Efficacy of maslinic acid and chloroquine on the co-infection of *Plasmodium berghei* and *Trichinella zimbabwensis* in Sprague-Dawley rats.

By

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PREFACE

The research contained in this dissertation/thesis was completed by the candidate while based in the College of Agriculture Engineering and Science, School of Life Sciences College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville campus, South Africa. The research was financially supported by South Africa National Research funds (Incentive funds) allocated to Professor S. Mukaratirwa.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by candidate.

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DECLARATION

I.....**Lorna Gcanga**..... declare that

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Lorna Gcanga

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Abstract

Many people living in Africa and elsewhere in the tropics suffer from combined malaria, soil-transmitted infections and tissue-dwelling nematodes. Most of these deaths are prevalent in sub-Saharan Africa (SSA) where the infections overlap, which often results in co-infection. Malaria and trichinellosis are one of the most important zoonotic diseases especially in sub-Saharan Africa caused by *Plasmodium* spp. and *Trichinella* spp., respectively. New drugs targeting malaria and trichinellosis have been examined with little success. The aim of our study was to determine and compare the efficacy of maslinic acid and chloroquine on the co-infection of muscle-dwelling larvae of *Trichinella zimbabwensis* and *Plasmodium berghei* in rats. Fifty-four Sprague-Dawley rats with an average weight of 150g and 200g for males and females respectively were infected with *T. zimbabwensis* and *P. berghei*. Infected rats were randomly assigned to nine groups which were subjected to treatments of maslinic acid and chloroquine and a combination of maslinic acid and chloroquine. Co-infected groups were infected with *T. zimbabwensis* on day 0, and then infected with *P. berghei* on day 30 post-infection (pi). Treatment was administered for 3 consecutive days on day 9 pi with *P. berghei*. Groups infected with *P. berghei* only were infected on day 0 and were treated on day 9 pi for 3 consecutive days. Groups infected with *T. zimbabwensis* only were infected on day 0 and treated on day 25 pi. Untreated control groups were a placebo (distilled water) on day 25 pi infected with *T. zimbabwensis* and from day 9 pi infected with *P. berghei*. In *Trichinella*-infected groups, the efficacy of each treatment measured by the rate of the reduction in muscle larvae was significant ($P < 0.05$) for both drugs compared to the untreated control group. In malaria-infected groups, the efficacy of each treatment, measured by the rate of reduction in parasitaemia, was significant ($P < 0.05$) for both drugs compared to the untreated control group. There was no apparent synergistic effect due to the combination of the two drugs in reducing the muscle larval burden and in reducing malaria parasitaemia. In all the treatment regimens, the reductions were significant when compared to the untreated control groups and not significant to each other ($P > 0.05$). From these results we can conclude that the efficacy of maslinic acid on the co-infection of *T. zimbabwensis* and *P. berghei* was comparable to that of chloroquine, making maslinic acid a promising drug to be used as an anthelmintic and anti-malaria against muscle larval stages of *Trichinella* spp. and malaria parasitaemia and no side effects were observed.

List of contents **Page**

TITLE PAGE

PREFACE i

DECLARATION OF PLAGIARISM ii

ACKNOWLEDGMENTS iii

ABSTRACT iv

LIST OF CONTENTS v

LIST OF TABLES AND FIGURES viii

ABBREVIATIONS AND ACRONYMS ix

DEFINITIONS x

ADDENDUM- ETHICAL CLEARANCE 38

CHAPTER 1: INTRODUCTION 1

1.1 Rational for the research 1
1.2 Justification 2
1.3 Aim 3
1.4 Objectives 3

CHAPTER 2: LITARATURE REVIEW 5

2.1 Introduction 5

2.2 Co-infection of malaria and tissue-dwelling nematodes in sub-Saharan Africa 7

2.3 Epidemiology of co-infection of malaria and trichinellosis 8

2.4 Conventional drugs for malaria 9

2.4.1 Chloroquine 9

2.4.2 Quinine 10

2.4.3 Mefloquine 11

2.4.5 Artemisin 12

2.5 The emergence of chloroquine resistance 12

2.6 Conventional drugs for trichinellosis 13

2.7 Potential drug for malaria and trichinellosis 14

2.7.1 Maslinic acid 14

2.7.2 Biosynthesis and role as phytoalexin 15

2.7.3 Natural source 16

2.7.4 Antimalarial anthelmintic properties of maslinic acid 16

2.8 Conclusion 18

CHAPTER 3: MATERIALS AND METHODS 19

3.1 Ethical statement 19

3.2 Experimental animals 19

3.3 Parasite strains 19

3.4 Experimental design 19

3.5 Determination of *P. berghei* and *T. zimbabwensis* muscle larvae 20

3.6 Drug efficacy determination 21

3.7 Termination of animals 21

3.8 Data analysis 21

CHAPTER 4: RESULTS 22

4.1 Larval reduction after treatment 22

4.2 Parasitaemia reduction after course of treatment 24

4.3 Drug efficacy determination 26

CHAPTER 5: DISCUSSION AND CONCLUSION 29

5.1 Discussion 29

5.2 Conclusion 31

REFERENCE LIST 33

List of tables and figures

Table 1. Summary of experimental study on the efficacy of maslinic acid and chloroquine on the co-infection of *Plasmodium berghei* and *Trichinella zimbabwensis* in rats.

Table 2. Log-transformed least square means (LSM) of *Trichinella zimbabwensis* larval counts following a single treatment (on day 25pi) and *Plasmodium berghei* parasitaemia following treatments (on days 9, 10, 11, 12pi) respectively, and % reduction following treatment regimen with maslinic acid, chloroquine and the combination of the two. CI, 95% confidence interval

Table 3. Multiple pairwise comparisons of least square means derived from the Bonferroni test of treatment regimen of *Trichinella zimbabwensis* larvae (on day 25pi) in rats, * significance $P < 0.05$

Table 4. Multiple pairwise comparisons of means derived from the Bonferroni test of subsequent treatment (on days 9, 10, 11, 12pi) regimen of *Plasmodium berghei* parasitaemia in rats, * significance $P < 0.05$

Table 5. Prediction equations for the rate of change in parasiteamia following treatments (on days 9, 10, 11, 12 pi) with maslinic acid, chloroquine and the combination of maslinic acid and chloroquine. CI, 95% confidence interval; SE, standard deviation; $P < 0.05$, significant; NS, not significant.

Figure 1. Chemical structure of chloroquine.

Figure 2. Chemical structure of quinine.

Figure 3. Chemical structure of mefloquine.

Figure 4. Chemical structure of artemisinin.

Figure 5. Chemical structure of maslinic acid.

Figure 6. Mean percentage of parasitaemia of rats co-infected with *Plasmodium berghei* and *Trichinella zimbabwensis* and treated from day 9 to day 13 post-infection.

Figure 7. Mean percentage of parasitaemia of rats infected with *Plasmodium berghei* only and treated from day 9 to 13 post-infection.

Figure 8. Log-transformed least square means of *Trichinella zimbabwensis* larvae on infected rats following treatment (on day 25pi).

Abbreviations and acronyms

UKZN- University of KwaZulu-Natal

SSA- sub-Saharan Africa

LSM- Log-transformed least square mean

CI- Confidence interval

SE- standard error

NS- Not significant

WHO- World Health Organization

ITNs- Insecticide Treated bed Nets

IRS- Indoor Residual Spraying

MDA- Mass Drug Administration

IPT – Intermittent preventative treatment

STH- Soil-transmitted helminths

DRC- Democratic Republic of Congo

MA- Maslinic acid

BRU- Biomedical resource unit

iRBC- infected red blood cell

SPSS- Statistical Package for Social Science

FDC- Fixed dose combination

PQP- Piperaquine phosphate

AIDS- Acquired Immune Deficiency Syndrome

Definitions

Malaria – infectious disease characterized by cycles of chills, fever and sweating, caused by the parasitic infection of red blood cells by the protozoan of the genus Plasmodium.

Trichinellosis – disease caused by the genus Trichinella, typically from infected meat, characterized by digestive disturbance, fever and muscular rigidity.

Benzimidazole – a crystalline base $C_7H_6N_2$ used especially to inhibit the growth of various viruses, parasitic worms or fungi.

Definitive host – host in which the parasites have sexual reproduction.

Intermediate host – host in which parasites grow and either do not reproduce or do so asexually.

Mass drug administration – administration of a full therapeutic course of an antimalarial drug to a whole population without screening for the presence of parasitaemia.

Intermittent preventative treatment – administration of full course antimalarial treatment at a specific time without parasite evaluation.

Soil-transmitted helminths - refers to the intestinal worms infecting humans that are transmitted through contaminated soil.

Chapter 1

Introduction

1.1 Introduction

Malaria is a common and life-threatening disease in many tropical and subtropical areas (WHO, 2012). It is a vector-borne disease, caused by protozoa of the genus *Plasmodium* (WHO, 2013). The species that cause human malaria are *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* (WHO, 2013) and one simian parasite, *P. knowlesi* transmitted by *Anopheles dirus* in communities living in or near forested areas of southeast Asia (WHO, 2012). In sub-Saharan Africa, *P. falciparum* parasite is transmitted to humans by female *Anopheles* mosquito during a blood meal (Gonçalves *et al.,* 2014). Children under 5 years of age (WHO, 2014), women in their first pregnancy (WHO, 2014) and travelers from areas that are malaria free are mainly affected by malaria caused by *P. falciparum* (WHO, 2014). The most deadly species are *P. falciparum*, which is widely spread geographically, and *P. vivax*, which is less infective, but relapsing (WHO, 2012). In 2010 it was estimated that 98% cases of malaria in Africa were caused by *P. falciparum*, while in South-East Asia 50% of the cases were caused by *P. vivax* (WHO, 2012). Between 2000 and 2013, an expansion of malaria interventions helped to reduce malaria incidence by 30% globally, and by 34% in Africa (WHO, 2014). *P. berghei* is the causative agent of rodent malaria and is widely used as a model system to study various stages of *Plasmodium* parasites that affect humans (van de Sand *et al*., 2005)

The biological diversity during the life cycle of the *Plasmodium* parasite is one of its vital characteristics (WHO, 2012). A human is infected with a bite from a mosquito carrying malaria. Parasites which are in the form of sporozoites occupy the liver cells where they are able to multiply over a few weeks. Once in the liver, the sporozoites become merozoites. The merozoites will leave the liver and enter into the bloodstream. When they are in the bloodstream they start infecting erythrocytes and start replicating asexually. The replication results in clinical signs in infected people such as changes in blood flow and vascular endothelium, systemic complications due to anemia, fever and other flu-like symptoms due to the release of cytokines (WHO, 2012). Some merozoites remain in the erythrocytes and develop into sexual forms, called gametocytes (WHO, 2012). The gametocytes will remain in the bloodstream until a mosquito feeds on the infected human and ingests the infected blood. Once the gametocytes are on the mosquito, they will replicate to form sporozoites (WHO, 2012).The three forms of malaria parasites are biologically diverse, resulting in different responses to diagnostic tools, vaccines and pharmaceuticals (WHO, 2012).

