.*, 2012)
Annonaceae	*Uvaria scheffleri Diels*	Leaves	(Soejarto *et al.*, 2012)
Apocynaceae	*P. nitida*	Whole plant	(Randrianarivelojosia *et al.*, 2003)
Aristolochiaceae	*Aristolochia Bracteolata*	Leaves	(Fokunang *et al.*, 2011)
Asclepiadaceae	*Gongronema napalense (Wall.) Decne*	Whole flower	(Rahmatullah *et al.*, 2011)
Asparagaceae	*Dracaena reflexa Lamk*	Leaf and bark decoction	(Rahmatullah *et al.*, 2012)
Asteraceae	*Vernonia amygdalina*	Leaves, root bark	(Dell'Agli *et al.*, 2012)
Bombacaceae	*Adansonia digitata* L	Leaf, flower and roots	(Moshi *et al.*, 2012)
Brassicaceae	*Lepidium sativum* L.	Seeds	(Nguta *et al.*, 2011)
Burseraceae	*Canarium schweinfurtii* Engl	The bark scent	(Bickii *et al.*, 2007)
Gentianaceae	*Tachia guianensis*	Root	(Willcox and Bodeker, 2004)
Combretaceae	*Combretummolle Engl. & Diels*	Leaves	(Soejarto *et al.*, 2012)
Crassulaceae	*Kalanchoe pinnata*	Leaf	(Oliveira *et al.*, 2009)
Cucurbitaceae	*Citrullus colocynthis*	Seeds	(Fokunang *et al.*, 2011)
Euphorbiaceae	*Croton macrostachyus Del.*	Fresh/dry leaves	(Ahmed *et al.*, 2010)
Fabaceae	*Caesalpinia pluviosa*	stem bark	(Zofou *et al.*, 2011)
Gentianaceae	*Tachia guianensis*	Root	(Willcox and Bodeker, 2004)
Hydrangeaceae	*Dichroa Febrifuga*	Leaf and root	(Pattanayak *et al.*, 2010)
Icacinaceae	*Icacina senegalensis* A. Juss.	Leaf extracts	(Kayano *et al.*, 2011)
Indoodae	*Phytolacca dodecandra* L'Herit	Fresh/dry leaves	(Willcox and Bodeker, 2004)

Lamiaceae	*Ocimum sanctum* Linn.	Plant extract	(Willcox and Bodeker, 2004)
Leguminosae	*Desmodium mauritianum D.C*	A decoction of the leaf and bark	(Rahmatullah *et al.*, 2012)
Malvaceae	*Adansonia digitata L.*	Leaves	(Rahmatullah *et al.*, 2011)
Meliaceae	*Pseudocedrela kotschyi*	Leaves	(Soejarto *et al.*, 2012)
Moraceae	*Streblus asper Lour*	Bark	(Zofou *et al.*, 2011)
Myrtaceae	*M. communis*	Aerial parts	(Moshi *et al.*, 2012)
Nyctaginaceae	*Boerhavia hirsuta*	Leaf extracts	(Jorim *et al.*, 2012)
Oleaceae	*Nyctanthes arbor tristis L*	Leaf	(Willcox and Bodeker, 2004)
Poaceae	*Cymbogon citratus*	Leaves	(WHO, 2010)
Polygalaceae	*Polygala paniculata L.*	Leaves and fruit	(Jorim *et al.*, 2012)
Ranuneulaceae	*Nigella sativa*	Seeds	(Tunru *et al.*, 2012)
Rubiaceae	*Cuviera longiflora*	Leaves	(Fokunang *et al.*, 2011)
Sapindaceae	*Tristiropsis sp.*	Soft leaves	(Dell'Agli *et al.*, 2012)
Solanaceae	*Solanum aculeastrum* Dunal	Fruits	(Krettli *et al.*, 2001)
Thomandersiaceae	*T. hensii*	Plant	(Bickii *et al.*, 2007)
Verbenaceae	*Lantana camara* L	Leaf, root, flower	(Randrianarivelojosia *et al.*, 2003)
Violaceae	*Viola canescens*	Plant	(Krettli *et al.*, 2001)
Zingiberaceae	*Aframomum* sp	Plant	(Sarr *et al.*, 2011)

1.5.1 *Syzygium aromaticum*

S. aromaticum [(Linnaeus) Merrill & Perry] (Figure 2A) is traditionally used for the aphrodisiac, stomachic, carminative, nervous stimulant and tonic activities. In addition to these properties, *S. aromaticum* has also been reported to possess, anti-bacterial, anti-fungal and anti-oxidant properties (Lee and Shibamoto, 2001; Alqareera and Andersson, 2006; Mkhwanazi *et al.*, 2014). *S. aromaticum* belongs to the family Myrtaceae and is native to the North Moluccas islands of Indonesia. Also known as the clove tree *S. aromaticum* is cultivated in Mauritius, Madagascar, India, Sri Lanka, Zanzibar, Brazil, the West Indies and Pemba. The clove is the unopened flower bud that turns red when ready for harvest (Figure 2B). The *S. aromaticum* cloves are hand-picked and dried until brown in colour (Figure 2C). Phytochemical investigations of dried *S. aromaticum* buds led to the isolation of triterpenes (OA and MA) with various pharmacological properties. In our laboratory we have shown that OA possess hypoglycaemic, hypotensive, reno-protective and cardio-protective effects in experimental diabetic animal models (Mapanga *et al.*, 2009; Musabayane *et al.*, 2010; Ngubane *et al.*, 2011). In this study however the triterpene of interest was MA.

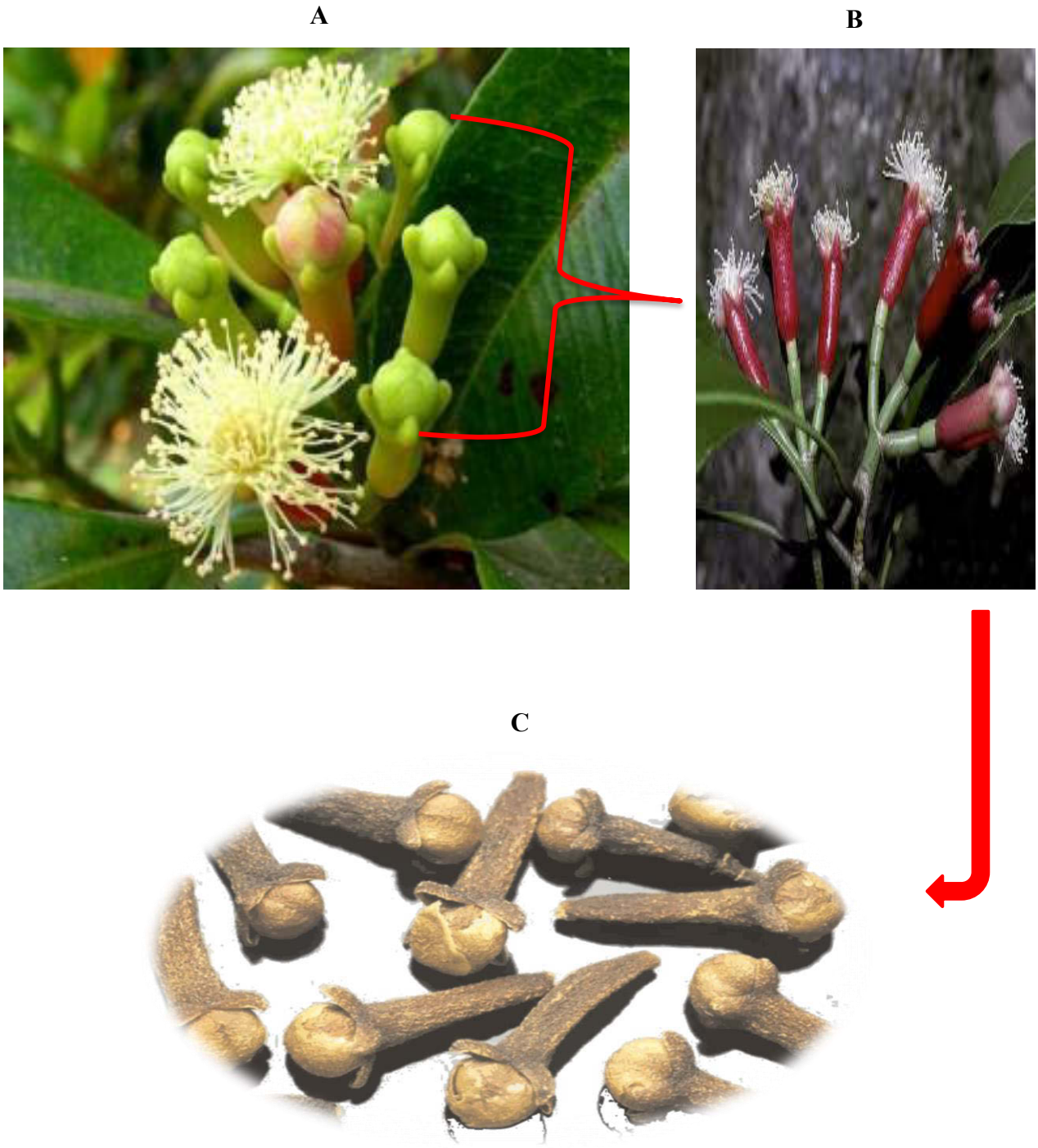

Figure 2: Picture showing (**A**) the evergreen *S. aromaticum* leaves, (**B**) maturing buds which turn bright red when ready for harvesting and (C) harvested buds which are dried until brown. The dried cloves are then grounded and accordingly soaked to begin the isolation of MA. (Adapted from (Kamatou *et al.*, 2008; Madlala *et al.*, 2012)).

1.6 *Syzygium aromaticum*-derived MA

Studies have reported that MA possesses biological activities such as anti-tumor, anti-diabetic, anti-oxidant, cardioprotective, neuroprotective and anti-parasitic effects (Juan *et al.*, 2008; Wen *et al.*, 2008; De Pablos *et al.*, 2010; Mkhwanazi *et al.*, 2014). In addition MA has recently emerged as a novel anti-plasmodial compound (Moneriz *et al.*, 2011a). *In vitro* experiments by Moneriz *et al*., 2011 demonstrated anti-plasmodial effects of MA in *P. falciparum*-infected cell lines (Moneriz *et al.*, 2011a). Furthermore, Moneriz *et al* 2011 reported on the anti-plasmodial effects of MA in *P. yoelli*-infected mice (Moneriz *et al.*, 2011b). Malaria infection and/ treatment is associated with pathophysiological manifestations such as impaired glucose homeostasis and renal function as well as oxidative stress. The evaluation of the effects of MA on physiological parameters of malaria infected models is imperative as this would help widen the spectrum on the biological activities of this triterpene. In this study we evaluated the ability of MA to clear malaria parasites in *P. berghei*-infected male Sprague-Dawley rats with the primary aim of evaluating the effects of MA on blood glucose concentrations, renal fluid and electrolytes handling which are parameters altered during malaria infection and/treatment. We envisaged that the antioxidant property of MA may ameliorate the malaria induced oxidative stress which could improve the malaria and/ treatment-induced impaired glucose homeostasis and renal function. Evidence in the preceding chapter suggests that MA could be a highly potent antimalarial compound.

1.7 Objectives of the study

The objective of this study was to investigate the ability of MA to clear malaria parasites in *P. berghei*-infected male Sprague-Dawley rats. Additionally we evaluated the effects of MA on blood glucose homeostasis as well as renal fluid and electrolyte handling in malaria infected animals.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Drugs and chemicals

Drugs and chemicals were sourced as indicated:

chloroquine diphosphate ($C_{18}H_{26}ClN_3 \cdot 2H_3PO_4$), Giemsa stain, acetonitrile (C_2H_3N), thiobarbituric acid (TBA) and butylated hydroxytoluene (BHT) (Sigma-Aldrich Chemical Company, St Louis, Missouri, United States of America);

sodium sulphate (Na_2SO_4), potassium dihydrogen phosphate (KH_2PO_4) and methanol (CH_3OH) (C_2H_5OH) (Merck Chemicals (PTY) LTD, Johannesburg, South Africa);

diethyl ether ($C_4H_{10}O$) (NT Laboratory Supplies (PTY) LTD, Johannesburg, South Africa); sulphuric acid (H_2SO_4) (BDH Chemicals LTD, Poole, Dorset, England);

isofor inhalation anaesthetic (Safeline Pharmaceuticals (PTY) LTD, Weltevreden Park, Rooderport, South Africa);

The ultrasensitive rat insulin ELISA kit (DRG Instruments, GmbH, Marburg, Germany);

Vasopressin ELISA kit, aldosterone ELISA kit (Abcam, Cambridge, Massachusetts, United States of America) and

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

All chemical reagents were of analytical grade.

2.1.2 Animals

Male Sprague-Dawley rats (90-120 g) bred and housed in the Biomedical Resource Unit of the University of KwaZulu-Natal (UKZN) Westville campus were used in the study. The animals were maintained under standard laboratory conditions of constant temperature (22 ± 2 °C), CO_2 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.

2.2 Ethical consideration

Ethical clearance was obtained from the University of KwaZulu-Natal's Ethics committee (References 085/13 Animal and 030/14 Animal) (Appendix I and II).

2.3 Experimental design

Sub-chronic effects of MA on blood glucose and renal fluid and electrolyte handling were assessed in separate groups of non-infected and *P. berghei*-infected male Sprague-Dawley rats (n= 6 in each group). The experimental design is summarised in (Figure 3) below.

