Repositioning of orally administered compounds for the treatment of African trypanosomosis

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Nthatisi Innocentia MOLEFE

Doctoral Program in Animal and Food Hygiene

Graduate School of Animal Husbandry

Obihiro University of Agriculture and Veterinary Medicine

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Abbreviations

%	Percent
<	Less Than
>	Greater Than
±	Plus/Minus
° N	Degrees North
° S	Degrees South
°C	Degree Celsius
µg	Microgram
µL	Microliter
AAT	Animal African Trypanosomosis
ANOVA	Analysis Of Variance
ATP	Adenosine Triphosphate
BSF	Blood Stream Form
BVAT	Blood Stream Variant Antigenic Type
CATT	Card Agglutination Test For Trypanosomiasis
CCK-8	Cell Counting Kit-8
CDC	Centres For Disease Control
CNS	Central Nervous System
DDT	Dichloro-Diphenyl-Trichloroethane
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ENR	Enoyl-Acyl Carrier Protein Reductase

FAO	Food And Agriculture Organization
FAS	Fatty Acid Synthesis
FBS	Fetal Bovine Serum
Fig	Figure
g	Grams
h	Hour
H^+	Proton
HAT	Human African Trypnosomosis
HCT	Haematocrit
HFF	Human Foreskin Fibroblast
HGB	Haemoglobin
HHT	β-Hexachlorohexane
HI-FBS	Heat Inactivated Fetal Bovine Serm
IC	Inhibitory Concentration
ICT	Immunochromatographic Test
IFAT	Indirect Fluorescent Antibody Test
ILCA	International Liver Cancer Association
kDNA	Kinetoplast DNA
Kg	Kilogram
Km^2	Square Kilometer
L	Litre
LAMP	Loop Mediated Isothermal Amplification
LD	Lethal Dosage

MDBK	Madin - Darby Bovine Kidney
Mg	Milligram
mL	Millilitre
MVAT	Metacyclic Variant Antigenic Type
NECT	Nifurtimox - Eflornithine Combination Therapy
NIH 3T3	Mouse Embryonic Cells
nm	Nanometre
OIE	Office International Des Epizooties
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PSG	Phosphate Buffered Saline with Glucose
S.D	Standard Deviation
SIT	Sterile Insect Technique
US$	United States Dollar
USA	United States of America
VAT	Variant Antigenic Type
VSG	Variant Surface Glycoprotein
WHO	World Health Organization

Introduction and literature review

I. Characterization of the *Trypanosoma* parasites

Aetiology and lifecycles

Trypanosomes are microscopic unicellular haemoflagellate protozoan organisms consisting of a nucleus, microtubules (cytoskeleton and flagellum), endoplasmic reticulum, golgi apparatus and a single mitochondrion. In addition, trypanosomes possess a kinetoplast, functioning as a self-replicating organelle, containing mitochondrial DNA information (Feder *et al.*, 2014). Trypanosomes are classified as follows:

Kingdom : Protista

Phylum : Sarcomastigophora

Class : Zoomastigophorea

Order : Kinetoplastida

Family : Trypanosomatidae

Genus : *Trypanosoma*

The genus *Trypanosoma* is divided into two sections, the salivarian trypanosomes which are transmitted through the saliva of biting flies and the stercorarian trypanosomes that are transmitted by bugs and/or fleas through faecal contamination of the wounds opened by the vector during the blood meal (Maudlin *et al.*, 2004).

Salivarian trypanosomes

Most salivarian trypanosomes are pathogenic, to both human and animals. This section includes the subgenus *Duttonella* (*T. vivax*), *Nannomonas* (*T. congolense, T simiae*)

and *Trypanozoon* (*T. brucei brucei, T. b. rhodensiense* and *T. b. gambiense*) and less likely subgenus *Pycnomonas* (*T. suis*).

T. congolense, T. vivax, and *T. b. brucei* are the causative agents of the animal African trypanosomosis (AAT) also known as nagana in the central, eastern and southern Africa. AAT affects a wide range of hosts; namely, bovines, ovines, caprines, camelids, swine and equids (Osorio *et al.,* 2008; Steverding, 2010; Morrison *et al.,* 2016). Amongst all trypanosomosis agents, *T. congolense* is reported as the most pathogenic and virulent species. Cattle remains the main host of the *T. congolense* infection which has been reported to be the most virulent in dogs, leading to the acute death (Nimpaye *et al.,* 2011; Watier-Grillot *et al.,* 2013; Lisulo *et al.,* 2014). Pigs are well known as reservoir host for *T. coongolense* infections. Nonetheless, few reports have observed in *T. congolense* infected pigs signs such as acute, mild chronic to chronic, anaemia and low levels of parasitaemia in apparently healthy pigs but abortion and death were observed in pregnant sows (Katunguka-Rwakishaya, 1996; Simukoko *et al.,* 2007). Rarely, *T. suis* has been reported in pigs causing surra-like infection, in addition to a pathogenic *T. simiae*. *T. b. rhodensiense* and *T. b. gambiense* cause human African trypanosomosis (HAT) or the African sleeping sickness in the Eastern and Western Africa, respectively (Kennedy, 2013; Franco *et al.,* 2014).