One of the leading causes in morbidity and mortality in people leaving in sub-Saharan Africa is malaria however; the gravity of the disease varies from person to person (Legesse *et al*., 2004). Populations living in the same areas affected by malaria may show differences in susceptibility to *Plasmodium* infection and development of acute malaria (Leggese *et al*., 2004). Some of the factors such as age, pregnancy, host genetic factors, immunity, inoculation dose and virulence of the parasite strains have been contemplated to influence the acute phase of the disease (WHO, 2012). The WHO recommended a coherent approach to control and prevent the disease by using insecticide treated bed nets (ITNs) and indoor residual spraying (IRS), chemoprevention and case management of infected people (White *et al*., 2014). All these methods have managed to decrease the occurrence of malaria, morbidity and mortality globally (WHO, 2014). However, because of the rising emergence of drug resistant *Plasmodium* parasites and insecticide resistant mosquitoes the goal towards eliminating and eradicating malaria is being compromised (White *et al*., 2014).

Trichinellosis is a widespread zoonosis, acquired through ingestion of raw or undercooked meat infected with the first stage larvae of the parasite (Soliman *et al.,* 2011). The infected meat contains infective larvae of *Trichinella spp*. (Soliman *et al.,* 2011). Human infections are mostly accidental and caused by *Trichinella spiralis* (Momoh *et al*., 2012). Between 1986 and 2009, an average of 2739 cases and two deaths were reported per year (Murrell and Pozio, 2011). The first known estimate of the global burden of trichinellosis expressed in DALYs was estimated to be 76 per billion persons per year (Devleesschauer *et al*., 2015). The common signs and symptoms of trichinellosis in man include fever, diarrhea, periorbital oedema and myalgia (Dupouy-Camet *et al*., 2007). According to Soliman *et al*. (2011) trichinellosis is regarded as a significant disease globally.

The normal life cycle of *Trichinella* involves humans, pigs, rodents and reptiles. Pigs are infected by ingesting raw or undercooked pork or rats, and humans become infected by ingesting raw or undercooked pork meat (Momoh *et al*., 2012). All stages of development occur in a single host. However there are two hosts required in completing the life cycle, primary host is the pig and the natural hosts are rodents, carnivores and various other species of omnivorous animals (Momoh *et al*., 2012). Man is the accidental host and when infected the adult worms which develop within 48hours will reside in the small intestine (Soliman *et al*., 2011). After fertilization the gravid female discharges the larvae which enters most organs but only persists in the skeletal muscles (Soliman *et al*., 2011). The larvae will be encapsulated (depending on the species) in the muscles (Yadav and Temjenmongla, 2011).

The disease is significantly high in prevalence globally and it is reported that prevalence is increasing in developing and developed countries (Bruschi, 2012). Globally, eight species and four genotypes of the genus *Trichinella* have been recognized (Gottstein *et al*., 2009). Until recently, reports only show that *Trichinella spiralis* was known to be found in sub-Saharan Africa (Mukaratirwa *et al*., 2013). Recent data shows that *Trichinella britovi*, *Trichinella nelsoni*, *Trichinella zimbabwensis* and *Trichinella T8* also occur in sub-Saharan Africa (Mukaratirwa *et al*., 2013). *Trichinella zimbabwensis* is classified as a non-encapsulating *Trichinella* species due to the absence of a collagen capsule surrounding the larvae in host musculature (Mukaratirwa *et al.,* 2013). Experimental studies with *T. zimbabwensis* in mammals and reptiles showed larvae of this parasite species to be larger in poikilothermic hosts than in mammalian hosts and is probably due to the host metabolic rate (Le Grange and Mukaratirwa, 2012). This species is similar to *T. papuae* and has been found only in wild and farmed reptiles of Africa (Zimbabwe, Mozambique, South Africa and Ethiopia) (Pozio *et al*., 2007). Human infections by *T. papuae* are not known so far (Gottstein *et al*., 2009). *Trichinella zimbabwensis* natural infection has only been reported in *Crocodylus niloticus*, *Varanus niloticus* and *Panthera leo* (Mukaratirwa *et al*., 2013). According to Mukaratirwa *et al*., (2012) *Trichinella zimbabwensis* infects caimans, varans, pythons, turtles, rodents and domestic pigs experimentally.

High prevalence of *Trichinella zimbabwensis* infections have been described among a diversity of mammals and reptiles in Southern Africa (Mukaratirwa *et al.,* 2008; 2013). This poses a public health risk of future human epidemic outbreaks of trichinellosis in poor communities who depend on bush meat for protein (Mukaratirwa *et al.,* 2013). *Trichinella zimbabwensis* is a tissue-dwelling nematode that has a complex life cycle in one host. The migrating first stage larvae get localized in the host striated muscles for long periods of time (Mukaratirwa *et al.*, 2008) contrary to the case of soil-transmitted helminths whose larvae settle in the lumen of the gastrointestinal tract.

Human trichinellosis can be treated with various benzimidazole derivatives, such as mebendazole, albendazole and flubendazole (Mukaratirwa *et al*., 2013). However, none of these drugs are believed to be fully effective against the encysted or newborn larvae of *T. spiralis*, because of their low bioavailability (Soliman *et al.,* 2011). Thus, new effective drugs are needed to help prevent and control this important zoonotic disease.

Epidemiological studies and laboratory experiments have been conducted over the last three decades to elucidate the mechanisms and outcomes of parasite-host-parasite and host-parasite interactions in malaria and helminth co-infections but the results obtained do not show a general consensus (Brooker *et al.,* 2007;

Degarege *et al*., 2012). Furthermore, chronic intestinal helminthic infections in humans have also become the subject of hypothesizing and exploration in malarial severity (Legesse *et al*., 2004). In a subset of experiments with *P. berghei* on cerebral malaria models, helminth co-infection significantly reduced death (Knowles, 2011). It is has also been declared that intestinal helminthic infections are related with protection from cerebral malaria, malaria-related acute renal failure and jaundice (Ojurongbe, 2011). The conflicting findings may be attributed to multiple confounding factors like; discrepancy in study designs, parasite polymorphisms, human population diversity and genetic polymorphisms, exposure history, antibody cross reactivity and nutritional status (Brooker *et al.*, 2012). In addition, the majority of the studies have focused on soil-transmitted helminths, schistosomes and filarial worms neglecting the effect of tissue-dwelling helminths and effect of deworming or mass drug administration (MDA) programs that are in place in malaria endemic subtropical and tropical areas. There is a need to develop effective vaccines, sensitive diagnostic tools and identification of drug candidate molecules through understanding host-parasite interactions elicited during co-infections with tissue-dwelling nematodes like *T. zimbabwensis.*

The aim of this study is to determine the anthelmintic and anti-malarial effect of maslinic acid and chloroquine on the co-infection *T. zimbabwensis* and *P. berghei* on Sprague-Dawley rats. The general objective is to determine efficacy of maslinic acid on Sprague-Dawley rats co-infected with *P. berghei* and *T. zimbabwensis* and if there any synergistic effect on the combination maslinic acid and chloroquine on co-infected rats.

Specific objectives of the study:

1. Determine the efficacy of maslinic acid against the co-infection of *Trichinella zimbabwensis* muscle larvae and *Plasmodium berghei* parasitaemia in laboratory rats.
2. Determine the efficacy of chloroquine against the co-infection of *Trichinella zimbabwensis* muscle larvae and *Plasmodium berghei* parasitaemia in laboratory rats.
3. Determine the efficacy of the combination of maslinic acid and chloroquine against the co-infection of *Trichinella zimbabwensis* muscle larvae and *Plasmodium berghei* parasitaemia in laboratory rats.

Chapter 2

Literature review

2.1 Introduction

Malaria poses a large health burden and Africa has more than 70% of the global clinical cases caused by *Plasmodium* (WHO, 2014). It is still one of the most significant infection diseases causing over 655 000 deaths per year, of which the majority of those deaths are children under 5 years of age (Sauerwein *et al*., 2012). There has been a decrease of more than 50% of either confirmed malaria cases or morbidity and mortality cases in eleven countries of the WHO African region through intense malaria control regions (WHO, 2011a). However, increase of malaria cases in 2009 in Rwanda, Sao Tome and Principe and Zambia has shown the weakness of the current successes (Sauerwein *et al*., 2012). This emphasizes the importance for additional and inventive strategies to control malaria (Sauerwein *et al*., 2012).

Plasmodium falciparum, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi* parasites infect humans under normal conditions (WHO, 2012) with *P. falciparum* and *P. vivax* being the major species that cause morbidity and mortality in children under five years of age, pregnant women and travelers from non-malarious areas (Warimwe *et al.,* 2013). *P. berghei* is a strain that affects rodents and mostly used as model for malaria experiments. In sub-Saharan Africa, morbidity and mortality due to malaria is decreasing despite a lack of a malaria vaccine, emergence of parasite resistance to available anti-malarial drugs, the female anopheles mosquito being resistant to IRS and a poor socio-economic situation that hinders malaria control and management (WHO, 2012). New drug discovery and vaccine development are delayed by limited knowledge of the underlying cellular and molecular mechanisms of host-parasite interactions during co-infection and polyparasitism (Good and Doolan, 2010; Nossal, 2011). This is also worsened by the emergence of zoonotic *P. knowlesi* malaria infections (Cox-Singh, 2012; Fan *et al*., 2013) as well as other zoonotic infectious diseases.

Trichinellosis is an emerging and re-emerging zoonotic disease the geographical distribution of which overlaps with malaria in endemic areas of Tanzania, Uganda, Kenya, Ethiopia, Zimbabwe, South Africa and Mozambique (Gottstein *et al.,* 2009; Le Grange *et al.,* 2012, 2010, 2009; Mukaratirwa *et al.,* 2013; Murrell and Pozio, 2011). More recent studies have reported on more species other than the most commonly known, *Trichinella spiralis* (Pozio, 2013). The genus *Trichinella* now encompasses eight species (Pozio, 2013). The twelve identified taxa are genetically and biologically delineated by two clades distinguished by encapsulated and non-encapsulated larvae in the host muscle tissue (Zarlenga *et al*., 2006). The encapsulated species are found in mammals only and the non-encapsulated species are found

in mammals, birds (one species) and some reptiles (Pozio and Murell, 2011). *T. spiralis* is the most common species to domestic and wild pigs but it can also involve synathropic rats in its life cycle (Gottstein *et al*., 2009). *T. spiralis* has a wide vast and global distribution and also is the important causative agent of human trichinellosis (Pozio and Murell, 2011).