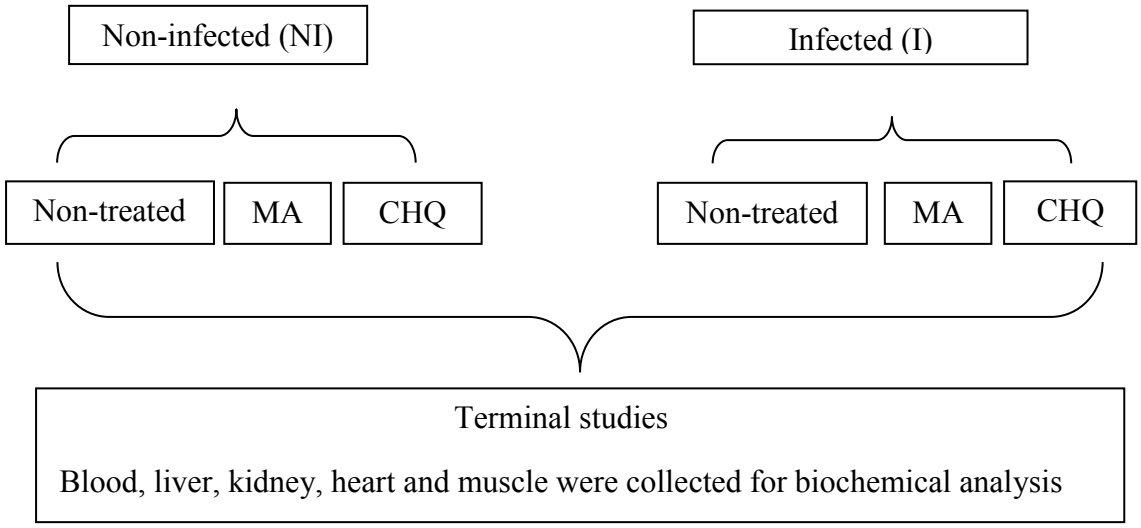

Figure 3: Flow diagram showing the summary of the experimental design. The animals were divided into malaria infected and non-infected. Various doses of MA and CHQ were administered to observe the effects of treatment on parasitaemia and physiological parameters.

2.4 Methods

2.4.1 Isolation of MA

The extraction process was conducted at the UKZN (Pietermaritzburg) chemistry laboratory under the supervision of Prof van-Heerden. MA was extracted using a validated protocol in our laboratory (Mkhwanazi *et al.*, 2014). Briefly (500 g) air-dried *Syzygium aromaticum* cloves were soaked twice at 24 h intervals in 1 L of dichloromethane (DCM) and 720 mL ethyl acetate (EA) to remove DCM soluble impurities and EA solubles. The resulting homogenate was filtered and concentrated in *vacuo* at 55 ± 1 °C using a laboratory 4000 efficient rotary evaporator (Laboratory consumables and chemical suppliers, Johannesburg, South Africa), yielding DCM solubles (63 g) and EA solubles (85 g). A silica-gel packed column chromatography was used to separate the EAS dissolved MA from oleanolic acid (OA) using an increasing polarity hexane-ethyl acetate ratio 9:1, 8:2 and 6:4. Fractions were closely monitored by thin layer chromatography (TLC) plates (Merck products, Dermstads, Germany). The most polar ratio 6:4 provided MA yield which was recrystallized from chloroform-methanol (1:1, v/v). Spectroscopic analysis using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) techniques were used to confirm the structure of MA yield.

2.4.2 Induction of malaria

A CHQ susceptible *P. berghei* strain was used for the induction of malaria. The *P. berghei* parasite was supplied by Prof Peter Smith (University of Cape Town, Division of Clinical Pharmacology, South Africa). This *Plasmodium* parasite is one of the four species of malaria parasites that infects murine rodents of West Africa (Clark and Cowden, 2003). *P. berghei* has been used in many studies as an experimental model for malaria (Goodman *et al.*, 2013). The *P. berghei* life cycle is similar to that of human infecting *Plasmodium* parasite making this *Plasmodium* species a good experimental model to study human malaria. Malaria was induced in male Sprague-Dawley rats via a single intra-peritoneal injection of *P. berghei* (10^5 parasitized red blood cells) (Gumede *et al.*, 2003). Percentage parasitaemia ranging from 15 - 20 % was considered as a stable malaria state before commencing any experimental procedures.

2.4.3 Parasite monitoring

Parasitaemia was scored on Giemsa-stained tail-blood films. Briefly, a small incision 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 sec and then stained in Giemsa stain for 20 minutes (min). Finally, the stained slide was air-dried and viewed under a microscope (Olympus cooperation, Tokyo, Japan) with an x50-100 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 Short-term studies

The effects of CHQ and MA on malaria parasites, physical parameters, blood glucose and renal function were investigated in male SD rats (90-120 g). Studies were carried out on non-infected and *P. berghei*-infected animals for a period of 3 weeks divided into pre-treatment (days 0-7), treatment (days 8-12) and post treatment (days 13-21) periods. During the 5 days treatment period separate groups of non-infected and *P. berghei*-infected animals were administered CHQ (30 mg/kg, p.o.) or MA (40, 80 and 160 mg/kg, p.o.) by means of a ball-tipped 18-gauge gavage needle (Kyron Laboratories (Pty) LTD, Benrose, South Africa) attached to a 1 mL syringe (Naumann *et al.*, 2009)twice daily at 9h00 and 17h00. These doses were selected based on our preliminary findings. The animals were individually housed in Makrolon polycarbonate metabolic cages to monitor 24 h food consumption, water intake, % body weight, blood glucose concentrations and urine volume output, these parameters were monitored daily during the treatment period and every third day during the treatment period. The overnight urine samples were collected for the measurement of Na^+, K^+ and Cl^-.

2.5.1 Effects of MA treatment on parasitaemia

Rats were monitored for % parasitaemia from the day of infection and days of survival relative to the non-infected control. % parasitaemia was monitored daily in the *P. berghei*-infected control and *P. berghei*-infected animals treated with CHQ or MA.

2.5.2 Blood glucose and physico-metabolic parameters

Blood glucose concentrations were measured 6 h after treatment in all groups of animals using a glucometer (OneTouch select glucometer, Lifescan, Mosta, Malta, United Kingdom).

Haematocrit levels were also monitored in all groups of animals at 9h00 every 3rd day during the pre-treatment, post- treatment periods and daily during the treatment period. Briefly, blood was collected into heparinised capillary tubes using the tail prick method (Parasuraman *et al.*, 2010). The sealed capillary tubes were then centrifuged for 3 min at 906 x g with a Micro-haematocrit centrifuge (346 MSE Centrifuge Manufacturer, London, UK). Haematocrit was determined by calculating the percentage of the total blood volume to packed cell volume.

2.6 Terminal studies

The percentage parasitaemia of the infected control animals peaked at day 12, this group of animals was therefore sacrificed on day 12 of the experimental period based on preliminary results. Separate groups of non-infected and *P. berghei*-infected rats treated with CHQ (30 mg/kg, p.o.) or MA (40, 80 and 160 mg/kg, p.o.) (n=6) were sacrificed during pre-treatment at day 0, treatment period at days 1, 5 and post-treatment period at day 14 for biochemical analysis. The animals were anaesthetized in an anaesthetic chamber with 100 mg/kg of isofor inhalation anaesthetic for 3 min. Acute and short term effects of CHQ and MA on plasma insulin concentrations were investigated in blood samples collected by cardiac puncture at 0.25, 0.5, 1, 5 and 14 days following treatment with CHQ (30 mg/kg, p.o.) or MA (80 mg/kg, p.o.). Blood and urine collected at pre-treatment, treatment and post-treatment periods was analyzed for hormones (AVP and aldosterone) and electrolytes respectively. Liver and kidney were collected and weighed gravimetrically (Mettler balance PC 180-insruments, Protea Laboratory Services, South Africa were stored in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands). All samples were stored at -80 °C until assayed.

2.7 Laboratory analysis

2.7.1 Measurement of electrolytes

Urine 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 (C_{cr}) was calculated using a standard formula [C_{cr}=Urine creatinine concentration x Urine flow rate (V) / Plasma creatinine concentration] based on the measurements of the plasma and urinary concentrations of creatinine and urine flow rate.

2.7.2 Hormone analysis

Plasma insulin assay

Ultra-sensitive rat ELISA kit was used to measure plasma insulin concentrations. The 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 micro titration 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 450 nm. Each determination was done in duplicate for standards (0.00, 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. Plasma samples (50 µL) 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 h. This was followed

by multiple wash using a wash buffer (350 µL). After the final wash, the plates were inverted against absorbant paper to remove all the liquid in the plates. The substrate, TMB was then added to all wells and incubated for 30 min. The reaction was stopped by adding 50 µL of stop solution to all wells and mixing for 5 min. Absorbance was measured using Spectrostar Nano microplate 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 1.39 pmol/L and 960 pmol/L, respectively. The intra-assay analytical coefficient of variation ranged from 4.4 - 5.5 % and the inter-assay coefficient variation from 4.7 - 8.9 %.

Arginine vasopressin (AVP) assay

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 microtubes. The mixture was vortex and centrifuged at 12000 x g for 20 min. 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 min to obtain the ether and aqueous layer. The remaining aqueous layer was transferred into new glass test tubes and dried under gas for 48 hrs. 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 Arg^8-Vasopressin ELISA Kit, using reagents purchased from the manufacturer (Abcam, Cambridge, Massachusetts, USA). The kit components included a 96 well plate coated goat antibodies, vasopressin conjugate, vasopressin antibody, assay buffer, wash buffer concentrate, vasopressin standards, p-nitrophenyl phosphate substrate (pNpp) and a stop solution. The Arg^8-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 an acid to give a

colorimetric endpoint that is read spectrophotometrically on a microplate reader (BMG Labtech GmbH, Ortenberg, Germany) at 405 nm.

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 4 ºC for 24 hrs. Following incubation, the reaction volume was emptied. 400 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. 200 µL of substrate pNpp was added to all wells and incubated at 37 ºC for 1 h. The reaction was stopped by adding 50 µL of stop solution to all wells and mixing on the shaker for 5 min. The absorbance was read at 405 nm using a Spectrostar Nano microplate 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 GraphPad Prism Instat Software (version 5.00). The respective AVP concentrations of the unknown samples were then extrapolated from the standard curve. The lower and upper limits of detection were 4 pmol/L - 923 pmol/L respectively. The intra assay analytical coefficient of variation ranged from 5.9 - 10.6 % and the inter-assay coefficient variation from 6.0 - 8.5 %.

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 aldosterone ELISA kit is a solid phase enzyme-linked ELISA based on the principle of competitive binding. The microtiter wells are 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 nmol/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 min and then incubation for 1 h 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 min 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 Nano microplate 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 GraphPad Prism Instat Software (version 5.00). The respective aldosterone concentrations of the unknown samples were then extrapolated from the standard curve.

2.7.3 Measurement of oxidative stress

MDA, a marker of oxidative stress and the activity of antioxidant defense enzymes (SOD and GPx) in the liver and kidney of *P. berghei*-infected control and *P. berghei*-infected animals treated with MA (80 mg/kg, p.o.) or CHQ (30 mg.kg, p.o.) were measured. In addition protein content in the above mentioned organs was quantified using the Lowry method (Lowry *et al.*, 1951).

The principle behind the Lowry method of protein measurement lies in the reactivity of peptide with Cu^{2+} ions under alkaline conditions. Protein is reacted with alkaline cupric sulfate in the presence of tartrate for 10 minutes at room temperature. During this incubation, a tetradentate copper complex forms from four peptide bonds and one atom of copper. A phosphomolybdic-phosphotungstic acid solution is added. This compound (called Folin-phenol reagent) becomes reduced, producing an intense blue color. The colour enhancement occurs when the tetradentate copper complex transfers electrons to the phosphomolybdic-phosphotungstic acid complex. The blue color continues to intensify during a 30 min room temperature incubation. The 30 min incubation, a rearrangement of the initial unstable blue complex leads to the stable final blue colored complex which has higher absorbance (Legler *et al.*, 1985). Sample preparation is summarised below.

Liver and kidney samples (0.5 g) were homogenized using isolation buffer (1 mL). The homogenate (0.2 mL) was made up to 0.5 mL using distilled water and 5 mL alkaline reagent was then added and the mixture incubated for 15 min at 40 C. After 15 min 0.5 mL of Folin Ciocalteu reagent (diluted with 0.5 mL of deionized water) was added. 2mg/mL of BSA (0-1 mg/mL) was used as the standard. The absorbance was read at 600 nm after standing for 30 min. An alkaline reagent consisted of 100 volumes of 4% sodium carbonate, 1 volume of 4% copper sulphate and 4% of sodium potassium tartrate. A calibration curve was set up using BSA standards.

Thiobarbituric acid reactive substances (TBARS) assay

TBARS assay measures levels of malondialdehyde (MDA) which is the product of lipid peroxidation. MDA reacts with thiobarbituric acid to form a colored complex which can be measured spectrophotometrically at 532 nm. Tissue (0.50 g) was homogenized in 400 µL of 2 % phosphoric acid. The homogenate was then separated into two equal halves, 200 µL of 7 % phosphoric acid was added in both halves followed by addition of 400 µL of butylated hydroxytoluene in the first half (sample test) and 400 µL of 3 mM hydrochloric acid (HCl) added in the second half (blank), pH of 1.5 was maintained in both solutions by adding 1M HCl (200 µL). Both solutions were heated at 100 °C for 15 min followed by addition of 1.5 mL of butanol then vortexing. Solutions were then centrifuged at 1300 g for 15 min and the organic phase was used to read absorbance at 532 and 600 nm. The absorbance from these wavelengths were used to calculate the concentration of malondialdehyde using Beer's Law.

$$\text{Concentration of MDA (mM)} = \frac{\text{Average Absorbance}}{\text{Absorption coefficient (156/ mM)}}$$

Superoxide dismutase (SOD) assay

In the presence of SOD, tetrazolium salt (WST-1) is reduced into a dye which can be measured photometrically. This assay was performed as by Biovision laboratory protocol. Briefly tissue (0.10 g) was homogenized in ice cold 0.1 M HCL and a buffer containing 0.5 % triton X, β methyl ethyl and 0 PMFS (0.1 mg/kg). Sample solution (20 µL) was added in a sample and blank 2 wells, H_2O (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.

$$\text{SOD activity (\%inhibition rate)} = \frac{(A_{\text{blank 1}} - A_{\text{blank 3}}) - (A_{\text{sample}} - A_{\text{blank 2}})}{(A_{\text{blank1}} - A_{\text{blank3}})}$$

Glutathione (GPx) assay

The GPx assay is based on the conversion of a luciferin derivative into luciferin in the presence of glutathione, catalysed by glutathione S-transferase (GST). The signal generated in a coupled reaction with firefly luciferase is proportional to the amount of GSH present in the sample. GSH assay was performed as by Promega laboratory protocol. 5 Mm GSH was diluted in water (1:100), and then followed by serial dilution (1:1). Each dilution of GSH (10 µL) was dispensed into well of opaque luminometer 96 well plate in triplicate, serving as standards. Tissues (0.10 g) were perfused with PBS, followed by homogenization in PBS containing 2 mM of EDTA. Homogenized tissue (50 µL) was dispensed in a well in duplicate. GSH-GLO reagent 2x (50 µL) was dispensed in both standards and samples wells followed by incubation for 30 min at room temperature. Luciferin detecting reagent (100 µL) was added followed by incubation for 15 min at room temperature. Afterwards the luminescence was read using luminometer (Promega, gloma multi direction system).

$$\text{GPx activity} = \frac{(B - B^0)}{(T2 - T1) \times V} \times \text{sample dilution}$$

Where the ΔA_{340nm} was used to extrapolated the values of B and B^o from the NADPH standard curve.

2.8 Data analysis

All data were expressed as means ± standard error of means (SEM). Data for untreated non-infected and *P. berghei* infected rats were used as baseline. For chronic studies the calculation of mean daily fluid voided and urinary amounts of electrolytes excreted were used to assess renal function. GFR was evaluated by creatinine clearance as assessed by 24 h urinary excretion rates of creatinine in relation to plasma concentration. GraphPad InStat Software version 5 (GraphPad Software, San Diego, California, USA) was used for all statistical analyses. One way 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 taken to imply statistical significance.

CHAPTER 3

RESULTS

3.1 General results

3.1.1 MA structure elucidation

The percentage yield of MA varied from 0.02 to 0.03 % while the purity was 98 %. The structure of MA was elucidated using ^{1}H and ^{13}C-NMR spectroscopic data obtained from white powder following recrystallization with chloroform-methanol (Figure 4). The ^{1}H NMR and ^{13}C NMR spectroscopic data and a table showing the location of olefenic bonds (carbon 2, 12 and 13) are given as supporting data below (Figure 5 and Table 2).

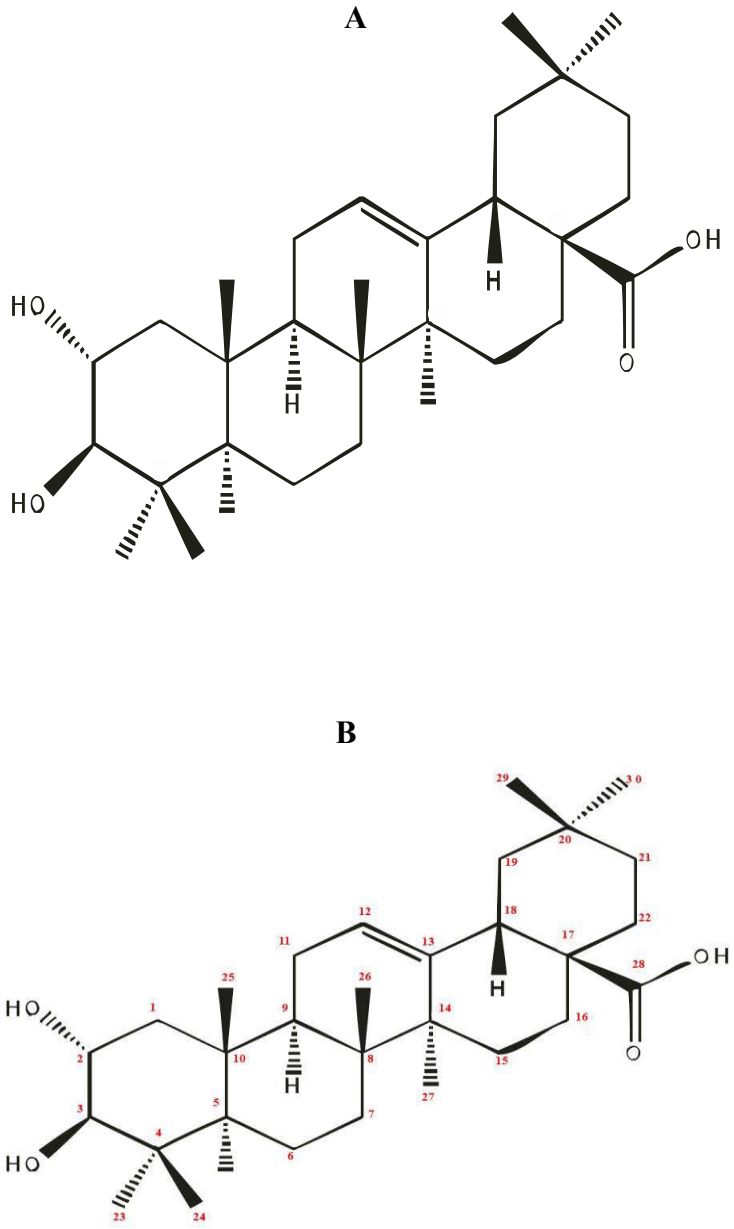

Figure 4: (**A**) The chemical structure and (**B**) numbering of carbons of maslinic acid (MA) according to the International Union of Applied Chemistry (IUPAC).

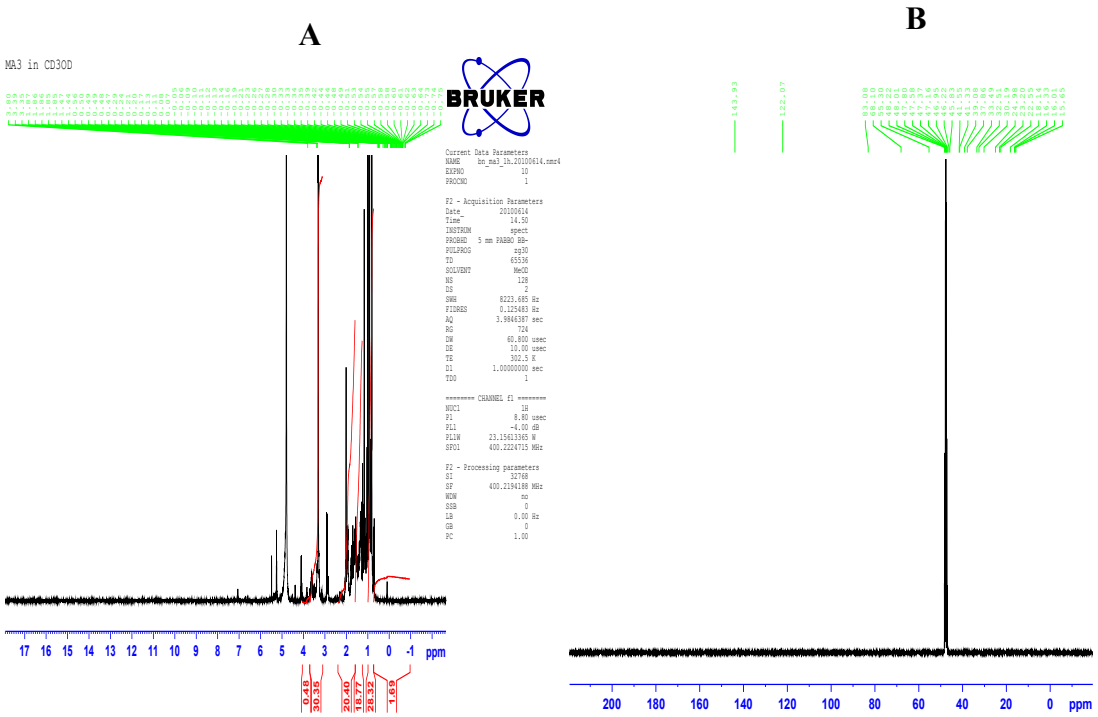

Figure 5: Supporting data showing **(A)** ^{1}H NMR and (**B**) ^{13}C NMR spectroscopic analysis of MA dissolved in deuterated methanol.

Table 2: ^{13}C Bruker NMR spectra showing the location of olefenic bonds of *S. aromaticum*-derived MA.

Carbon position	*S. aromaticum*-derived MA
1	46.2
2	**68.3**
3	83.3
4	39.1
5	55.0
6	18.1
7	32.7
8	39.0
9	47.4
10	38.0
11	23.2
12	**121.9**
13	**143.7**
14	41.6
15	27.4
16	23.0
17	46.2
18	41.0
19	45.7
20	30.4
21	33.6
22	32.3
23	28.3
24	16.6
25	16.5
26	16.4
27	23.2
28	178.5
29	32.2
30	23.2

3.2 Short-term studies

The effects of twice daily treatment for 5 consecutive days with MA (40, 80 and 160 mg/kg, p.o.) or CHQ (30 mg/kg, p.o.) on parasitaemia, physical parameters, glucose homeostasis, renal fluid and electrolytes handling were evaluated in separate groups of non-infected and *P. berghei*-infected rats.

3.2.1 Effects of MA on parasitaemia

Figure 6 shows pictures of parasite density during the pre-treatment, treatment and post-treatment period of animals treated with the most potent dose of MA (160 mg/kg, p.o.) or CHQ (30 mg/kg, p.o.). The lower dose MA (40 mg/kg, p.o.) significantly ($p<0.05$) decreased parasite density in comparison with the *P. berghei*-infected control however this dose did not eliminate the malaria parasites by the end to the experimental period. The higher doses MA (80 and 160 mg/kg, p.o.) decreased parasite density and eliminated by the end of the experimental period (Figure 6). Treatment with CHQ (30 mg/kg, p.o.) decreased parasite density and cleared the malaria parasites from the systemic circulation by day 5 following treatment (Figure 6).

Following infection with *P. berghei*, % parasitaemia in the infected animals gradually increased reaching a peak of 55 ± 8 % on day 12 of the experimental period (Figure 7). These animals were therefore sacrificed on day 12 as per ethics guidelines. As such all the subsequent results showing the untreated *P. berghei*-infected control will have no post treatment period (days 13 - 21). Figure 7 shows percentage parasitaemia of the *P. berghei*-infected control, MA and CHQ treated *P. berghei*-infected rats. Treatment with MA (40 mg/kg, p.o.) significantly ($p<0.05$) reduced the parasite in comparison with the *P. berghei* -infected control however this dose did not eliminate the malaria parasites until the end of the 21 days experimental period (Figure 7). The higher doses MA (80 and 160 mg/kg, p.o.) significantly ($p<0.05$) reduced and cleared the malaria parasites from systemic circulation by day 9 following treatment (Figure 7). CHQ (30 mg/kg, p.o.) eliminated the malaria parasites by day 5 following treatment (Figure 7).