Until recently, all *Trichinella* infections found in animals and humans in SSA were regarded to be caused by *T. spiralis* (Mukaratirwa *et al*., 2012). The existing data shows that *T. britovi*, *T. nelsoni*, *T. zimbabwensis* and T8 are found in SSA (Mukaratirwa *et al.,* 2012). Regardless of being reported as found globally, *T. spiralis* has not yet been reported in SSA (Mukaratirwa *et al*., 2012). *Trichinella nelsoni* is only found on the Eastern and Southern part of the sub-Saharan region (Mukaratirwa *et al*., 2012); reported in Kenya, South Africa and Tanzania (Marucci *et al*., 2008). *Trichinella* T8 has been described to occur in Southern African countries (Marucci *et al*., 2008). The presence of this species in South Africa and Namibia has been accredited to possible introduction from Europe but research in Guinea and the successive re-evaluation of the distribution of encapsulated *Trichinella* spp. and specifically *T. britovi* suggests a more natural explanation (Marucci *et al.,* 2009). Reports earlier describing encapsulated *Trichinella* spp. larvae in South Africa (Young and Kruger, 1967; Young and Whyte, 1975) could possibly have been *T. nelsoni* or *Trichinella* T8 now known to be found in that country (Marucci *et al*., 2009). Earlier investigations of *Trichinella* spp. in Kenya and Tanzania (Forrester *et al*., 1961; Forrester, 1964; Nelson, 1970) could possibly have been *T. nelsoni* and *T. zimbabwensis* which circulate in Southern and Eastern areas of SSA and have so far been found in Zimbabwe, South Africa, Mozambique and Ethiopia (La Grange *et al*., 2009, 2010, 2012).

Many drugs have been used to treat trichinellosis, however there is lack of an effective drug against tissue-dwelling nematodes due to increasing drug resistance. The development of vaccines against parasitic infections has been complicated due to the fact that co-infecting parasites have life cycles that are either direct or complex. Direct life cycles involve cycling of mature parasites from one definitive host to another while complex life cycles involve cycling of distinct developing life stages through a number of intermediate hosts (Jackson *et al.,* 2009).

2.2 Co-infection of malaria and tissue-dwelling nematodes in sub-Saharan Africa

Epidemiological studies have shown that the largest burden of malaria infections is felt by communities living in poor regions of developing countries *(*Boraschi *et al.,* 2008; Brooker *et al.,* 2007; Shankarkumar *et al.,* 2011). In these areas, the wide spread of soil-transmitted helminthic infections have also been documented (Mwangi *et al.,* 2006). This results in co-infections, multi-parasitism or polyparasitism (Supali *et al.,* 2010). In the past three decades, many studies have been undertaken to determine the nature of interaction that occurs between soil-transmitted helminths (STHs) and malaria during co-infection scenarios. The studies have mainly focused on immunological aspects and disease outcomes neglecting non-immunological mechanisms that may explain the heterogeneity observed in these studies (Fenton *et al.,* 2014; Knowles, 2011). Varying conclusions have been made from both epidemiological studies and laboratory experiments. Some studies have established that helminths may confer protection against cerebral malaria, others indicate that helminths exacerbate malaria, others report a reduction or increase in prevalence and transmission of malaria, while a few others report no association between the parasites (Bejon *et al.,* 2008; Nacher, 2011; Roussillon *et al.,* 2010).

The lack of general consensus in the studies is evidence that malaria immunity is not well understood. However, it is argued that STHs influence clinical malaria disease presentation or confer malaria tolerance through the establishment of chronic infections, induction of adaptive immunity and immunosuppression of immune responses to unrelated antigens and parasites (Taylor *et al.,* 2012). These result in an induction of host regulatory immunity, and signaling and effector mechanisms *(*Ashour, 2013*;* Brindley *et al.,* 2009; Maizels *et al.,* 2009) that are beneficial to co-infecting parasites. This is mainly due to host's failure to regulate the immune responses induced by the parasites. During co-infections, one parasite does not have direct influence on disease outcome and establishment of another parasite, however, the concept of parasite-host-parasite interactions plays a key role. One parasite influences the host to induce immune responses that will favor its establishment which in the long run, become beneficial to the co-infecting parasite. This immunological phenomenon is parasite-driven to make the host susceptible to infection and not favor the establishment of the co-infecting parasite. The amelioration or exacerbation of the disease outcome of the co-infecting parasites is a spill-over effect.

In the majority of co-infection studies, tissue-dwelling parasites, prevalent in sub-Saharan Africa, have not been adequately considered. The hypothetical arguments presented are sketchy, making it difficult to clearly predict disease outcomes during malaria interaction with tissue-dwelling parasites. Therefore there is little that has been done on studies on the co-infection of malaria and *Trichinella*.

There are few studies that are directed towards elucidating the host-parasite interactions and disease outcomes that are caused by tissue-dwelling parasites during co-infection with malaria. This has created a glaring paucity of data on understanding the mechanisms and outcomes of tissue-dwelling parasites and mono-and co-infection with malaria. This has also hampered diagnosis, vaccine development, drug discovery, and management and control of these emerging and re-emerging parasites.

2.3 Epidemiology of co-infection of malaria and trichinellosis

Sequences of single parasite species infection of helminth or *P. falciparum* have been well reported during classical epidemiology (Brooker *et al*., 2007). Both types of parasites show clearly that age is dependent on infection sequences (Brooker *et al*., 2007). Epidemiological studies based on age have shown that that the prevalence of asymptomatic *Plasmodium* infections increases in early childhood, and starts to decrease with the moderate acquired immunity (Brooker *et al*., 2007). The rate at which this immunity is acquired is relative to exposure, but in areas of low transmission infections in adulthood are generally low (Brooker *et al*., 2007).

Helminths are widely distributed, with most people having little or no worms and the majority of the worm population found in a minority of individuals (Knowles, 2011); it is within the minority group that most relative incidence occurs. The same patterns of wide distribution are also seen for *P. falciparum* infections (Brooker *et al*., 2007). Earlier modeling of the prevalence of the parasite and its reproduction rate of infection implies that 20% of children in a community get 80% of new infections in a community (Smith *et al*., 2005). Both malaria and helminth infections have a likelihood to occur within certain families and households (Knowles, 2011).

Currently, human trichinellosis has been reported in only four countries (8.7%, 4/46) of SSA; Ethiopia, Kenya, Senegal and Tanzania (Mukaratirwa *et al*., 2012). Four species reported in SSA are *T. britovi*, *T. nelsoni*, *T.* T8 and *T. zimbabwensis* (Mukaratirwa *et al*., 2012). Ethnic minorities and tourists are the ones mainly reported to have human trichinellosis, with less than 100 reported human infections in the region (Pozio, 2007). The low prevalence of human trichinellosis in the region has been ascribed to the consumption of well-cooked meats and religious beliefs prohibiting the consumption of pork (Pozio *et al*., 2013. The causative agents of human trichinellosis in sub-Saharan Africa have not yet been recognized to the species or genotype level (Mukaratirwa *et al*., 2013). The zoonotic potential of both *Trichinella* T8 and *T. zimbabwensis* is currently unknown (Mukaratirwa *et al*., 2013). However, *T. zimbabwensis* was displayed to infect non-human primates and the clinical signs described were similar to those described in human trichinellosis due to the non-encapsulated species, *T. pseudospiralis* (Mukaratirwa *et al*., 2008).

At present, there are no studies on the epidemiological details of the co-infection of malaria and trichinellosis. However, what is currently known about the epidemiology of co-infection, it is proposed that school-going children, rather than pre-school children or adults, are most at-risk of *Plasmodium*-helminth co-infection, and to bear greatest risk of the consequences of co-infection (Knowles, 2011).

2.4 Conventional drugs for malaria

The resistance of drugs to malaria has become an important issue in malaria control. Drug resistance *in vivo* has been reported against almost all antimalarial drugs currently used for malaria control (Valecha *et al*., 2016). *P. falciparum* and *P. vivax* have been reported to be resistant against antimalarial drugs (Valecha *et al*., 2016). *P. falciparum* is not only resistant to chloroquine alone, but also to the other currently used antimalarial drugs and is widespread (Valecha *et al*., 2016).

2.4.1 Chloroquine

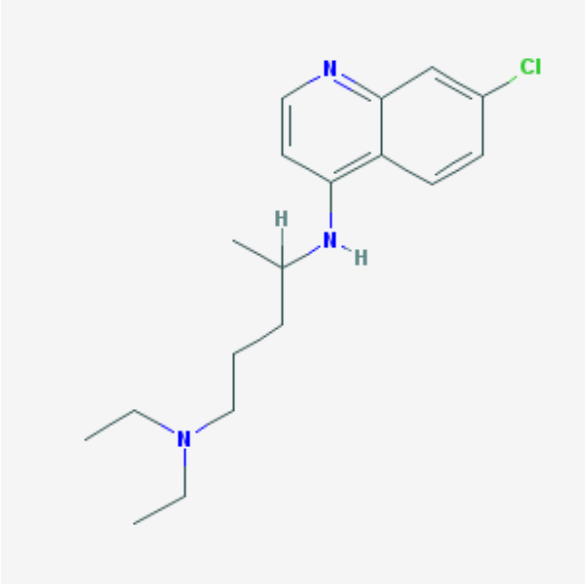

Figure 1. Chemical structure of chloroquine.

Chloroquine-resistant *P vivax* was first reported in 1989, almost 30 years after chloroquine-resistant *P falciparum* was first noted (Baird *et al.*, 1991). The absence of reliable, robust, sensitive methods for detection, mapping, and monitoring of antimalarial drug efficacy in *P vivax* has almost certainly contributed to the delayed recognition of this emerging problem (Price *et al*., 2011). This delay has had important public health implications. The resistance of *P. falciparum* malaria to chloroquine has been

described to be widespread (The Lancet, 2014). The first cases of *P. falciparum* resistance to chloroquine were reported in Southeast Asia and South America (Colombia) in late 1950s (Spencer, 1985; Young and Moore, 1961; Wernsdorfer and Payne, 1991). Since then chloroquine resistance has spread and is now found in all parts of the world where malaria is endemic (The Lancet, 2014). The first case of chloroquine resistance in Africa was first reported in the eastern part in 1978 (Wernsdorfer and Payne, 1991; Peters, 1987), which then spread to the central and southern parts before being described in West Africa in 1983 (Peters, 1987; Pickard and Wernsdorfer, 2002). By 1989 chloroquine resistance was already widely distributed in sub-Saharan Africa (Peters, 1987). The seriousness of resistance was more in east Africa than in west and central Africa, but even in West Africa, the potency of resistance varied from a progressed stage with severe effects on morbidity and mortality in focal areas of Senegal to a moderate degree in Ghana, Cameroon and low level in Mali (The Lancet, 2014).