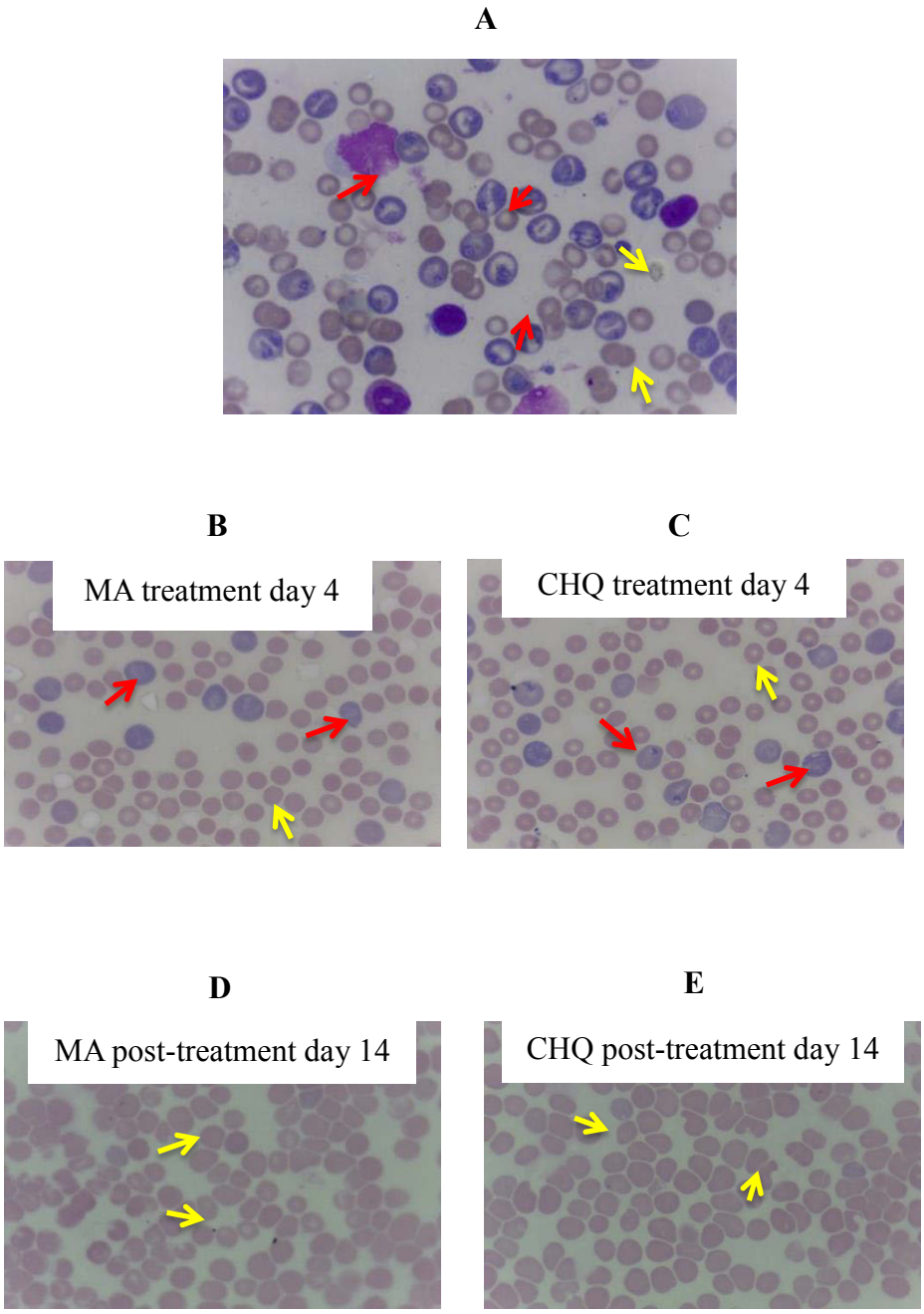

Figure 6: (**A**) Pictures showing parasite density during pre-treatment, (**B**) treatment with MA (160 mg/kg, p.o.) or (**C**) CHQ (30 mg/kg, p.o.) and (**D**) post-treatment with MA (160 mg/kg, p.o.) or (**E**) CHQ (30 mg/kg, p.o.) of *P. berghei*-infected animals (Magnification, 100 x10). (Leica SCN 400, Leica Microsystems CMS GmbH, Watzlar, Germany).

- ✓ Parasitized red blood cells are denoted by red arrows
- ✓ Non-parasitized red blood cells are denoted by yellow arrows

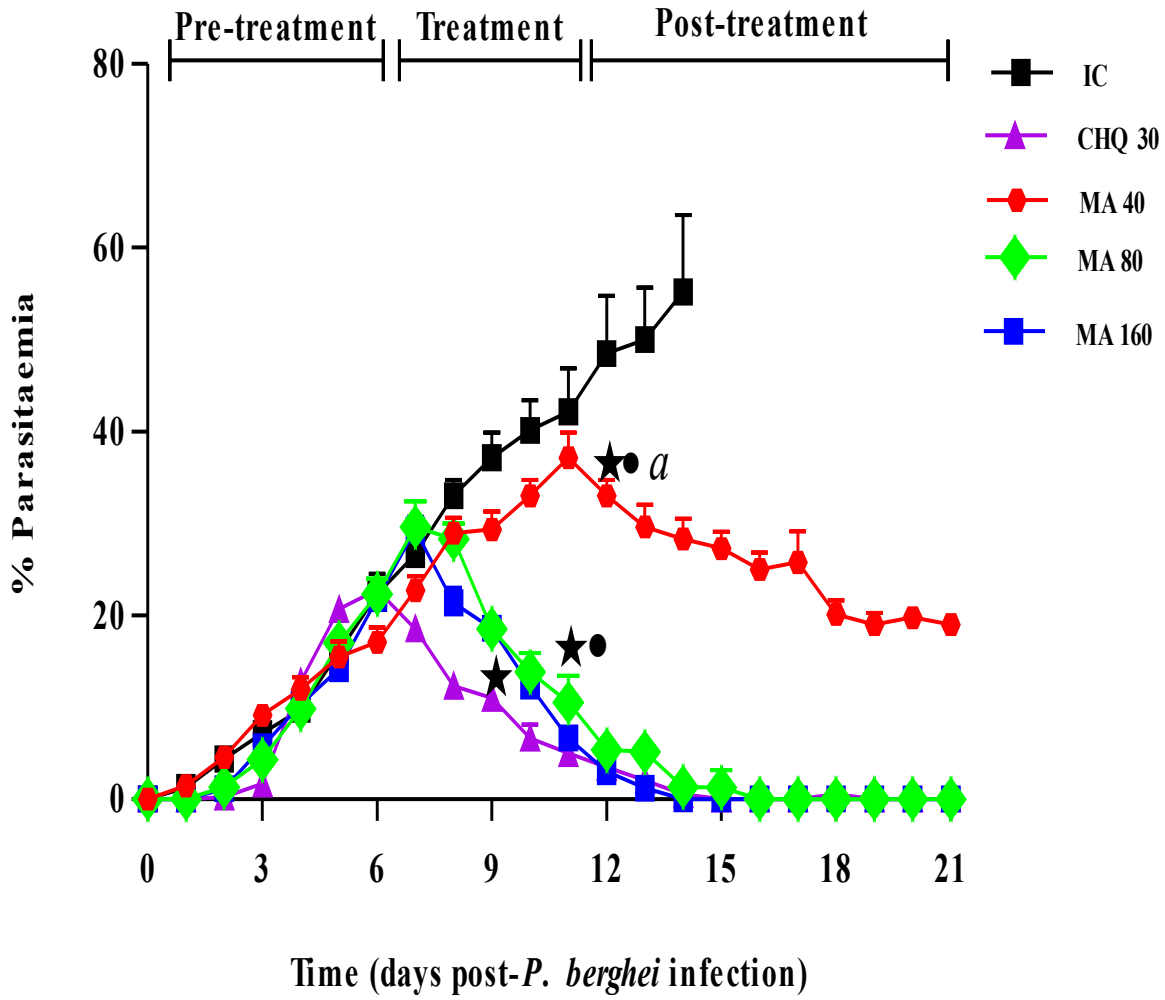

Figure 7: Comparison of the effects of twice daily treatment with MA (40, 80 and 160 mg/kg, p.o.) or CHQ (30 mg/kg, p.o.) on percentage parasitaemia in *P. berghei*-infected animals with the *P. berghei*-infected control. Values are presented as mean ± S.E.M, where columns represent means and vertical bars represent SEM (n=6 in each group).

★$p<0.05$ by comparison with the infected control

●$p<0.05$ by comparison with CHQ treated animals

a $p<0.05$ by comparison with MA (80 mg/kg, p.o.) treated animals

3.3 Effects of MA on physical parameters

Food consumption and water intake of the non-infected control were not altered, these animals progressively gained weight throughout the experimental period (Table 3). The above mentioned parameters were significantly ($p<0.05$) decreased in the untreated *P. berghei*-infected control. These animals showed severe wasting when compared with the non-infected control (Table 3). Non-infected rats treated with MA (40, 80 and 160 mg/kg, p.o.) progressively gained weight with no change in food and water intake (Table 3). Treatment of *P. berghei*-infected animals with various doses of MA (40, 80 and 160 mg/kg, p.o.) significantly ($p<0.05$) increased food and water intake as well % body weight change animals (Table 3). Administration of CHQ (30 mg/kg, p.o.) to non-infected and *P. berghei*-infected animals significantly ($p<0.05$) reduced the above mentioned parameters by comparison with non-infected control (Table 3).

Table 3: Food consumption, water intake and % body weight changes of non-infected and *P. berghei*-infected animals treated twice daily with MA (80 mg/kg, p.o.) or CHQ (30 mg/kg, p.o.). Values are presented as means ± SEM (n = 6 in each group).

Parameter	Groups	Baseline	Treatment	Post-treatment
Food intake (g/100g)	NIC	9 ± 1	10 ± 1	9 ± 1
	NI MA 40	9 ± 2	9 ± 1♦	8 ± 2
	NI MA 80	10 ± 1	9 ± 1♦	9 ± 1
	NI MA 160	9 ± 1	10 ± 1	9 ± 1
	NI CHQ 30	9 ± 1	7 ± 1#	9 ± 2
	IC	10 ± 1	7 ± 2#	N/A
	I MA 40	9 ± 1	9 ± 2★♦	9 ± 2
	I MA 80	9 ± 1	9 ± 2★♦	9 ± 2
	I MA 160	10 ± 1	9 ± 2★♦	8 ± 3
	I CHQ 30	10 ± 1	6 ± 1#	9 ± 1
Water intake (mL/100g)	NIC	15 ± 2	14 ± 1	15 ± 2
	NI MA 40	14 ± 1	14 ± 1#	14 ± 3
	NI MA 80	14 ± 1	14 ± 1#	14 ± 3
	NI MA 160	14 ± 1	14 ± 1#	14 ± 3
	NI CHQ 30	11 ± 3	9 ± 1#	14 ± 1
	IC	8 ± 3#	8 ± 2#	N/A
	I MA 40	13 ± 1♦	13 ± 2★♦	15 ± 2
	I MA 80	13 ± 1♦	13 ± 2★♦	15 ± 2
	I MA 160	14 ± 1♦	11 ± 4★♦	14 ± 2
	I CHQ 30	8 ± 2	7 ± 2#	12 ± 2
% b.wt change	NIC	8 ± 1	12 ± 1	18 ± 1
	NI MA 40	7 ± 1	10 ± 1#	19 ± 1♦
	NI MA 80	8 ± 1	11 ± 1#	18 ± 1♦
	NI MA 160	8 ± 2	10 ± 1#	19 ± 1♦
	NI CHQ 30	7 ± 1	-2 ± 1#	8 ± 1
	IC	-6 ± 2#	-4 ± 2#	N/A
	I MA 40	-6 ± 1	-1 ± 1★♦	2 ± 1♦
	I MA 80	-6 ± 1	-1 ± 1★♦	2 ± 1♦
	I MA 160	-6 ± 2	-1 ± 1★♦	2 ± 1♦
	I CHQ 30	-8 ± 1	-5 ± 1#	-1 ± 1

# $p<0.05$ by comparison with the non-infected control (NIC)

★$p<0.05$ by comparison with the infected control (IC)

♦ $p<0.05$ by comparison with CHQ treated animals

3.4 Effects of MA on metabolic processes

3.4.1 Effects of MA on glucose homeostasis

Blood glucose and plasma insulin concentrations in the non-infected control were not altered throughout the study (Figure 8 and Table 4). *P. berghei*-infected control group exhibited a significant ($p<0.05$) reduction in blood glucose concentrations by comparison with the non-infected control at corresponding time periods, however plasma insulin concentrations remained unaltered in these animals (Figure 8 and Table 4). Treatment with MA (40, 80 and 160 mg/kg, p.o.) had no significant ($p>0.05$) influence on blood glucose concentrations of the non-infected animals (Figure 8). The lower dose of MA (40 mg/kg, p.o.) had no significant ($p>0.05$) influence on the blood glucose concentrations of *P. berghei*-infected animals however the higher doses of MA (80 and 160 mg/kg, p.o.) significantly ($p<0.05$) increased blood glucose concentrations of *P. berghei*-infected animals to values comparable with the non-infected control (Figure 8). MA did not influence plasma insulin concentrations in the non-infected and *P. berghei*-infected animals (Table 4). Administration of CHQ (30 mg/kg, p.o.) significantly ($p<0.05$) decreased blood glucose concentrations with concomitant increase in plasma insulin concentrations in the non-infected and *P. berghei*-infected animals in comparison with the non-infected control (Figure 8 and Table 4).

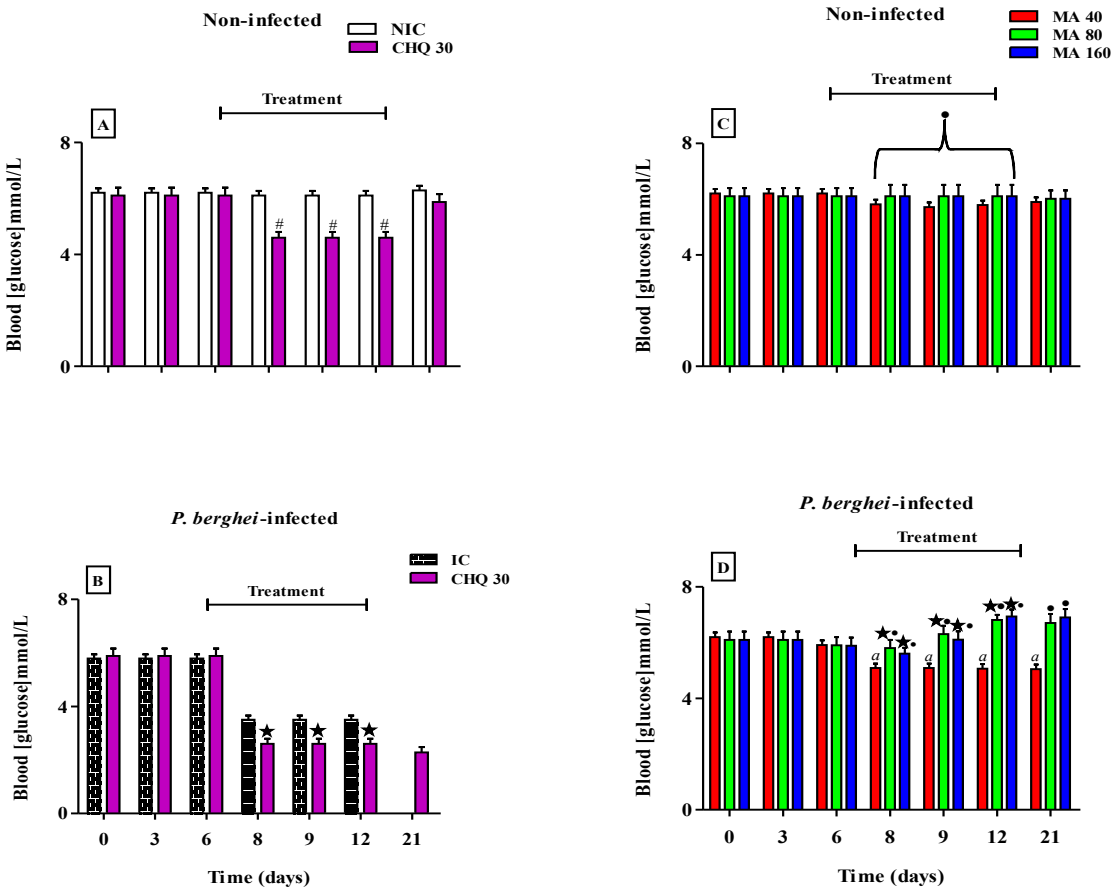

Figure 8: Comparison of blood glucose concentration profiles in (**A**) non-infected animals, (**B**) *P. berghei*-infected animals treated twice daily with CHQ (30 mg/kg, p.o.) and (**C**) non-infected animals, (**D**) *P. berghei*-infected animals treated with MA (40, 80 and 160 mg/kg, p.o.) with respective controls. 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

★$p<0.05$ by comparison with the infected control

● $p<0.05$ by comparison with CHQ treated animals

a $p<0.05$ by comparison with MA (80 mg/kg, p.o.) treated animals

Table 4: Comparison of plasma insulin and blood glucose concentrations of non-infected and *P. berghei*-infected rats treated with MA (80 mg/kg, p.o.) or CHQ (30 mg/kg, p.o.) with respective control animals. Data are expressed as means ± SEM (n = 6 in each group).

Treatment	Time (days post treatment)	Insulin concentration (pmol/L)	Blood glucose (mmol/L)	% parasitaemia
NIC	0	10.00 ± 0.60	6.41 ± 0.36	N/A
NI MA (80 mg/kg, p.o.)	0.50	11.48 ± 0.41♦	5.36 ± 0.48♦	N/A
	1	10.16 ± 0.67♦	5.46 ± 0.07♦	N/A
	5	11.51 ± 0.15♦	5.02 ± 0.07♦	N/A
	14	10.40 ± 0.52♦	5.96 ± 0.07♦	N/A
NI CHQ (30 mg/kg, p.o.)	0.50	18.57 ± 1.00#	2.83 ± 0.27#	N/A
	1	28.00 ± 2.00#	3.98 ± 0.25#	N/A
	5	39.00 ± 4.00#	2.03 ± 0.19#	N/A
	14	13.00 ± 2.00#	4.32 ± 0.13#	N/A
IC	0	11.00 ± 0.47	5.73 ± 0.28	22.96 ± 2.16
I MA (80 mg/kg, p.o.)	0.50	11.39 ± 0.20♦	6.06 ± 0.44♦	20.05 ± 1.87★♦
	1	10.83 ± 0.37♦	6.56 ± 0.09♦	18.50 ± 1.08★♦
	5	10.56 ± 0.37♦	5.68 ± 0.06♦	5.30 ± 1.58★♦
	14	11.95 ± 1.19♦	5.95 ± 0.09♦	0.00 ± 0.00
I CHQ (30 mg/kg, p.o.)	0.50	19.57 ± 1.50★	2.29 ± 0.51★	18.05 ± 1.87★
	1	27.91 ± 2.63★	2.16 ± 0.40★	11.00 ± 0.22★
	5	29.00 ± 4.00★	2.29 ± 0.13★	3.50 ± 0.76★
	14	16.00 ± 4.00★	3.40 ± 0.28★	0.00 ± .0.00

# $p<0.05$ by comparison with the non-infected control (NIC)

★$p<0.05$ by comparison with the infected control (IC)

♦ $p<0.05$ by comparison with CHQ treated animals

N/A: Not available. The animals were not infected

3.4.2 Effects of MA renal function

Effects of MA on renal fluid and electrolyte handling

Urine volume outputs and electrolyte handling of the non-infected control group remained unchanged throughout the 21 days experimental period (Figure 9). MA (40 mg/kg, p.o) had no significant ($p>0.05$) influence on urinary Na^+ excretion of the non-infected animals. However, administration of MA (80 and 160 mg/kg, p.o.) significantly ($p<0.05$) increased urinary Na^+ output from the 1st day of treatment until the end of the experimental period (Figure 9). Various doses of MA had no significant ($p>0.05$) influence on urinary K^+ and Cl^- outputs of non-infected (Figure 9). MA (40, 80 and 160 mg/kg, p.o.) maintained urine volume outputs of non-infected animals at normal values (Figure 9). Administration of CHQ to non-infected and *P. berghei*-infected animals significantly ($p<0.05$) increased urinary Na^+ outputs on day 1 following treatment. However, urinary Na^+ and urine volume outputs were significantly ($p<0.05$) decreased from day 2 of the treatment period until the end of the study (Figure 9). Urinary K^+ outputs were significantly ($p<0.05$) increased in these animals while Cl^- outputs remained unaltered (Figure 9).

Urine volume and urinary Na^+ outputs of the *P. berghei*-infected control were significantly ($p<0.05$) reduced in comparison with the non-infected control while urinary Cl^- outputs remained unchanged. However a significant ($p<0.05$) increase in urinary K^+ outputs was observed in these animals (Figure 10). MA (40 mg/kg, p.o) had no significant ($p>0.05$) influence on urinary Na^+ excretion of *P. berghei*-infected animals in comparison with the non-infected control. However, administration of MA (80 and 160 mg/kg, p.o.) significantly ($p<0.05$) increased urinary Na^+ output from the 1st day of treatment until the end of the experimental period (Figure 10). Various doses of MA had no significant ($p>0.05$) influence on urinary K^+ and Cl^- outputs of *P. berghei*-infected animals (Figure 10). MA (40, 80 and 160 mg/kg, p.o.) restored urine volume outputs of the *P. berghei*-infected animals to values comparable with the non-infected control (Figure 10). Administration of CHQ to *P. berghei*-infected animals significantly ($p<0.05$) increased urinary Na^+ outputs on day 1 following treatment. However, urinary Na^+ and urine volume outputs were significantly ($p<0.05$) decreased from day 2 of the treatment period until the end of the study (Figure 10). Urinary K^+ outputs were significantly ($p<0.05$) increased in these animals while Cl^- outputs remained unaltered (Figure 10).

% haematocrit in the non-infected control remained unchanged at (48 ± 4 %) for the duration of the study (Figure 11). The *P. berghei*-infected control animals exhibited a significant ($p<0.05$) reduction in % haematocrit when compared with the non-infected control (Figure 11). MA (40, 80 and 160 mg/kg, p.o.) had no significant ($p>0.05$) effect on % haematocrit of the non-infected animals (Figure 11). The lower dose MA (40 mg/kg, p.o.) had no significant ($p>0.05$) influence on % haematocrit of *P. berghei*-infected animals. However, the higher doses MA (80 and 160 mg/kg, p.o.) significantly ($p<0.05$) increased % haematocrit of animals to values comparable with the non-infected control (Figure 11). Administration of CHQ (30 mg/kg, p.o.) to non-infected and *P. berghei*-infected animals significantly ($p<0.05$) reduced % haematocrit by comparison with the non-infected control (Figure 11).

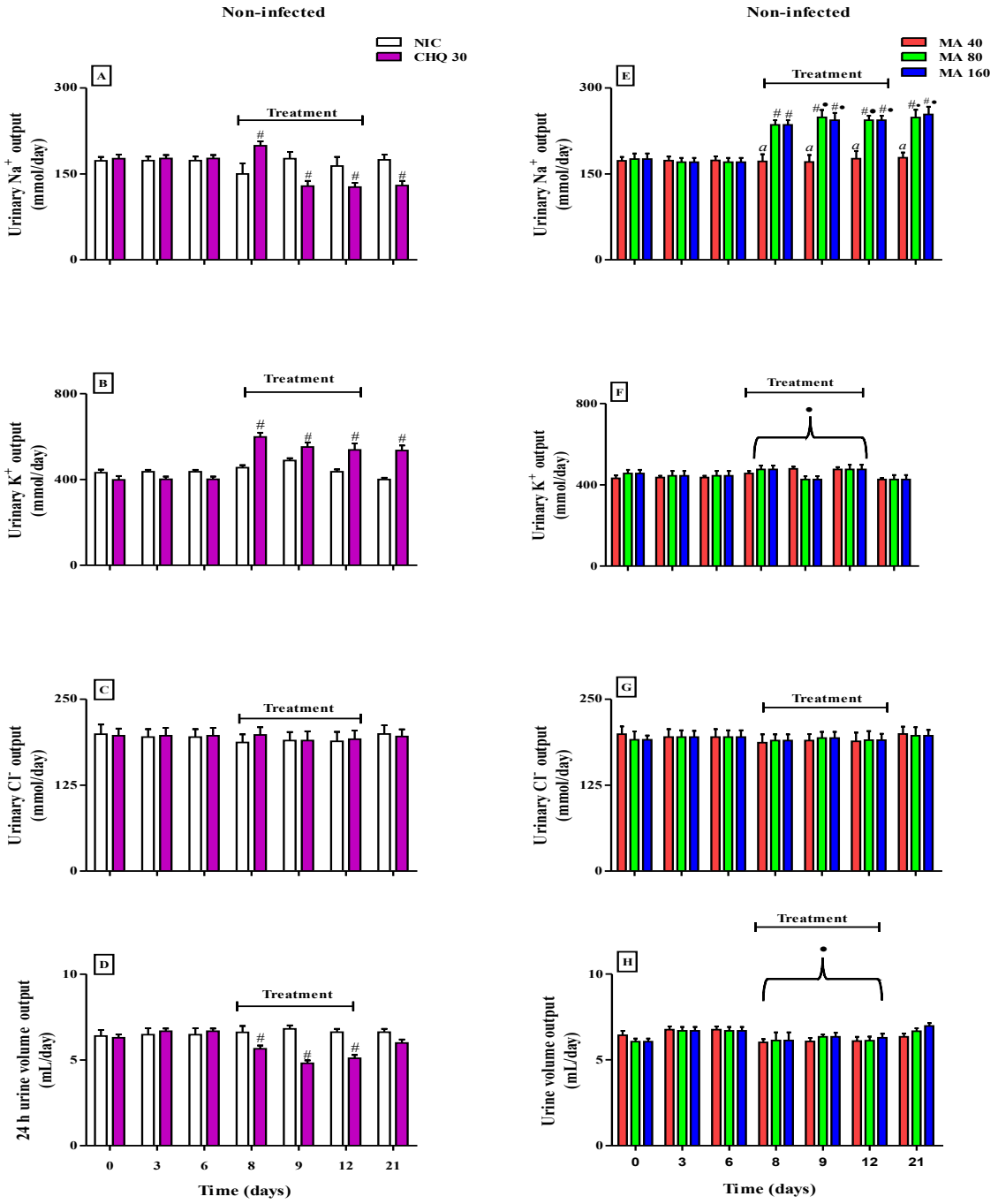

Figure 9: Comparison of 24 h urinary (**A**) Na^+, (**B**) K^+ (**C**) Cl^-, (**D**) urine volume outputs and (**E**) Na^+, (**F**) K^+ (**G**) Cl^-, (**H**) urine volume outputs of non-infected animals treated twice daily with CHQ (30 mg/kg, p.o.) and MA (40, 80 and 160 mg/kg, p.o.) respectively with the non-infected control. 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