2.4.2. Quinine

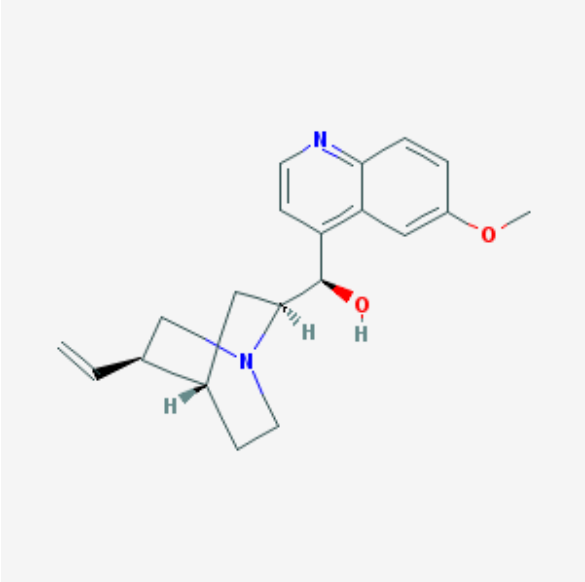

Figure 2. Chemical structure of quinine.

The initial case to quinine resistance was reported about a century in South America (Farooq and Mahajan, 2004). It was reported from the Thai-Cambodian border in mid 1960s (Pickard and Wernsdorfer, 2002). Quinine is now used to treat severe cases of malaria and, as a second line treatment, in combination with antibiotics to treat resistant malaria. Its short half-life of 8– 10 h likely contributed to the scarcity of widespread quinine resistance (Petersen, 2011). The molecular mechanism by which quinine acts against *P. falciparum* is only partially understood. Similar to chloroquine, quinine has been demonstrated to accumulate in the parasite's digestive vacuole (DV) and can inhibit the detoxification of

heme, an essential process within the parasite (Petersen, 2011). Recent studies show that the genetic basis for resistance to quinine is complex, with multiple genes influencing susceptibility (Petersen, 2011). Quinine has since been used in combination with tetracycline or doxycycline to improve its effectiveness (Farooq and Mahajan, 2004).

2.4.3. Mefloquine

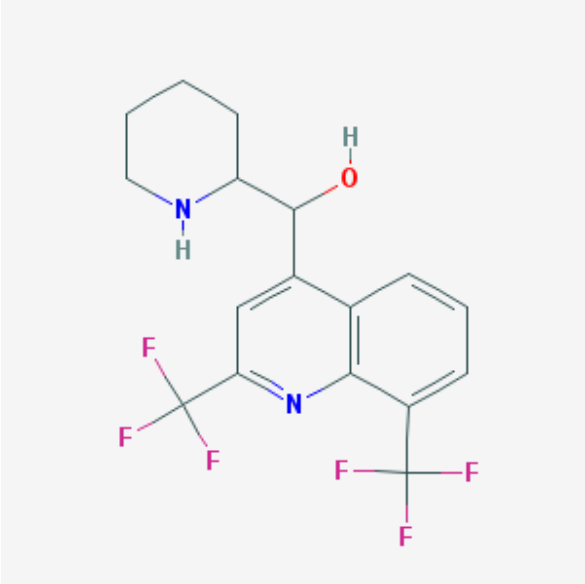

Figure 3. Chemical structure of mefloquine.

Mefloquine resistance was initially described in late 1980s near the Thai-Cambodian border (Shanks, 1994). Resistance to mefloquine is mediated by amplification of pfmdr1, leading to overexpression of this resident DV membrane transporter (Petersen *et al*, 2011). Although the exact mechanism of action remains unclear, in vitro experiments demonstrate that mefloquine can bind to heme and exert some antimalarial activity by inhibiting heme detoxification (Petersen *et al*, 2011). However, studies on transgenic parasites expressing different pfmdr1 copy numbers, observed a reduced parasite susceptibility to mefloquine with increased PfMDR1-mediated import into the DV (Petersen *et al*., 2011), suggesting a primary mode of action outside of the DV (Petersen *et al*, 2011). Additionally, it has been shown that mefloquine inhibits the import of other solutes into the DV and might therefore also target the PfMDR1 transport function itself (Petersen *et al*., 2011).

2.4.4 Artemisinin

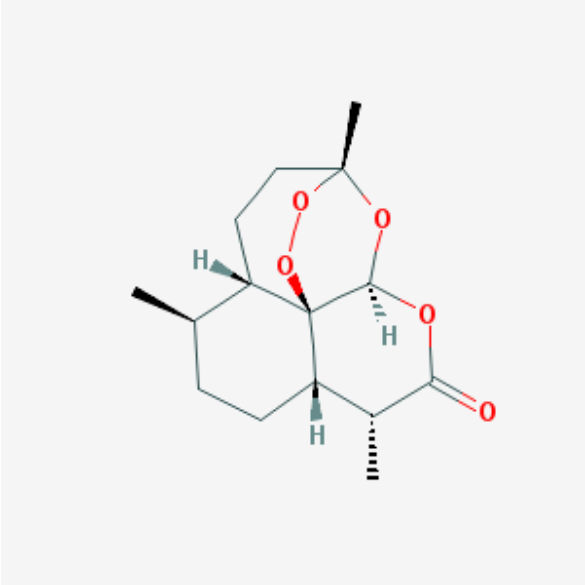

Figure 4. Chemical structure of artemisinin.

Artemisinin and its derivatives were most efficacious antimalarial drug (Farooq and Mahajan, 2004). These drugs exert influence on the protein synthesis of the parasite (Farooq and Mahajan, 2004). In recent years, parasite resistance to artemisinin has been detected in five countries of the Greater Mekong sub region: Cambodia, the Lao People's Democratic Republic, Myanmar, Thailand and Viet Nam. In areas along the Cambodia–Thailand border, *P. falciparum* has become resistant to most available antimalarial medicines, and multi-drug resistance is a major concern (WHO, 2014).

2.5 The emergence of chloroquine resistance

Chloroquine has been the most researched drug but its method of action still remains to be explained (Farooq and Mahajan, 2004). The method of the antimalarial action of quinolone containing drugs such as chloroquine has been researched by many workers and many therapeutic targets have been proposed (Farooq and Mahajan, 2004). The acid food vacuole of the parasite has most of the drug targets (Petersen *et al.*, 2011). The resistance of *P. falciparum* to chloroquine is suspected to be caused by the increased capacity for the parasite to remove chloroquine at a rate that does not allow chloroquine to reach levels required for inhibition of heamepolymerization (Petersen *et al.*, 2011). This chloroquine efflux occurs at a rate that is 40 to 50 fold faster among resistant parasites than that in sensitive ones(Petersen *et al.*, 2011). Further, proof which supports this mechanism is given by the fact that chloroquine resistance would be altered by drugs which interfere with this efflux system (Petersen *et al.*, 2011) but the biochemical basis of this efflux is lacking of discussion. The efflux of chloroquine and in fact the entire chloroquine

resistant phenotype can be altered with Ca+ channel blocker, such as verapamil and diltiazem (Petersen *et al*., 2011).

2.6 Conventional drugs for trichinellosis

There is no effective vaccine for controlling trichinellosis diseases; therefore the treatment and prophylaxis is based on anthelmintic drugs (Taman and Azab, 2014). These drugs are aimed at eliminating helminthes from the host body through affecting different targets with various mechanisms (Taman and Azab, 2014). Drugs administered for trichinellosis include anthelmintics, glucocorticosteroids and preparations that compensate for protein and electrolyte deficits (Gottstein *et al*., 2009). Anthelmintics are the primary drugs for treatment of trichinellosis (Gottstein *et al*., 2009). They include primarily albendazole (Zentel; Smith-KlineBeecham) and mebendazole (Vermox, Jansen pharmaceutical, and Beerse, Belgium) (Gottstein *et al.,* 2009). Albendazole is one of the most commonly used drugs for trichinellosis (Taman and Azab, 2014). It acts on all developmental forms of the alimentary tract of the parasite (Taman and Azab, 2014). Mebendazole is also used to treat trichinellosis, though is poorly absorbed from the intestinal lumen, but it is well tolerated (Taman and Azab, 2014). Both albendazole and mebendazole are contraindicated during pregnancy and not recommended for children aged less than 2 years old (Taman and Azab, 2014). Pyrantel (Combantrin) paralyzes parasite muscles by blocking their nerve endings. It is not absorbed from the intestinal lumen (Taman and Azab, 2014). It interferes with neurotransmission by inhibiting acetylcholine esterase enzyme in helminthes and induces persistent activation of nAChR, resulting in worm irreversible spastic paralysis (Taman and Azab, 2014). More than 50% of the drug is excreted in stools in the form of metabolites and around 7% is excreted in urine, in original form or in the form of metabolites (Taman and Azab, 2014). A study done by Mukaratirwa *et al*. (2014), showed that fenbendazole and levamisole are effective against *Trichinella zimbabwensis* even though the parasite was not completely cleared from the host.

The administration of anthelmintics at the intestinal stage invasion with the aim of removing intestinal forms of *Trichinella* sp. from the lumen of the gastrointestinal tract is of significance importance for an early and effective therapy, especially in the first 3 days following the infection. If administered early, such intervention prevents successive muscular invasion and development of disease. Efficacy of these anthelmintics at various stages of trichinellosis in humans continues to be the topic of many studies and discussions because of increasing resistance of the parasite to the drugs (Taman and Azab, 2014). There is no known effective treatment for trichinellosis especially drugs targeting all three stages and as a result more studies targeting new and effective drugs against *Trichinella* species are needed.

2.7 Potential drug for malaria and trichinellosis

2.7.1 Maslinic acid

Maslinic acid (MA) is a natural olenane-type pentacycle triterpene found in the olive fruit and from acquired from olive pomace oil (Gradia- Granados *et al*., 1998). Reports have shown that this compound has activities such as anti-tumor, anti- oxidant, HIV protease inhibitor, antimicrobial, vasorelaxation and anti- diabetic action (Moneriz *et al*., 2011). It has been reported to show low toxicity in anti-tumor cells, an indication that it is safe to use for humans (Juan *et al*., 2008; De Pablos *et al*., 2010). Several reports have shown that MA has an anti-malarial activity (Carlos *et al*., 2011). Maslinic acid has also been shown to inhibit growth in *Toxoplasma gondii* cultures (Moneriz *et a*l., 2011) and of recent anthelmintic activity against the muscle larval stage of *Trichinella zimbabwensis* in Sprague-Dawley rats (Mukaratirwa *et al*., 2014).