● $p<0.05$ by comparison with CHQ treated animals

a $p<0.05$ by comparison with MA (80 mg/kg, p.o.) treated animals

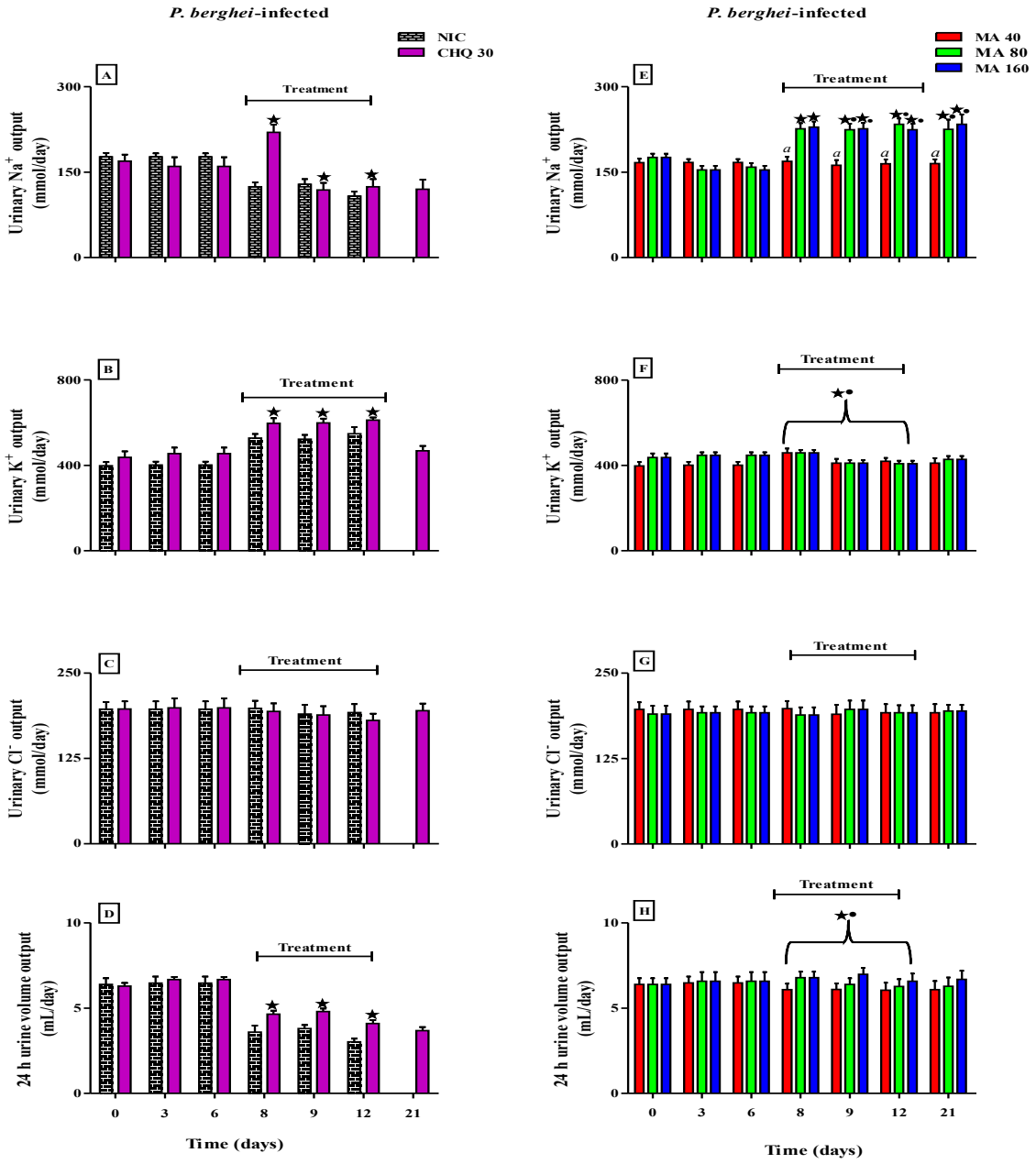

Figure 10: Comparison of 24 h urinary (**A**) Na^+, (**B**) K^+ (**C**) Cl^-, (**D**) urine volume outputs and (**E**) Na^+, (**F**) K^+ (**G**) Cl^-, (**H**) urine volume outputs of *P. berghei*-infected animals treated twice daily with CHQ (30 mg/kg, p.o.) and MA (40, 80 and 160 mg/kg, p.o.) respectively with the untreated *P. berghei*-infected control. 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

● $p<0.05$ by comparison with CHQ treated animals

a $p<0.05$ by comparison with MA (80 mg/kg, p.o.) treated animals

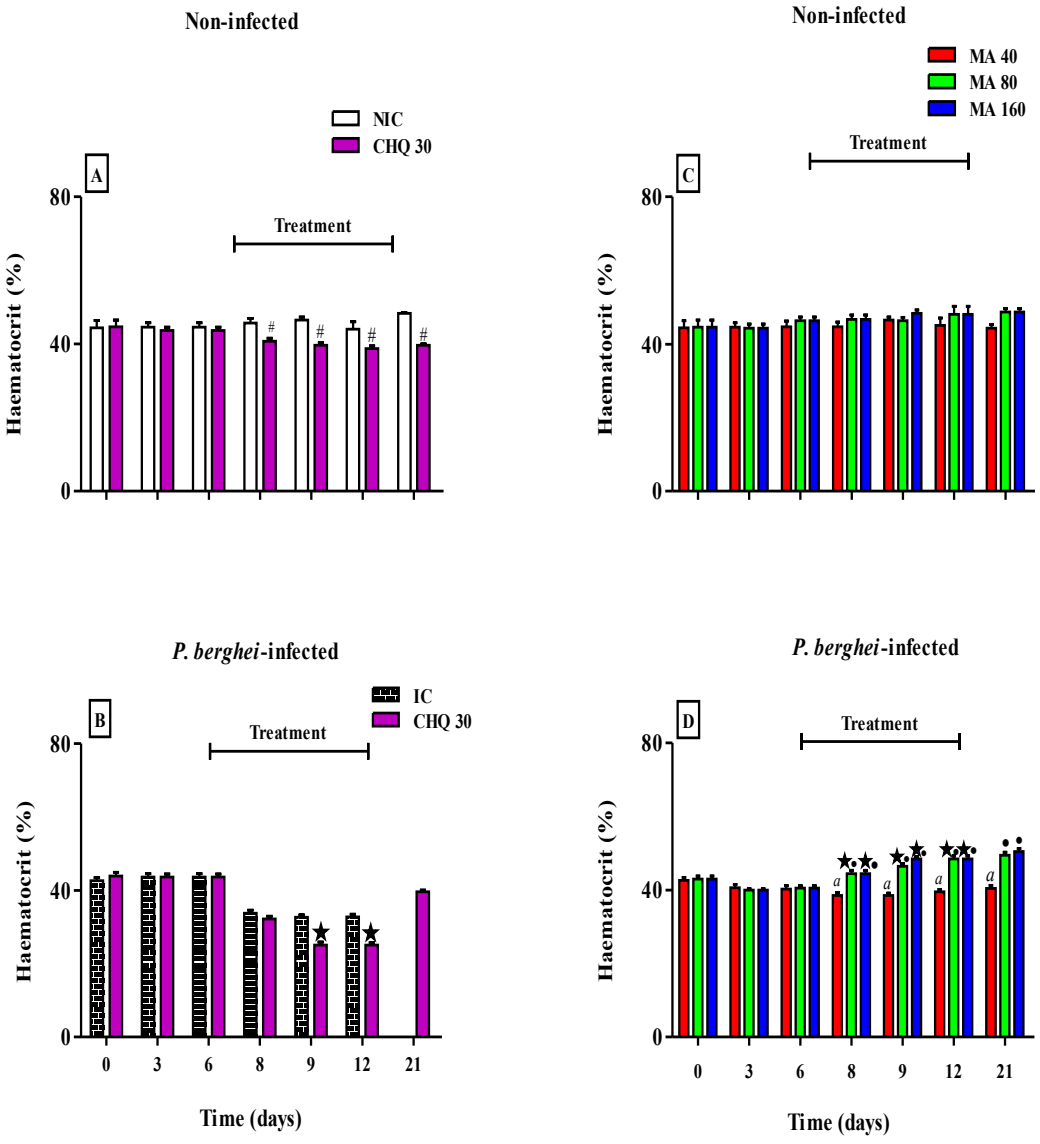

Figure 11: Comparison of effects of twice daily treatment with CHQ or MA on % haematocrit in (**A**) non-infected animals and (**B**) *P. berghei*-infected animals treated twice daily with CHQ (30 mg/kg, p.o.) and (**C**) non-infected animals and (**D**) *P. berghei*-infected animals treated with MA (40, 80 and 160 mg/kg, p.o.). 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

★$p<0.05$ by comparison with the infected control

♦ $p<0.05$ by comparison with CHQ treated animals

a $p<0.05$ by comparison with MA (80 mg/kg, p.o.) treated animals

Effects of MA on plasma electrolytes

Table 4 shows the effects of treatment twice daily with MA or CHQ on plasma biochemical parameters of non-infected and *P. berghei*-infected rats. Plasma biochemical parameters, GFR and kidney mass of the untreated *P. berghei*-infected animals were not altered (Table 4). MA (40, 80 and 160 mg/kg, p.o.) had no significant ($p>0.05$) effect on plasma Na^+, K^+, Cl^- and kidney mass of non-infected and *P. berghei*-infected animals. GFR in the non-infected and *P. berghei*-infected animals remained unchanged following treatment with MA. Treatment with CHQ (30 mg/kg, p.o.) did not alter plasma Na^+, K^+, Cl^-, kidney mass and GFR of non-infected animals. Administration of CHQ to *P. berghei*-infected animals did not influence Na^+, Cl^-, kidney mass and GFR, however plasma K^+ concentrations in these animals were significantly ($p<0.05$) increased when compared with the non-infected control (Table 5).

Table 5: Plasma biochemical parameters of non-infected animals treated twice daily with MA (40, 80 and 160 mg/kg, p.o.) for 5 consecutive days. Data are expressed as means ± SEM (n = 6 in each group).

Protocol	Parameter	Groups				
		NIC	**NI CHQ 30**	**NI MA 40**	**NI MA 80**	**NI MA 160**
Pre-treatment	Na^+ (mmol/L)	134 ± 3	138 ± 2	135 ± 2	136 ± 1	136 ± 1
	K^+ (mmol/L)	5.89 ± 0.80	4.33 ± 0.82	5.07 ± 0.37	5.37 ± 0.27	5.68 ± 0.47
	Cl^- (mmol/L)	102 ± 8	102 ± 9	104 ± 1	106 ± 2	106 ± 2
	GFR (mL/min/ 100g)	0.80 ± 0.02	0.90 ± 0.02	0.82 ± 0.05	0.83 ± 0.06	0.84 ± 0.06
	Kidney mass (g/100g)	0.80 ± 0.02	0.89 ± 0.03	0.74 ± 0.02	0.75 ± 0.02	0.75 ± 0.02
Treatment	Na^+ (mmol/L)	131 ± 2	134 ± 2	130 ± 4	130 ± 4	130 ± 4
	K^+ (mmol/L)	5.51 ± 0.30	4.63 ± 0.40	5.52 ± 0.56	5.62 ± 0.36	5.51 ± 0.76
	Cl^- (mmol/L)	104 ± 3	103 ± 3	104 ± 2	102 ± 2	104 ± 1
	GFR (mL/min/ 100g)	0.91 ± 0.03	0.80 ± 0.02	0.89 ± 0.08	0.89 ± 0.04	0.87 ± 0.04
	Kidney mass (g/100g)	0.90 ± 0.04	0.89 ± 0.02	0.98 ± 0.03	0.98 ± 0.02	0.98 ± 0.02
Post-treatment	Na^+ (mmol/L)	130 ± 3	131 ± 3	134 ± 2	135 ± 1	134 ± 2
	K^+ (mmol/L)	5.39 ± 0.10	3.93 ± 0.30	4.05 ± 0.51	4.02 ± 0.47	4.05 ± 0.47
	Cl^- (mmol/L)	101 ± 1	101 ± 3	108 ± 1	106 ± 3	106 ± 3
	GFR (mL/min/ 100g)	0.90 ± 0.01	0.92 ± 0.03	0.95 ± 0.06	0.93 ± 0.05	0.95 ± 0.07
	Kidney mass (g/100g)	0.83 ± 0.03	0.90 ± 0.01	0.80 ± 0.07	0.82 ± 0.05	0.87 ± 0.02

# $p<0.05$ by comparison with the non-infected control (NIC)

♦ $p<0.05$ by comparison with CHQ treated animals

Table 6: Plasma biochemical parameters of *P.berghei*-infected animals treated twice daily with MA (40, 80 and 160 mg/kg, p.o.) for 5 consecutive days. Data are expressed as means ± SEM (n = 6 in each group).

Protocol	Parameter	Groups				
		IC	**I CHQ 30**	**I MA 40**	**I MA 80**	**I MA 160**
Pre-treatment	Na^+ (mmol/L)	136 ± 4	140 ± 4	136 ± 2	138 ± 1	138 ± 2
	K^+ (mmol/L)	6.70 ± 0.36	7.09 ± 0.33	6.30 ± 0.62	6.60 ± 0.62	6.60 ± 0.71
	Cl^- (mmol/L)	108 ± 3	98 ± 4	104 ± 5	105 ± 4	106 ± 5
	GFR (mL/min/ 100g)	0.90 ± 0.01	0.90 ± 0.02	0.87 ± 0.05	0.87 ± 0.05	0.87 ± 0.05
	Kidney mass (g/100g)	0.89 ± 0.01	0.83 ± 0.01	0.90 ± 0.02	0.92 ± 0.01	0.90 ± 0.01
Treatment	Na^+ (mmol/L)	133 ± 4	140 ± 4	134 ± 3	135 ± 2	135 ± 3
	K^+ (mmol/L)	4.50 ± 0.58	10.50 ± 1.56★	5.57 ± 0.82♦	5.67 ± 0.42♦	5.83 ± 0.62♦
	Cl^- (mmol/L)	101 ± 3	101 ± 2	102 ± 2	101 ± 3	104 ± 2
	GFR (mL/min/ 100g)	0.88 ± 0.20	0.90 ± 0.01	0.90 ± 0.02	0.90 ± 0.02	0.90 ± 0.02
	Kidney mass (g/100g)	1.01 ± 0.40	0.33 ± 0.04	0.84 ± 0.01	0.85 ± 0.02	0.85 ± 0.01
Post-treatment	Na^+ (mmol/L)	N/A	137 ± 4	138 ± 2	140 ± 2	140 ± 2
	K^+ (mmol/L)	N/A	10.93 ± 0.60	5.07 ± 0.65♦	5.87 ± 0.55♦	5.67 ± 0.72♦
	Cl^- (mmol/L)	N/A	103 ± 2	109 ± 4	108 ± 4	107 ± 4
	GFR (mL/min/ 100g)	N/A	0.96 ± 0.01	0.95 ± 0.04	0.98 ± 0.05	0.92 ± 0.07
	Kidney mass (g/100g)	N/A	0.90 ± 0.03	0.87 ± 0.05	0.96 ± 0.03	0.91 ± 0.08

★$p<0.05$ by comparison with the infected control

♦ $p<0.05$ by comparison with CHQ treated animals

Plasma AVP and aldosterone concentrations

Plasma AVP and aldosterone concentrations were measured in samples collected from non-infected and *P. berghei*-infected rats at day 5 and day 14 following treatment with MA (80 mg/kg, p.o.). Plasma AVP and aldosterone assays in samples collected from animals treated with CHQ (30 mg/kg, p.o.) were not conducted in the current study. Plasma samples collected on day 0 of the treatment period served as baseline. Plasma AVP and aldosterone concentrations in the *P. berghei*-infected control were significantly ($p<0.05$) increased by comparison with non-infected control animals. MA did not alter plasma AVP and aldosterone concentrations of non-infected animals by comparison with the non-infected control. However plasma AVP concentrations in *P. berghei*-infected animals were significantly ($p<0.05$) increased following MA administration while aldosterone concentrations were restored towards normal values by comparison with the non-infected control.