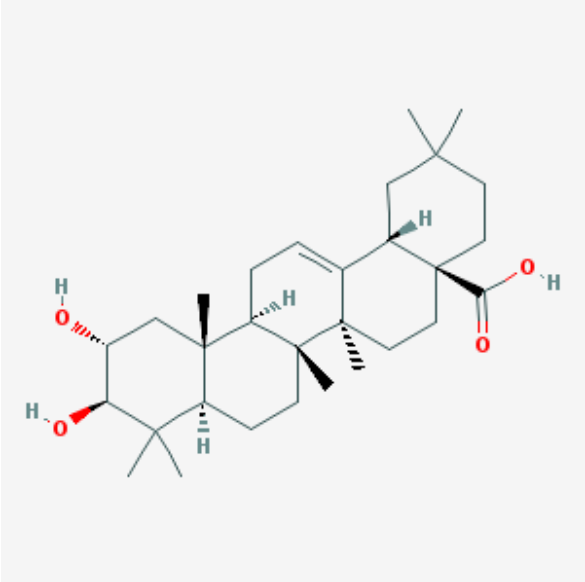

Figure 5. Chemical structure of maslinic acid.

Plants with medicinal properties, such as *Cornus kousa*, *Junillia aspera*, *Malus pumila*, and *Ulmus pumila* are known to have low concentrations of maslinic acid (Lozano-Mena *et al*., 2014), the waxy skin of *Olea europaea* L. is reported to have a concentration as high as 80% (Lozano-Mena *et al*., 2014). In previous decades, there has been growing interest in developing new natural products which would be beneficial on health. As a result maslinic acid has been extracted in various plants used in traditional herbal medicine, also in vegetables and fruits (Lozano-Mena *et al*., 2014).Similarly, the biological activities of maslinic acid have been evaluated in various experimental models, from tumor cell lines to animal models of several diseases, supported by the

lack of adverse effects *in vivo* after the oral administration of the triterpene (Sanchez-Gonzalez *et al*., 2013). In summary, maslinic acid is rising as a new natural and safe product with diverse biological goals, which might develop to considering it as a potential drug in the future.

2.7.2. Biosynthesis and role as phytoalexin

Triterpenoids, such as maslinic acid, are a group of secondary metabolites resulting from the cyclation of squalene, oxidosqualene or bis-oxidosqualene (Xu *et al*., 2004). These precursors (C30) are substrate of several types of triterpene synthases, which catalyze their cyclation through intermediate cations to a wide variety of triterpenes. Depending on the number of rings, the latter are classified as mono-, bi-, tri-, tetra- or pentacyclic triterpene alcohols (Xu *et al*., 2004). Lupeol, α- and β-amyrin are examples of pentacyclic triterpene alcohols, which not only constitute secondary metabolites themselves, but also might undergo oxidation reactions to yield other derivatives, such as betulinic, ursolic and maslinic acids (Xu *et al*., 2004).

Not long after *Crataegus oxyacantha* L. was identified, Tschesche *et al.* (1959) recognized maslinic acid as a derivative of the β-amyrin series, and then Stiti *et al.* (2007) suggested the biosynthetic pathway that leads to the formation of maslinic acid in the fruits of *Olea europaea* L., one of the main natural sources of this triterpene. Stiti *et al*. (2007) suggest that in the developing olive both the sterols (primary metabolites) and the non-steroidal triterpenoids (secondary metabolites) share oxidosqualene as a common precursor. The enzyme β-amyrin synthase catalyzes its cyclation into β-amyrin, and further oxidation steps give rise to the triterpenic dialcohol erythrodiol followed by the hydroxyl pentacyclic triterpenic acids oleanolic and maslinic (Stiti *et al*., 2007).

2.7.3. Natural sources

MA was initially identified in *Crataegus oxyacantha* L., but the rising attention in this triterpene because of its extensive range of health-boosting activities has led to its documentation in other natural sources, being existent in more than 30 plants globally (Lozano-Mena *et al*., 2014). On one hand, the triterpene has been found in plants used in traditional Asian medicine for the treatment of diverse affections. To reference only a few examples, the leaves of loquat (*Eriobotrya japonica*) (Lu *et al.,* 2009), which have been used as antitussive and anti-inflammatory for chronic bronchitis, and also as diuretic, digestive and antipyretic (Banno *et al.,* 2009); the flowers of *Campsis grandiflora*, employed for female disorders like uterine hemorrhage (Kim *et al*., 2005); the whole plant of *Geum japonicum* used as diuretic; and *Agastache rugosa*, for the treatment of anorexia, vomiting and other intestinal disorders (Shin and Khang,

2003). On the other hand, maslinic acid has newly been measured in edible vegetables, such as table olives, spinach and eggplant, aromatic herbs like mustard and basil (Lin *et al*., 2011; Yin *et al*., 2012), legumes such as chickpeas and lentils (Kologeropoulos *et al*., 2010), and to a lesser extent in some fruits like mandarin and pomegranate (Li *et al*., 2011). Therefore, plant-based diets might offer a constant supply of maslinic acid, which could be considered, among many other factors, partially responsible of the health-enhancing properties of these dietary habits.

2.7.4. Antimalarial and anthelmintic properties of maslinic acid

Xu et al. (1996) first published the reports that showed the biological activities of maslinic acid, and defined the anti-HIV properties of several triterpenic acids isolated from the methanolic extract of *Geum japonicum*. Although the study did not offer mechanistic details of the inhibitory effect on HIV-1 protease, it is clearly specified that maslinic acid was the most effective compound (Xu *et al*., 1996). Although neither the antiviral nor the antibacterial activities of maslinic acid have been further studied thoroughly, the protective effect of the triterpene against parasitic infections has given rise to much interest in recent years.

De Pablos *et al.* (2010) showed that maslinic acid inhibited the entrance of *Toxoplasma gondii* into Vero cells in a dose-dependent manner, with IC50 of 8 μM at 48 hours of treatment. The fundamental mechanism appeared to be the inhibitory activity of the triterpene against proteases secreted by the parasite, which are important for the proteolytic processing of other proteins that contribute in the invasion of host cells. The gliding motility was also blocked by up to 100% by maslinic acid (50 μM) (De Pablo *et al*., 2010). Furthermore, the triterpene induced morphological alterations in the endomembrane systems of the parasite, such as a greater amount of apparently empty spaces that De Pablo *et al*. (2010) attribute to a possible collapse of the Golgi apparatus. Disturbances in external and nuclear membranes were also shown and ascribed to a general obstruction of protein turnover, which would delay the functionality of those proteins necessary for the structural maintenance of the membranes. De Pablo *et al*. (2010) also presented the anti-parasitic effect of maslinic acid in *Gallus domesticus* chicks infected with *Eimeria tenella*. The animals were fed a maslinic-acid supplemented diet (90 ppm) for 21 days, and this treatment caused a reduction in the release of oocysts in the faeces by 80.1%, being more effective than the positive control with sodium salinomycin (60 ppm). Histological evaluation of the caeca exposed that the characteristic lesions of this coccidiosis were less evident in the animals that had received maslinic acid. Moreover, the body weight gain was significantly higher in treated animals compared to the positive control and the uninfected group, showing that besides the anticoccidial activity, the triterpene improved weight gain (De Pablo *et al*., 2010).

Maslinic acid has also been reported to be effective against different species of the genus *Plasmodium*, responsible of causing malaria (Moneriz *et al*., 2011). *In vitro* experiments using erythrocytes infected with *Plasmodium falciparum* established that maslinic acid (0.1−200 μM) inhibited the growth of the parasite dose-dependently (Moneriz *et al*., 2011). At a concentration of 30 μM (close to the IC50), parasitaemia was reduced to 4% by maslinic acid (compared to 8% in untreated red blood cells) and reduced the cell cycle, since only the infective (schizonts) and immature (new rings) forms, but not the mature forms (trophozoites), were observed in the erythrocytes. However, the exclusion of maslinic acid from the medium allowed the infection to recommence, meaning that the triterpene acts as a parasitostatic agent (Moneriz *et al*., 2011).

This effect was further recognized *in vivo* with ICR mice infected with the lethal strain of *Plasmodium yoelii* (Moneriz *et al*., 2011). The intraperitoneal injection of 40 mg/kg for 4 days increased the survival rate of the animals to 80%, compared to 20% found in animals without any experimental intervention, and this was related with a seizure of the maturation of the parasite in the erythrocytes. In addition, the animals that survived the primary infection were re-infected with an identical second infection 40 days later. Parasitaemia was observed for the following 30 days but no parasites were identified, signifying that mice were completely protected against the parasite (Moneriz *et al.,* 2011). Further research on the mechanism of action underlying the antimalarial activity of maslinic acid reported that the compound hinders the growth of the parasite inside the erythrocytes by hindering various proteins (Moneriz *et al*., 2011).

Anthelmintics targeting the intestinal adults and muscle-dwelling larvae of *Trichinella* spp. have been tried, with limited success. Mukaratirwa *et al*. (2014) aimed at determining the efficacy of maslinic acid and fenbendazole on muscle larvae of *Trichinella zimbabwensis* in laboratory rats. Sprague–Dawley rats, with an average weight of 270 g and 180 g for males and females respectively, were infected with *T. zimbabwensis* larvae. Infected rats were randomly allocated to three groups which were exposed to single treatments with each of maslinic acid, fenbendazole and a combination of both on day 25 post-infection (pi), and three groups which were exposed to double treatments with each of these drugs and a combination on days 25 and 32 pi. The untreated control group received a placebo. In single-treatment groups, the efficacy of each treatment, measured by rate of reduction in muscle larvae, was significant ($P < 0.001$) for both drugs compared to the untreated control group (Mukaratirwa *et al*., 2014). There was no apparent synergistic effect on the combination of the two drugs in reducing the muscle larval burden, either in single or double treatments (Mukaratirwa *et al*., 2014). In all the treatment regimens, the

reductions were significant ($P < 0.001$) when compared to the untreated control and not significant when the single treatments were compared with the double treatments ($P > 0.05$) (Mukaratirwa *et al*., 2014). Mukaratirwa *et al.* (2014) concluded that the efficacy of maslinic acid against larval stages of *T. zimbabwensis* in rats was similar to that of fenbendazole, with no side-effects observed, making maslinic acid a capable anthelmintic against larval stages of *Trichinella* species.