Table 7: Comparison of plasma AVP and aldosterone concentrations of non-infected and *P. berghei*-infected rats following 5 and 14 days of treatment with MA (80 mg/kg, p.o.) with respective controls. Data are expressed as means ± SEM (n = 6 in each group).

Treatment	Time (days post-treatment)	AVP (pmol/L)	Aldosterone (nmol/L)
NIC	0	2.82 ± 0.47	0.78 ± 0.04
NI MA (80 mg/kg, p.o.)	5	2.83 ± 0.90	0.76 ± 0.06
	14	2.34 ± 0.59	0.75 ± 0.04
IC	0	4.98 ± 0.62#	1.79 ± 0.08#
I MA (80 mg/kg, p.o.)	5	4.47 ± 0.52	0.79 ± 0.04★
	14	3.59 ± 0.66	0.78 ± 0.07★

# $p<0.05$ by comparison with the non-infected control

★$p<0.05$ by comparison with the infected control

Oxidative stress

The concentrations of MDA and antioxidant enzymes (SOD and GPx) of the non-infected control represent baseline/normal activity levels found in the liver and kidney tissues used (Table 7). In comparison with the non-infected control, MDA concentrations in the liver and kidney of *P. berghei*-infected control animals were significantly ($p<0.05$) increased while the activities of SOD and GPx were significantly ($p<0.05$) decreased (Table 7). Treatment with MA did not influence the levels of MDA in the liver and kidney of non-infected animals. However the activity of SOD and GPx were increased although this increases were not of statistical significance ($p>0.05$). MA significantly ($p<0.05$) decreased MDA concentrations in the liver and kidney of *P. berghei*-infected animals to values comparable with the non-infected control (Table 5). Furthermore, treatment with MA significantly ($p<0.05$) increased the activity of SOD and GPx in the liver and kidney of *P. berghei*-infected animals to values comparable with the non-infected control (Table 5). Administration of CHQ (30 mg/kg, p.o.) however, significantly ($p<0.05$) increased MDA concentrations in the liver and kidney in the non-infected and *P. berghei*-infected animals when compared with the non-infected control. In addition, SOD and GPx concentrations in these organs were significantly ($p<0.05$) decreased when compared with the non-infected control (Table 7).

Table 8: Comparison of MDA concentrations and antioxidants SOD and GPx activities in hepatic and renal tissues of non-infected and *P. berghei*-infected rats at day 5 following twice daily treatment with MA (80 mg/kg, p.o.) or CHQ (30 mg/kg, p.o.) with respective controls. Values are presented as means ± SEM (n=6 in each group).

Parameter measured	Group	Organ	
		Liver	Kidney
MDA (nmol/g protein)	NIC	1.94 ± 0.67	1.15 ± 0.13
	NI MA	1.63 ± 0.68♦	1.42 ± 0.17♦
	NI CHQ	5.30 ± 0.38#	5.88 ± 0.25#
	IC	4.07 ± 0.91#	6.07 ± 0.62#
	I MA	2.08 ± 0.56★♦	1.47 ± 0.62★♦
	I CHQ	5.92 ± 0.85★	7.75 ± 0.41★
SOD activity (nmol/min mL/g protein)	NIC	9.27 ± 1.80	18.64 ± 1.89
	NI MA	8.57 ± 2.29♦	19.9 ± 1.46♦
	NI CHQ	4.57 ± 1.29#	3.49 ± 1.46#
	IC	3.43 ± 0.90#	5.31 ± 0.66#
	I MA	8.98 ± 1.20★♦	18.28 ± 1.20★♦
	I CHQ	0.26 ± 0.01★	1.87 ± 0.04★
GPx activity (nmol/min mL/g protein)	NIC	2.08 ± 0.15	4.36 ± 0.25
	NI MA	2.06 ± 0.27♦	4.52 ± 0.35♦
	NI CHQ	1.33 ± 0.10#	2.27 ± 0.25#
	IC	0.92 ± 0.07	0.36 ± 0.05
	I MA	2.01 ± 0.15★♦	4.08 ± 0.15★♦
	I CHQ	0.45 ± 0.06★	0.19 ± 0.02★

# $p<0.05$ by comparison with the non-infected control

★ $p<0.05$ by comparison with the infected control

♦ $p<0.05$ by comparison with CHQ treated animals

CHAPTER 4

DISCUSSION

Findings from the current study show that MA possesses anti-malaria properties *in vivo.* Furthermore MA restored blood glucose concentrations of malaria infected animals to physiological ranges with concomitant improvement of renal fluid and electrolyte handling as well as anti-oxidant activity. These findings are of clinical relevance because malaria infection and/ treatment are associated with hypoglycaemia, impaired renal fluid and electrolyte handling as well as oxidative stress. Findings from the current study not only validate our indigenous knowledge systems, but provide scientific evidence that contributes to current knowledge about therapeutic effects of plant-derived MA in malaria. This study introduces the first *in vivo* evidence of anti-hypoglycaemic and antioxidant effects of MA on malaria and/treatment-induced adverse effects. In addition our results confirm the previously reported anti-plasmodial activity of MA on malaria infected cell line (Moneriz *et al.*, 2011a). *P. berghei* was used as our experimental model. This rodent infecting *Plasmodium* strain is considered to be a good experimental model to study human malaria. *P. berghei* is inexpensive, easy to maintain and has physiological similarities to human infecting *Plasmodium* parasites (Sinden *et al.*, 2002). Furthermore a study by Jense and Waters, 1995 reported that the *P. berghei* species does not infect humans (Janse and Waters, 1995). *P. berghei* is therefore considered safe to be handled by humans (Janse and Waters, 1995). Weanling male Sprague-Dawley rats (90 - 120 g) were used in the present study. *Plasmodium* parasites have been reported to have high affinity for developing RBC's (Cowman and Crabb, 2006). Indeed clinical studies have reported malaria cases in infants and children (Maina *et al.*, 2010). Peak percentage parasitaemia was therefore achieved in the rat model creating an opportunity to investigate the potential curative effects of MA in malaria (Dow *et al.*, 1999). In the current study MA of high purity was used to treat the animals. The purity of MA was assessed by NMR spectroscopy. Furthermore the absolute stereostructure of MA elucidated from the ^{1}H and ^{13}C-NMR spectra was comparable to previously reported data (Júlio *et al.*, 2003).

MA treatment administered twice daily 8 h apart for 5 consecutive days significantly decreased percentage parasitaemia during the treatment period and cleared the malaria parasites in *P. berghei*-infected animals during the post-treatment period. The ability of MA to eliminate the malaria parasites was comparable with the standard malaria drug CHQ. The anti-plasmodial property of MA may be attributed to the anti-parasitic activity. Indeed a study by De Pablos *et al*., 2010 reported anti-parasitic activity of MA against *T.gondii* parasites (De Pablos *et al.*, 2010). Our findings therefore suggest that MA could be a potent anti-plasmodial compound.

The malaria parasite and treatment with CHQ have been associated with adverse effects on glucose homeostasis (Davis, 1997; Onyesom and Eagho, 2011). *P. berghei*-infected control animals exhibited a significant reduction of blood glucose concentrations. Studies have suggested that the malaria parasite lowers blood glucose concentrations via multiple mechanisms (Krishna *et al.*, 1999; Uyemura *et al.*, 2000; Ménard, 2005; Roe and Pasvol, 2009). Uyemura and colleagues (2000) suggested that the utilisation of the host glucose stores by the malaria parasites is one of the main factors that precipitate hypoglycaemia in malaria patients (Uyemura *et al.*, 2000). The depletion of vital gluconeogenic substrates such as thiamine by the malaria parasite has been suggested to play a major role in the reported malaria parasite-induced hypoglycaemia (Krishna *et al.*, 1999). A study by Dekker *et al*., 1997 however suggested that hepatocellular damage by the malaria parasites is responsible for malaria-induced hypoglycaemia (Dekker *et al.*, 1997). This damage to the liver is suggested to be due to invasion of hepatic cells by the parasites during replication (Dekker *et al.*, 1997). Indeed studies have shown that the liver plays a pivotal role in the breakdown of the activated insulin-receptor complex therefore hepatocellular damage may result in slow insulin receptor recycling which in turn may induce hypoglycaemia (Onyesom and Eagho, 2011). Histological techniques were not conducted in the current study therefore the mechanism by which malaria parasite induced hypoglycaemia cannot be fully explained. Our results however show a significant decrease in food and water intake by the *P. berghei*-infected control animals (Table 3). Indeed previous studies have reported a significant decrease in food intake during infection with malaria (Djimdé and Lefèvre, 2009). We therefore speculate that the reduced food intake may be responsible for the significant decrease in blood glucose concentrations. MA had no significant effect on blood glucose concentrations of non-infected animals. The ability of MA to maintain blood glucose of non-infected animals indicates that MA alleviates adverse effects on glucose homeostasis that are

often observed following infection with malaria and/ treatment with CHQ. *P. berghei*-infected animals treated with MA exhibited a significant increase in blood glucose concentrations in comparison with the *P. berghei*-infected control and CHQ treated animals. This could be attributed to increased food intake of the *P. berghei*-infected animals to levels comparable to the non-infected control following MA administration. MA administration showed no significant effect on plasma insulin concentrations of non-infected and *P. berghei*-infected animals. This is another mechanism by which MA could have maintained the blood glucose concentrations at physiological levels. Our findings are of novelty in that MA is well established as an anti-diabetic compound through the inhibition of glycogen phosphorylase (GP) which catalyses the first step of glycogen breakdown (Liu *et al.*, 2007; Wen *et al.*, 2008). In addition, a recent study by Liu et al., 2014 reported that MA modulates glycogen metabolism by enhancing the insulin signaling pathway and inhibiting glycogen phosphorylase (Liu *et al.*, 2014). Furthermore, Khathi and colleagues (2013) reported that MA reversed the higher expression of Na^+-dependent glucose co-transporter (SGLT1) and glucose transporter 2 (GLUT2) found in diabetic animals (Khathi *et al.*, 2013). These transporters are implicated in the intestinal absorption of glucose, thus their down-regulation contributed to diminishing plasma glucose concentrations (Khathi *et al.*, 2013). The limitation of the current study however is that glycogen content and western blot analysis of glucose transporters were not conducted and therefore this study cannot provide the mechanism by which MA improved blood glucose homeostasis of malaria infected animals. The role of MA in glucose metabolism of malaria-infected models is of clinical relevance because of malaria and/treatment induced hypoglycaemia. Current literature has only investigated the effects of MA on diabetic models (Liu *et al.*, 2007; Mkhwanazi *et al.*, 2014). Findings from the current study suggest that MA is able to avert the malaria and/treatment induced hypoglycaemia perhaps through increasing food intake (Table 3). Non-infected animals treated with CHQ exhibited a significant reduction in blood glucose concentrations. These results suggested that CHQ lowers blood glucose concentrations independent of the malaria parasites. Our findings correlated with previous studies which reported that CHQ possesses hypoglycaemic properties (Davis, 1997; Sibiya, 2013). A study by Davis, 1997 attributed the CHQ-induced hypoglycaemia to increased insulin secretion (Davis, 1997). Furthermore studies have suggested that CHQ-induced increase insulin secretion could be due to rapid accumulation of the drug in the pancreas thereby having a direct effect on the beta cells of islets of Langerhans to secrete insulin (Asamoah *et al.*, 1990). Some studies however have suggested that the increased plasma insulin concentration following CHQ

administration is due to the lysosomal property of CHQ which compromises the optimal activity of lysosomal hydrolases that are responsible for the degradation of insulin (Knutson *et al.*, 1985). Based on our findings, the current study complements the notion that CHQ-induced increase of plasma insulin concentrations may be responsible for this reduction in blood glucose concentrations (Table 4). A recent study by Jarzyna *et al*., 1997 reported that CHQ causes hypoglycaemia through insulin-independent pathways (Jarzyna *et al.*, 1997). Jarzyna and colleagues (1997) reported that CHQ inhibits glutamate dehydrogenase (GLUD), a key enzyme in the metabolism of amino acids in the liver and kidney. The inhibition of GLUD results in a decrease in hepatic glucose output which leads to hypoglycaemia. Blood glucose concentrations of *P. berghei*-infected rats treated with CHQ significantly decreased with a concomitant increase in plasma insulin concentrations in comparison to the *P. berghei*-infected control. These results indicated that indeed CHQ causes hypoglycaemia via increased insulin secretion. Our findings therefore show that malaria parasites and/treatment with CHQ induce hypoglycaemia.