To sum up, numerous lines of data show maslinic acid as an antiparasitic and/or anthelmintic agent. More research is necessary in order to confirm its efficacy in target species, which would permit the use of maslinic acid either alone or in combination with other therapeutic strategies for the treatment of parasitoses. Maslinic acid has shown that is has potential antimalarial and anthelmintic properties, which is why our main goal on this study is to determine if it is effective on the co-infection of malaria and tissue-dwelling nematode.

2.12 Conclusion

There are few studies that focus on host-parasite interaction and disease outcomes that are elicited by tissue-dwelling parasites during co-infection with malaria. This hinders data on understanding the mechanisms and outcomes of tissue-dwelling nematodes and co-infection with malaria. This lack of data makes it hard to develop diagnosis, vaccine development, drug discovery and management and control of these emerging and re-emerging parasites. Therefore, more studies are significant in addressing the lack of data and heterogeneity of results reported during STHs, schistomes and filarial worm co-infection with malaria. These future studies should be focused on developing new drug strategies designed at controlling the co-infection f tissue-dwelling helminths and protozoa especially in sub-Saharan Africa. The use of the future different study designs and approaches, as well as different tissue-dwelling helminths and protozoa will provide very important information that can be used in humans. The data from this study will useful in providing a new potential drug that has an effect on the co-infection tissue-dwelling helminths and malaria.

Chapter 3

Materials and methods

3.1 Ethical statement

All animal procedures and protocols were approved by University of KwaZulu-Natal (UKZN) Animal Ethics committee (ref. no. 064/14/Animal) in accordance with South African National Legislation Regulations in respect to animal husbandry, experimentation and welfare of laboratory animals for biomedical research.

3.2 Experimental animals

Fifty-four Sprague-Dawley male and female rats with an average weight of 150 and 200g were sourced from University of KwaZulu-Natal, South Africa. The rats were maintained at the Biomedical Resources Unit (BRU) of UKZN, Westville campus, under specific pathogen free conditions in individually ventilated metabolic cages. They were randomly assigned into nine groups with males and females kept separate, and fed daily heat sterilized pelleted food (Meadow Feeds RSA) and clean water was supplied *ad libitum.*

3.3 Parasite strains

Plasmodium berghei ANKA strain which was kindly donated by the University of Cape Town malaria research group was used to induce malaria infections in rats. Stock rats were sacrificed and blood was collected by cardiac puncture. The blood was collected into EDTA tubes and spun at maximum speed for 5 minutes. Serum was separated from the red blood cells which was then mixed with freeze mix for storage at a -80ºC freezer for further use.

Trichinella zimbabwensis (Code ISS1209) was isolated from stock rats previously infected and maintained at the BRU (UKZN) by repeated passages in rats. Carcasses of rats were digested with tissue digestive fluid according to the method described by Pozio *et al.* (2002) and the dose of larvae administered orally to each experimental animal was 3 larvae per gram of animal body weight administered as described by Mukaratirwa *et al.* (2001).

3.4 Experimental design

The study design is shown in Table 1. Rats were randomly assigned into nine equal groups six rats each; (A) Co-infected and treated with maslinic acid, (B) Co-infected and treated with chloroquine, (C) Co-

infected and treated with maslinic acid and chloroquine, (D) malaria infected and treated with chloroquine, (E) malaria infected and treated with maslinic acid, (F) malaria infected and given placebo (distilled water) (control), (G) *Trichinella*-infected and treated with maslinic acid, (H) *Trichinella*-infected and treated with chloroquine, and (I) *Trichinella*-infected and given placebo (control). At day 0 post infection (pi), rats in groups A, B, C, G, H and I were infected with 3 muscle larvae per gram of animal body weight through oral dose as described by Mukaratirwa *et al*. (2001). At day 25 pi, rats in groups G and H were treated with maslinic acid at 2.5mg/kg body weight and chloroquine at 100mg/kg body weight, respectively. At day 0, rats in groups D, E and F and at day 30 pi, rats in groups A, B and C were inoculated with 1 x 10^5 infected red blood cells (iRBC) *P. berghei* parasites according to procedures described by Wang *et al*. (2014).

Table 1. Summary of experimental design on the efficacy of maslinic acid and chloroquine on the co-infection of *Plasmodium berghei* and *Trichinella zimbabwensis* in rats.

Group	Day of infection		Parasite		Treatment day(s)	Drug		Day of sacrifice
	T. zimbabwensis	*P. berghei*	*T. zimbabwensis*	*P. berghei*		Maslinic acid	Chloroquine	
A	0	30	+	+	9-12	+	-	42
B	0	30	+	+	9-12	-	+	42
C	0	30	+	+	9-12	+	+	42
D		0	-	+	9-12	-	+	13
E		0	-	+	9-12	+	-	13
F		0	-	+	-	-	-	13
G	0		+	-	25	+	-	42
H	0		+	-	25	-	+	42
I	0		+	-	-	-	-	42

3.5 Determination *P. berghei* parasitaemia and *T. zimbabwensis* muscle larvae

Rats infected with *P. berghei* were monitored daily for parasitaemia by Giemsa-stained thin blood smears prepared using tail blood starting from day 4 pi until day 13 pi. The number of malaria parasites per µL of whole rat blood was derived from counting of iRBCs in at least 10 high power fields. Agility, demeanor, appetite, posture and fur scores were used to determine experimental humane end points according to UKZN animal experimentation standard operating procedures. Muscle larvae in rats infected with *T.*

zimbabwensis were determined from whole carcasses of rats at the endpoint of the experiment by the standard HCl-pepsin digestion method (Pozio *et al*., 2002).

3.6 Drug efficacy determination

Treatment schedules of rats infected with maslinic acid and chloroquine are shown on Table 1. Starting on day 9 till day 12 post malaria-infection, rats in groups A and E were treated with maslinic acid at 2.5mg/kg body weight administered orally. Starting from day 9 till day 12 post malaria-infection, rats in groups B and D were treated with chloroquine at 100mg/kg body weight administered orally. Starting from day 9 till day 12, rats in group C were treated with a combination of maslinic acid and chloroquine at 2.5mg/kg and 100mg/kg body weight administered orally. At day 25 post *Trichinella*-infection, rats in groups G and H were treated with maslinic acid and chloroquine at 2.5 mg/kg and 100mg/kg body weight administered orally, respectively.

3.7 Termination of animals

On day 13 post treatment all *P. berghei* and co-infected rats were sacrificed using halothane inhalation in line with ethical guideline, including *P. berghei* control group. All rats infected with *T. zimbabwensis* only were sacrificed 42 days post infection, including *T. zimbabwensis* control group. Carcasses of rats infected with *Trichinella* were digested with tissue digestive fluid according to the method described by Pozio *et al.* (2002) for the recovery of larvae in the muscle.

3.8 Data analysis

General Linear Models Procedure was implemented in the SPSS version 23 program (2015) to assess the efficacy of the drug regimens against the control using the log transformed mean larval counts and parasitaemia mean. Multiple pairwise Bonferroni was used to assess the efficacy between the treatment regimens. Graphs were constructed on Graph Prism version 6 (2015). Efficacy rates for each drug and combination were calculated as a percentage of recovered larvae versus those recovered from the control rats for *Trichinella* and for *P. berghei* it was calculated as a percentage of parasitaemia at day 13 post treatment (pt) versus parasitaemia at day 9 post infection for malaria (De la Rossa *et al*., 2007). A Regression Linear model was implemented in the SPSS version 23 program (2015) to assess the prediction equation of the parasitaemia slope from day 9 pi till day 13 pt.

Chapter 4

Results

Table 2. Log-transformed least square means (LSM) of *Trichinella zimbabwensis* larval counts following a single treatment (on day 25pi) and *Plasmodium berghei* parasitaemia following treatments (on days 9, 10, 11, 12pi) respectively, and % reduction following treatment regimen with maslinic acid, chloroquine and the combination of the two. CI, 95% confidence interval, N/A, not applicable.

Treatment regimens	Larval reduction (%)	LSM	CI	Parasitaemia reduction (%)	Mean	CI
Maslinic acid (co-infection)	91.3	2.11	1.862 - 2.366	74	10.34	7.935-12.797
Chloroquine (co-infection)	95.4	2.54	2.285 - 2.789	95.8	11.55	9.123-13.985
Maslinic acid + chloroquine (co-infection)	95.3	2.25	2.001- 2.505	95.5	8.36	5.639-11.082
Maslinic acid (single infection)	89.8	1.90	1.647 - 2.151	93.3	10.93	8.340-13.515
Chloroquine (single infection)	88.7	2.88	2.663 - 3.137	93.7	9.30	6.762-11.831
Untreated control	N/A	3.73	3.473 - 3.977	N/A	15.75	12.879-18.629

4.1 Larval reduction after treatment

Maslinic acid (*P. berghei* + *T. zimbabwensis*), chloroquine (*P. berghei* + *T. zimbabwensis)*, maslinic acid and chloroquine (*P. berghei* + *T. zimbabwensis*), maslinic acid (*T. zimbabwensis*) and chloroquine (*T. zimbabwensis*) single treatments cleared 91.3%, 95.3%, 89.8% and 88.7% of the larvae, respectively (Table 2). All treatments infected with *T. zimbabwensis* after day 25 pi from each drug regimen reduced the larvae. There was no significant difference in the reduction rate among the treated groups ($P > 0.05$). The Bonferroni pairwise comparison of the efficacy of the five drug regimens showed a significant reduction in larval counts compared to the untreated control group ($P < 0.05$) (Table 3). However, there was no significant difference in the larval counts among the treated groups, except among maslinic acid (*P. berghei* + *T. zimbabwensis*) and chloroquine (*T. zimbabwensis*), chloroquine (*P. berghei* + *T. zimbabwensis*) and maslinic acid (*T. zimbabwensis*), maslinic acid and chloroquine (*P. berghei* + *T. zimbabwensis*) and chloroquine (*T. zimbabwensis*), and chloroquine (*T. zimbabwensis*) and maslinic acid

(*T. zimbabwensis*) ($P < 0.05$) (Table 3). There was no apparent synergistic effect observed in the combination of maslinic acid and chloroquine in reducing larval burden (Table 2). While there was no significant difference in the efficacy between most drug regimens, co-infected groups treated with maslinic acid, chloroquine and the combination of maslinic acid and chloroquine, were slightly more effective than the groups infected with *T. zimbabwensis* only and treated with maslinic acid and chloroquine (Table 2). The group infected with *T. zimbabwensis* only and treated with chloroquine had the highest mean of larval counts compared to other treatment regimens (Table 2). Figure 8 shows the log-transformed square means of *Trichinella* and the control group had the highest mean square.

Table 3. Multiple pairwise comparisons of least square means derived from the Bonferroni test of treatment regimen of *Trichinella zimbabwensis* larvae in rats (on day 25 pi), *significance $P < 0.05$

	Larval reduction (%)					
	Untreated control	Maslinic acid (co-infection)	Chloroquine (co-infection)	Maslinic acid + chloroquine (co-infection)	Maslinic acid (single infection)	Chloroquine (Single infection)
Untreated control		0.000*	0.000*	0.000*	0.000*	0.001*
Maslinic acid (co-infection)	0.000*		1	1	1	0.002*
Chloroquine (co-infection)	0.000*	1		1	0.017*	1
Maslinic acid + chloroquine (co-infection)	0.000*	1	1		1	0.019*
Maslinic acid (single infection)	0.000*	1	0.017*	1		0.000*
Chloroquine (single infection)	0.001*	0.002*	1	0.019*	0.000*	

Table 4. Multiple pairwise comparisons of means derived from the Bonferroni test of subsequent treatment (on days 9, 10, 11, 12pi) regimen of *Plasmodium berghei* parasitaemia in rats, *significance P < 0.05

	Malaria reduction (%)					
	Untreated control	Maslinic acid (co-infection)	Chloroquine (co-infection)	Maslinic acid + chloroquine (co-infection)	Chloroquine (single infection)	Maslinic acid (single infection)
Untreated control		0.002*	0.032*	0.000*	0.003*	0.003*
Maslinic acid (co-infection	0.002*		1	1	1	1
Chloroquine (co-infection)	0.032*	1		1	1	1
Maslinic acid + chloroquine (co-infection)	0.000*	1	1		1	1
Chloroquine (single infection)	0.003*	1	1	1		1
Maslinic acid (single infection)	0.003*	1	1	1	1	

4.2 Parasitaemia reduction after treatment

The reduction rate in parasitaemia in rats infected with *P. berghei* alone and treated with maslinic acid and chloroquine was 93.3% and 93.7%, respectively, while the reduction rate in parasitaemia in rats co-infected with *P. berghei* and *T. zimbabwensis*, treated with maslinic acid, chloroquine and a combination of maslinic acid and chloroquine was 74%, 95.8% and 95.5%, respectively (Table 2). There was no significant difference in the reduction rate among the treated groups (P > 0.05). The Bonferroni pairwise comparison of the efficacy of the five drug regimens showed that there was a significant reduction in parasitaemia compared to the untreated control group (P < 0.05), however there was no significant difference in parasitaemia among the treated groups (P > 0.05) (Table 4). There was no apparent synergistic effect observed in the combination of maslinic acid and chloroquine in reducing parasitaemia

(Table 2). The reduction rate in parasitaemia for the co-infected group treated with chloroquine and the combination of maslinic and chloroquine was slightly more effective compared to other treated groups (Table 5).

Nine rats died during the course of the experiment. Of the nine rats that died, three were from the group co-infected with *P. berghei* and *T. zimbabwensis* and treated with the combination of maslinic acid and chloroquine, three from the untreated control group, one from the group infected with *P. berghei* only and treated with maslinic acid and two from the group infected with *P. berghei* only and treated with chloroquine. No rats died from the two groups co-infected with *P. berghei* and *T. zimbabwensis* and treated with maslinic acid and chloroquine, respectively.

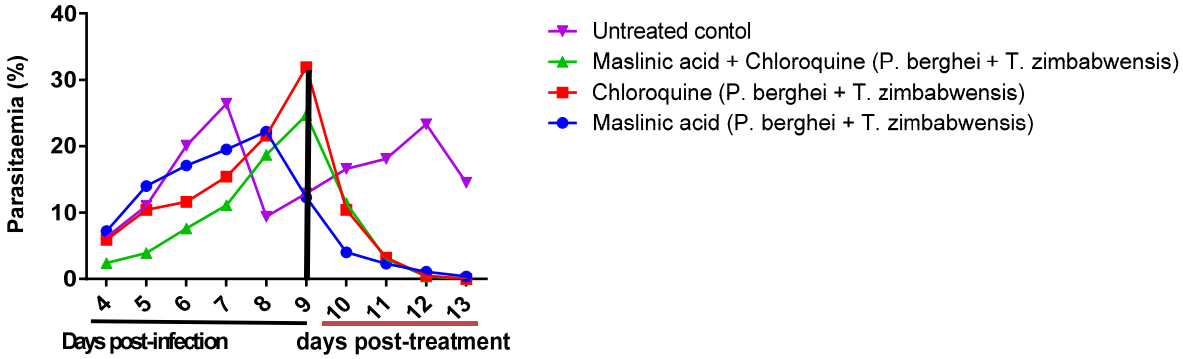

Figure 6. Mean percentage of parasitaemia of rats co-infected with *Plasmodium berghei* and *Trichinella zimbabwensis* and treated from day 9 to 13 post-infection.

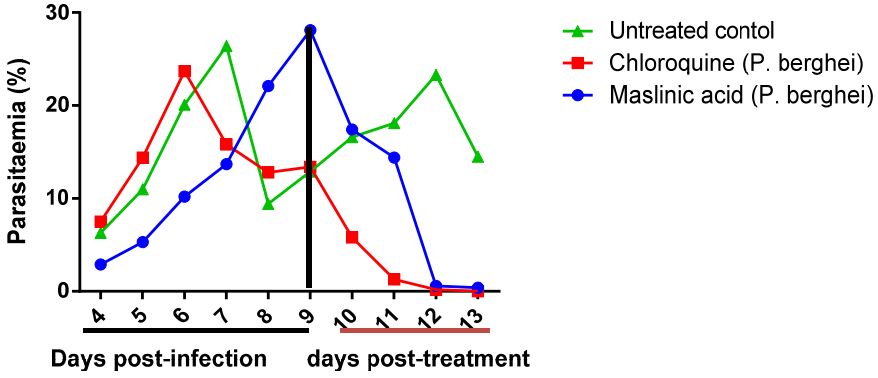

Figure 7. Mean percentage of parasiatemia of rats infected with *Plasmodium berghei* only and treated from day 9 to 13 post-infection.

4.3 Drug efficacy determination

Blood-stage of *P. berghei* parasite was observed on the thin blood smears collected from treatment regimens including the untreated control group on day 4 post-infection (Figure 6). All animals were positive for the parasite by day 4 post-infection (Figure 6). The mean parasitaemia peaked on day 6 post-infection (23.7%) on rats infected with *P. berghei* only and treated with chloroquine, while the mean parasitaemia peaked on day 9 post-infection for rats infected with *P. berghei* only and treated with maslinic acid (28.1%) (Figure 7). In rats co-infected with *P. berghei* and *T. zimbabwensis* treated with maslinic acid, mean parasitaemia peaked on day 8 post-infection (22.2%) (Figure 6). However the mean parasitaemia recorded peaked on day 9 post-infection in rats co-infected with *P. berghei* and *T. zimbabwensis* and treated with chloroquine and the combination of maslinic acid and chloroquine (31.9% and 24.7) (Figure 6). A similar trend was followed by all treatment regimens, except for the untreated control group where mean parasitaemia peaked on days 6, 8, 9, and 12 post-infection (Figure 6 and 7). On the day treatment was administered to the rats the mean parasitaemia started decreasing drastically post-treatment on all treatment regimens (Figure 6 and 7). The untreated control group however had a different trend where mean parasitaemia decreased significantly from 26.4% to 9.4% from day 7 to day 8 post-infection (Figure 6 and 7). After day post-infection mean parasitaemia increased again and reached a peak at day 12 post-infection (Figure 6 and 7).

Table 5 shows that maslinic acid (*P. berghei* + *T. zimbabwensis*) had the fastest decrease in parasitaemia after treatment was administered from day 9 pi with a regression slope of -2.67±0.85, compared to the untreated control which had a regression slope of 0.99±1.35. Chloroquine (*P. berghei* + *T. zimbabwensis*) and maslinic acid (*P. berghei*) both had the slowest decrease in parasitaemia after treatment was administered with regression slopes of -7.37±2.40 and -7.22±1.11, respectively (Table 5).

Table 5. Prediction equations for the rate of change in parasitaemia following treatments (on days 9, 10, 11, 12 pi) with maslinic acid, chloroquine and the combination of maslinic acid and chloroquine. CI, 95% confidence interval; SE, standard error; P < 0.05, significant; NS, not significant.

Treatment regimens	**Constant ± SE**	**Regression Coefficient ± SE**	**Goodness of Fit (%) (R^2 value)**	**95 % Confidence Interval for Regression coefficient**	**Significance level**
Maslinic acid (*P. berghei)*	91.60 ± 12.35	-7.22 ± 1.11	93.3	(-10.76) - (-3.68)	*** (P < 0.001)
Chloroquine (*P. berghei*)	39.78 ± 12.35	-3.24 ± 0.90	81.2	(-6.12) - (0.38)	* (P < 0.05)
Maslinic acid (*P. berghei* + *T. zimbabwensis*)	33.40 ± 9.47	-2.67 ± 0.85	76.5	(-5.39) – (0.05)	NS (P > 0.05)
Chloroquine (*P. berghei* + *T. zimbabwensis*)	90.30 ± 26.40	-7.37 ± 2.40	76.2	(-11.94) – (0.20)	NS (P > 0.05)
Maslinic acid + Chloroquine (*P. berghei* + *T. zimbabwensis*)	74.34 ± 17.2	-6.04 ± 1.55	83.5	(10.98) – (-1.10)	*(P < 0.05)
Untreated Control	6.19 ± 14.91	0.99 ± 1.35	15.3	(-3.29) – (5.27)	NS (P > 0.05)