Renal function studies were designed to investigate whether MA can avert the reported malaria parasite and/CHQ-induced adverse effects on renal fluid and electrolyte handling. The current study therefore investigated the effects of MA and CHQ on renal fluid, electrolyte handling, AVP and aldosterone concentrations as well as oxidative stress in non-infected and *P. berghei*-infected animals treated with MA or CHQ. Previous reports indicate that the malaria parasite and/CHQ treatment impair kidney function consequently resulting in inappropriate renal fluid and electrolytes handling (Musabayane *et al.*, 1993). These changes are believed to be attributed to hepatocellular damage by both the malaria parasite and/ CHQ due to increased deposition of CHQ in tissues such as the heart and kidney following treatment with CHQ administration (Dondo and Mubagwa, 1990; Baguet and Fabre, 1999). Urinary Na^+ and renal fluid handling in the non-infected animals remained unchanged throughout the study. The *P. berghei*-infected control animals showed a significant decrease in urinary Na^+ excretion and urine volume outputs when compared with the non-infected control. Previous studies have reported that parasitized RBC's stick to one another and tend to adhere to adjacent unparasitized erythrocytes and capillary endothelium (Elsheikha and Sheashaa, 2007; Das, 2008). This in turn results in the formation of intravascular clumps which impede the renal microcirculation (Sriamornsak, 1998). This constriction of the renal vascular bed often causes a decrease in oxygen supply. The resulting ischaemia causes a reduction urinary Na^+ and urine volume outputs (Elsheikha and Sheashaa,

2007; Das, 2008). The pathophysiology of hyponatraemia in severe malaria is incompletely understood, some studies found no changes in vasopressin release whereas other studies found evidence for increased vasopressin release (Holst *et al.*, 1994; Hanson *et al.*, 2009). Our results showed that plasma AVP concentrations of the *P. berghei*-infected control animals increased significantly in comparison with the non-infected control while aldosterone concentrations were not altered. Indeed previous studies have reported increased vasopressin concentrations during malaria infection (Holst *et al.*, 1994). Treatment with MA significantly increased urinary Na^+ outputs of the non-infected and *P. berghei*-infected rats. However, the loss of Na^+ was not reflected in the plasma (Table 5). Our results correlate with previous studies in our laboratory which showed that MA increases urinary Na^+ outputs in STZ-induced diabetic animals (Mkhwanazi *et al.*, 2014). Mkhwanazi *et al*., 2014 suggested that MA attenuated the diabetes induced kidney dysfunction via enhancement of the antioxidant status. Treatment with MA did not alter plasma AVP and aldosterone concentrations of non-infected animals. Plasma aldosterone of *P. berghei*-infected animals were not altered however AVP concentrations were significantly increased when compared with the non-infected control animals. These results suggest that the increase in AVP concentrations of *P .berghei*-infected animals following treatment with MA could perhaps be due to the malaria parasite not MA. The current study has shown for the first time that MA has no effects on plasma AVP and aldosterone concentrations of non-infected and *P.bergei*-infected animals. Our findings therefore suggest that MA improves kidney function by increasing urinary Na^+ excreation thus averting the malaria and/ treatment induced Na^+ retention. Results from the present study show that chronic administration of CHQ causes reduction of urinary Na^+ outputs and urine volume outputs of non-infected and *P. berghei*-infected animals. Indeed previous studies in our laboratory reported that CHQ causes renal Na^+ retention via increased plasma aldosterone concentration and reduced GFR (Musabayane *et al.*, 1994; Musabayane *et al.*, 2000a). However a study by Musabayane *et al*., 1993 reported no significant changes in aldosterone following acute CHQ administration. Musabayane and colleagues (1996) suggested CHQ-induced natriuresis may be attributed to CHQ stimulated increase in plasma AVP concentration (Musabayane *et al.*, 1996). A study by Musabayane *et al*., 2000b suggested that CHQ supresses the vasopressin-induced increase in cyclic adenosine monophosphate (cAMP) production in the isolated inner medullary collecting ducts consequently interfering with the normal antidiuretic response to vasopressin by reducing cAMP formation (Musabayane *et al.*, 2000b). Thus the CHQ-induced natriuresis could be due to the precursor effects of AVP on V_1 receptors which increase Na^+ excretion without

changing the urine flow rate (Musabayane *et al.*, 1997). In the current study however we observed a significant reduction in the urine output of non-infected and *P. berghei*-infected following treatment with CHQ. We speculate that this phenomenon may also be attributed to the reduced water intake that was recorded in animals treated with oral CHQ (Table 3). The present study did not measure aldosterone and AVP concentration of non-infected nor *P. berghei*-infected animals following chronic CHQ administration and therefore cannot provide the mechanism by which chronic administration of CHQ causes Na^+ retention. However a previous study in our laboratory suggested that the decreased Na^+ excretion following chronic administration of CHQ may be accredited to the CHQ-induced kidney damage (Musabayane *et al.*, 2000a). Urinary K^+ outputs of the *P. berghei*-infected control animals were significantly increased in comparison to the non-infected control animals. The *P. berghei*-induced hyperkalaemia is thought to be due to the increased release of K^+ from RBCs during cell lysis during which K^+ is released. Indeed, the present study recorded a concomitant reduction in haematocrit levels of the *P. berghei*-infected control. The reduced haematocrit values suggested that there was a significant reduction of RBC's due to the malaria parasite-induced hemolysis of infected and non-infected cells. Indeed, previous studies have reported increased plasma K^+ concentrations in malaria patients (Phillips and Pasvol, 1992). Urinary K^+ output of the *P. berghei*-infected animals increased following MA administration however this increase was not of statistical significance. This could be due to parasite reduction by MA which led to decreased cell lysis and concomitant improvement of haematocrit values. Our results therefore suggest that MA may improve urinary K^+ handling of *P. berghei*-infected animals via parasite clearance and subsequent improvement of haematocrit values.

MDA, a marker for oxidative stress was elevated in the *P. berghei*-infected control and CHQ treated groups. SOD and GPx activity in liver and kidney of these animals were significantly decreased. A number of studies have associated malaria infection with oxidative stress (Atamna and Ginsburg, 1993; Francis and Goldberg, 1997; Ricardo *et al.*, 2012). Ricardo and colleagues (2012) suggested that the oxidative stress that occurs in malaria is due to the degradation of haemoglobin by the *Plasmodium* parasite (Ricardo *et al.*, 2012). Furthermore studies have shown that the free haeme undergoes oxygenation resulting in the formation of oxygen radicals (Atamna and Ginsburg, 1993; Francis and Goldberg, 1997). Some studies however have suggested the oxidative stress observed during infection with malaria is due to treatment with CHQ (Magwere *et al.*, 1997; Atamna and Ginsburg, 2005). Indeed our results show increased MDA concentrations and diminished SOD and GPx activities following CHQ

administration (Table 7). A study by Ginsburg *et al*., 1998 suggested that CHQ decreases the availability of reduced glutathione to pathways involved in detoxification and reacts with ferriprotoporphyrin IX which produces highly reactive radicals that generate oxidative stress in the host (Ginsburg *et al.*, 1998). Although the mechanisms by which oxidative stress occurs in malaria and/treatment cannot be explained by the current study, our finding indicate that the malaria parasites and CHQ induce oxidative stress via independent mechanisms. Our results also indicate that treatment with CHQ exacerbates the malaria-induced oxidative stress (Table 7). Treatment with MA restored MDA concentrations in the liver and kidney of *P. berghei*-infected animals to normal values. The reduction of MDA levels could be due to increased antioxidant activity. Indeed our results show that MA increases the activity of SOD and GPx (Table 7). Findings from the current study are in correlation with a previous study in our laboratory which reported that MA improves renal function of diabetic animals by increasing antioxidant activity in the liver, kidney and heart (Mkhwanazi *et al.*, 2014). Furthermore a study by Montilla *et al*., 2003 reported that MA inhibits nitric oxide (NO) and reduces the susceptibility of plasma to lipid peroxidation (Montilla *et al.*, 2003). Allouche and colleagues (2010) reported that MA not only inhibits LDL oxidation but possesses peroxyl radical scavenging activity and metal chelating effects (Allouche *et al.*, 2010). Based on our findings we can therefore conclude that MA alleviates malaria-induced oxidative stress by improving antioxidant levels.

CHAPTER 5

CONCLUSIONS

The results described in this study demonstrate that the *S. aromaticum*-derived MA possesses antimalarial activity *in vivo*. In addition, our results show that MA ameliorates the malaria-induced hypoglycaemia by restoring blood glucose concentrations of *P. berghei*-infected animals to normalcy. The fluid and electrolyte handling of the malaria infected experimental rats were also restored following treatment with MA. In summary, this study has demonstrated the ability of MA to clear the malaria parasite with concomitant improvement of glucose homeostasis, fluid and electrolyte handling of the *P. berghei*-infected male Sprague-Dawley rats. The results presented in this thesis suggest that MA could be a potent compound in the management of malaria. The strategy of investigating bioactive compounds isolated from anti-malaria herbal medicine at pre-clinical followed by clinical studies could yield a much desired efficacious and safe class of antimalarial drugs for the management of malaria.

5.1 Shortfalls of the study

MA does not completely dissolve in water. Dissolution studies should be conducted to find the best solvent or mixture of solvents to dissolve MA. The transdermal delivery of MA could also be investigated to improve the solubility of this compound. Previous studies in our laboratory have reported successful delivery of CHQ and OA via dermal patches. This could perhaps improve the efficacy of this highly promising triterpene. Hepcidin was not measured in the current study this hormone has recently been found to play a major role in malarial anaemia. The measurement of hepcidin would help determine whether malarial anaemia is caused by the malaria parasites or by hepcidin thus aiding in developing new approaches for malaria management. Some studies suggest that the malaria induced hypoglycaemia is due to hepatocellular damage however histology of the liver was not conducted in this study. Therefore this study cannot fully provide a mechanism by which hypoglycaemia occurs in malaria. The mechanism by which MA affects glucose metabolism in the malaria model also needs to be investigated.

5.2 Recommendations for future studies

We have demonstrated that MA clears the malaria parasite in a dose dependant manner. Future studies should investigate higher doses of MA so as to perhaps improve the rate of parasite clearance. Pharmacokinetics of MA should be conducted to determine the half-life and concentrations of MA in various organs and in plasma.

CHAPTER 6

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CHAPTER 7

APPENDICES

APPENDIX I

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
Research Office
Animal Ethics Research Committee

Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
Email: animalethics@ukzn.ac.za

13 March 2013

Reference: 085/13/Animal

Miss T Thaane
School of Laboratory Medicine
and Medical Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Miss Thaane

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:

"Evaluation of transdermal delivery of combined maslinic acid and chloroquine on malaria parasites, blood glucose, renal electrolyte handling in *Plasmodium berghei-infected* male Sprague-Dawley rats."

Yours sincerely

HT Coetzer

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar – Prof. J Meyerowitz
Research Office – Dr N Singh
Supervisor, Prof. C Musabayane
Head of School – Prof. W Daniels
BRU, Dr S Singh

APPENDIX II

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

20 December 2013

Reference: 030/14/Animal

Miss T Thaane
Discipline of Physiology
School of Laboratory Medicine &
Medical Sciences
WESTVILLE Campus

Dear Miss Thaane

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 transdermal delivery of maslinic acid alone and in combination with chloroquine on malaria parasites, blood glucose, renal electrolyte handling in *Plasmodium berghei*-infected male Sprague-Dawley rats."

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

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

Animal Ethics Committee
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APPENDIX III

COLLEGE OF HEALTH SCIENCES RESEARCH SYMPOSIUM

12-13 September 2013

K-RITH TOWER BUILDING

CHLOROQUINE PROFILES IN PECTIN-CHLOROQUINE HDROGEL PATCHES FORMULATION IN MALE SPRAGUE-DAWLEY RATS AND *IN VITRO* OVER A PERIOD OF TIME

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 becoming popular due to unique advantages the pectin-CHQ patch could be an alternative to conventional formulation of malaria management.

APPENDIX IV

PHYSIOLOGY SOCIETY OF SOUTHERN AFRICA

14-17 September 2014

THE GATEWAY HOTEL-UMHLANGA

ORAL PRESENTATION 13

CHLOROQUINE PROFILES IN PECTIN-CHLOROQUINE HDROGEL PATCHES FORMULATION IN MALE SPRAGUE-DAWLEY RATS AND IN VITRO OVER A PERIOD OF TIME

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ABSTRACT

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 delivery system of drugs which provides sustained controlled drug release thus averting the adverse effects observed with oral CHQ. Against this background we then investigated the stability of the pectin-CHQ patch formulation in male Sprague-Dawley rats and *in vitro* over a period of time. 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 a patch containing 14 mg of CHQ. The 21 days study was divided into pre-treatment; days 0-7, treatment 8-12 and post treatment 13-21. The *in vitro* studies revealed that the CHQ concentration in the various patches remained constant over the 28 days period with the percentage drug incorporation ranging from 44-58%. *In vivo* we found that plasma CHQ concentrations also remained constant for 21-days. The TDDS is becoming popular due to unique advantages, the pectin-CHQ patch could be an alternative to conventional formulation for malaria management.