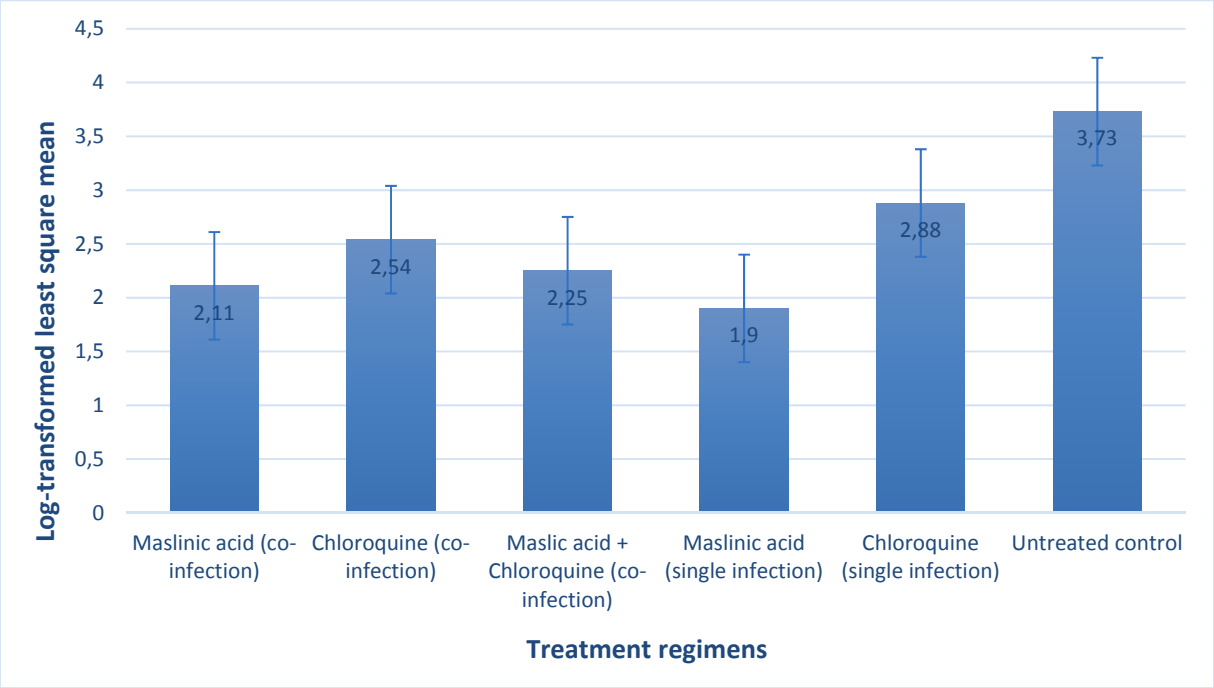

Figure 8. Log-transformed least square means of *Trichinella zimbabwensis* larvae on infected rats following treatment (on day 25 pi).

Chapter 5

Discussion and conclusion

5.1 Discussion

It is expected that a third of the world's population, mostly those living in the tropics and sub-tropics, are infected with STHs or one or more of the species of *Plasmodium* (Soliman *et al*., 2011). Malaria and intestinal helminths are parasitic diseases causing morbidity and mortality in most tropical parts of the world (Ojurongbe *et al*., 2011). These parasites are especially co-endemic in children who live in the endemic regions of sub-Saharan Africa, resulting in a high rate of co-infection (Ojuronge *et al*., 2011). Naturally associated infections could cause modifications of the specific immune response to each pathogen and thus modification of clinical expression (Ojuronge *et al*., 2004). Helminths can either improve (Ojuronge *et al*., 2004) or worsen (Nacher, 2011) malaria severity. The interaction between helminths and malaria could function in either way (Ojurongbe *et al*., 2011). Helminths may either change susceptibility to clinical malaria or malaria may affect the clinical consequences of helminth infection (Ojurongbe *et al*., 2011).

The results of the present study showed that maslinic acid and chloroquine were efficacious in reducing larval burden of *T. zimbabwensis* and malaria parasitaemia of *P. berghei* in both co-infected groups and in groups infected with *P. berghei* only and *T. zimbabwensis* only. However the efficacy of the combination of the two drugs did not surpass the efficacy of the individual drugs in clearing both *P. berghei* and *T. zimbabwensis*. This is in agreement with Mukaratirwa *et al*. (2014) who demonstrated that the combination of maslinic acid and fenbendazole had no apparent synergistic effect. Mukaratirwa *et al*. (2014) also showed that maslinic acid was efficacious in reducing *T. zimbabwensis* larvae after single or double treatment. Maslinic acid has been previously reported to be effective against different species of *Plasmodium* (Moneriz *et al*., 2011). The results found from this study further prove that maslinic acid is indeed effective against *Plasmodium* and *T. zimbabwensis* as it was able to reduce parasitaemia of *P. berghei* in co-infected groups and in groups only infected with *P. berghei* and reduce larval burden in all treatment regimens.

Moneriz *et al*. (2011) demonstrated that maslinic acid inhibited the growth of the parasite in *in vitro* experiments using erythrocytes infected with *P. falciparum*. Chloroquine also proved to be efficacious, especially in reducing malaria parasitaemia in co-infection of *P. berghei* and *T. zimbabwensis* and in groups infected with *P. berghei* only. Results from this study confirm previous studies (Farooq and

Mahajan, 2004; Wensdorfer and Payne, 1991). However chloroquine did not prove to be as efficious in reducing *T. zimbabwensis* larvae compared to other treatment regimens. A similar study by Danesvar *et al.* (2010) showed that chloroquine was associated with initial parasite clearance that is both faster in *knowlesi* malaria than *vivax* malaria. However they are also showed chloroquine resistance after early treatment failure (Danesvar *et al*., 2010). Similar results were also observed in another study where chloroquine was used to treat *P. vivax* in comparison with other drugs such as fixed dose combination (FDC) of arterolane maleate and piperaquine phosphate (PQP) (Valecha *et al.,* 2016). The results showed that 100% of patients treated with FDC of arterolane maleate and PQP were aparasitaemic and afebrile at day 3 compared to 99.3% of patients on chloroquine treatment. The efficacy was more than 95% at 72h in both treatment groups (Valecha *et al*., 2016). The efficacy of chloroquine to *Trichinella* cannot be compared to any previous literature. The literature available for the effect of chloroquine against ascariasis is more than 55 years and chloroquine was found to be not effect against this helminth.

Groups co-infected with *P. berghei* and *T. zimbabwensis* and treated with either maslinic acid and/or chloroquine had the highest reductions in terms of larvae and malaria parasitaemia, except for the group co-infected with *P. berghei* and *T. zimbabwensis* and treated with maslinic acid which had the lowest efficacy in malaria reduction. These results suggest that *T. zimbabwensis* and *P. berghei* were interacting with the drugs, in this case to reduce infection severity. A similar study showed tissue-dwelling nematodes and malaria interact to either reduce or increase the prevalence (Onkoba *et al*., 2015). There are no previous studies on the efficacy of a drug on the co-infection of tissue-dwelling nematodes and malaria. The influence of tissue-dwelling nematodes on the period of a malaria episode is not known (Nacher, 2011). However, they appear to decrease some symptoms of malaria that can lead patients to pursue treatment (Onkoba *et al.,* 2015).

Maslinic acid treated group which had been infected with *P. berghei* only did not show the same peaking and decline of parasitaemia prior to the treatment on day 9 pi as the control and chloroquine treated groups. This could be immunity had not been acquired as peak levels of parasitaemia was on day 9 pi. The untreated group, and co-infected groups reached peak levels of parasitaemia which reduced before treatment was administered on day 9 post-infection. This could be as a result of the rats acquiring immunity and the co-infection confers early protection. Similar studies have shown that immunization with sporozoit forms induces more than 90% protection in rodents and humans (Friesen and Matuschewski, 2011; Hoffman *et al*., 2002). Sauerwein *et al.* (2012) have explored the idea that using drugs with naturally acquired infection could affect the induction of protective immunity. An example of such drugs is IPT where parasite exposure is less to undesired sub therapeutic drug concentrations while at the same time allowing an effective generation of natural immunity (Schellenberg *et al*., 2006).

In this study we observed that early death occurred in the malaria infected groups compared to co-infected groups that had sustained parasitaemia. In our current study we have shown that the co-infection of *P. berghei* and *T. zimbabwensis* after treatment with maslinic acid and chloroquine reduced muscle larvae and parasitaemia. Therefore, we deduce that the use of maslinic acid as an anthelmintic and anti-malaria drug in Sprague-Dawley rats has an effect on the co-infection of *P. berghei* and *T. zimbabwensis*.

Rebound of malaria was not assessed in our study but studies have showed that rebound of malaria usually occur from 3 months to 1 year after discontinuation of chemoprophylaxis (Geerligs et al., 2003). Geerligs *et al*. (2003) also showed that of the twelve studies investigating rebound malaria or parasitaemia, nine did not show increased clinical malaria or parasitaemia. Similar results were also found in a Nigerian study that investigated the effects of chloroquine prophylaxis in children (Bradley-Moore *et al*., 1985).

5.2 Conclusion

Because of rapid growth in drug resistance, potential new drug strategies which are cost-effective are needed. A huge population especially in SSA are being infected with more than one pathogen and new drug targets to treat and control co-infections and not just one pathogen are needed. In view of many important needs and limited resources, a package of interventions to substantially decrease the co-infection of malaria and helminths is unfortunately not affordable to very low-income countries

This study observed that maslinic acid and chloroquine does in fact reduce muscle larval burden and parasitaemia co-infection of both *P. berghei* and *T. zimbabwensis* in Sprague-Dawley rats. Drug combination is most widely used in treating the most dreadful diseases, such as cancer and AIDS. The main aim of drug combination is to achieve synergistic therapeutic effect, dose and toxicity reduction. The present study did not achieve a synergistic effect on the drug combination. Therefore, more studies need to be done to evaluate drug combinations.

Humans are exposed to many pathogens on a basis. There are some suggestions that infections such as those caused by helminths and by the malaria parasite can interact and affect the immune responses and clinical outcomes, but more studies are needed on how tissue dwelling nematodes interacts with the immune system to help detoriate or increase malaria episode. Globally, many people are co-infected with malaria and helminths and the influence of helminth infections on the course of malaria infection needs more careful investigation. There also needs to be more studies on the co-infection of tissue-dwelling nematodes and malaria on either immune responses or host-pathogen interaction. Each type of tissue-dwelling helminth infection should be studied separately, since they might have different effects on the

course of malaria. Treatment of tissue-dwelling helminth infection followed by a detailed follow-up on the infection rate and on the symptoms of malaria will be important in establishing the effect of tissue-dwelling helminth infections on parasitaemia. A better understanding of the interactions between the two parasites will improve the evaluation of malaria vaccine trials, drug combinations studies and also reveal the implications of anti-helminth treatment programs for the course of malaria disease. Taking into account the results of this study and previous studies and the continuing problem of drug resistance, maslinic acid is one of the promising natural compounds to exhibit both anthelmintic and anti-malarial activities, and may be relevant in the treatment and control of helminths and malaria in endemic areas. These findings provide new important knowledge of co-infections with malaria parasites and tissue-dwelling nematodes and the protective effect of maslinic acid as an anthelmintic and anti-malaria drug against trichinellosis and malaria disease outcomes.

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ADDENDUM

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

28 February 2014

Reference: 064/14/Animal

Miss L Gcanga
School of Life Sciences
WESTVILLE Campus

Dear Miss Gcanga

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 projects:

"Efficacy of masilinic acid on the co-infection of *Trichinella zimbabwensis* and *Plasmodium berghei* ANKA on Sprague-Dawley rats."

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

Cc Registrar – Mr C Balovi
Research Office – Dr N Singh
Supervisor & HOS – Prof. S Mukaratirwa
BRU – Dr S Singh