Salivarian trypanosomes are transmitted biologically through a bite of a tsetse fly belonging to the genus *Glossina.* There is a wide range of these flies in Africa, found predominantly in the sub-Saharan region of Africa. The vectors are climatic condition restricted, and therefore are abundant in the warm places with the most fertile and arable land of the Africa (Simukoko *et al.,* 2007; Wamwiri and Changasi, 2016). There is still dearth of information regarding whether a specific *Glossina* species can transmit either a specific *Trypanosoma* species or more. The distribution of tsetse plays a major role in determining the possible pathogens rotating in the area (Wamwiri and Changasi, 2016).

According to Leak (2007), studies have shown that *G. palpalis* and *G. morsitans* mainly transmit *T. vivax* and the *Trypanozoon* trypanosomes, respectively, whilst *G. m. centralis, G. brevipalpis* and *G. fusca fuscipes* transmit *T. congolense.*

Furthermore, in contrast to other salivarian trypanosomes, there are monomorphic *T. evansi* and *T. equiperdum* species which cause surra and dourine, respectively. These two salavarian trypanosomes are known as the non-tsetse transmitted animal trypanosomes due to the loss of the kDNA (maxicircles and some of the minicircles) meaning that *T. evansi* and *T. equiperdum* have lost the ability to maintain the biological transmission by the tsetse. The *T. evansi* adapted to a mechanical transmission by biting flies of the genera *Tabanus* and *Stomoxys* amongst others. According to Desquesnes *et al.* (2013), *T. evansi* can be referred to as a blood and tissue parasite for it can exhibit high levels of parasitaemia in the blood of the hosts, but also has the ability to invade the tissues. *T. equiperdum* is a primarily a tissue parasite causing a venereal disease strictly in equids and is transmitted through coitus of the infected hosts resulting in signs of oedema, damage to the genitals, progressive anaemia, paralysis of the hind legs and death if left unattended. Dourine results in 50 to 70% mortality in equids (Claes *et al.,* 2005; Mitchell, 2017). Horses are generally susceptible to dourine infection while donkeys and mules are noted resistant and could therefore act as reservoirs. Zebra have also tested positive for dourine (Gizaw *et al.*, 2017).

The life cycle begins with an insect, tsetse fly of *Glossina* spp. taking a blood meal from a definite host of the *T. brucei* parasite. An infected tsetse injects metacyclic trypomastigotes into the skin tissue which will later enter the lymphatic system and pass into the bloodstream. In the bloodstream of a definite host the metacyclic trypomastigotes will differentiate into the long slender form and multiply by binary fission. The long slender form has the ability to invade other parts of the hosts' body such as the central nervous system (CNS). The long slender form differentiate into the non-proliferative short stumpy form, this

is infectious to insects. The tsetse fly ingests the stumpy form which will differentiate into the procyclic trypomastigate in the midgut. The parasites then migrate to the proventriculus and differentiate into the epimastigotes. Finally, the migration to the salivary glands takes place where the epimastigotes differentiates into the metacyclic trypomastigotes which are infectious to the definite host (Fig. 1) (Langousis and Hill, 2014).

Metacyclic trypomastigotes are injected into the mammals by tsetse flies during a blood meal. *Trypanosoma congolense* is monomorphic in the mammalian bloodstream form and it is therefore strictly intravascular. The bloodstream form is ingested by the tsetse fly during a blood meal. In the midgut of the tsetse fly the bloodstream form differentiates into the procyclic trypomastigotes. The parasites migrates into the proventriculus where they differentiates into the long trypomastigote form. Subsequently, migrate into the proboscis and/or the cibarium where they will differentiate into the epimastigotes and eventually metacyclic form, the infective form of the parasites. And the cycle will repeat itself (Fig. 2) (Peacock *et al.*, 2012).

Stercorarian trypanosomes

Stercorarian trypanosomes include the subgenus *Megatrypanum* (*T. theileri*), *Herpetozoma* (*T. lewisi* and *T. rangeli*) and *Schizotrypanum* (*T. cruzi*). Stercorarian trypanosomes can infect most of the vertebrates, the nucleated mammalian cells, if not all (Tyler and Engman, 2001). The *T. theileri* is a widely distributed parasite of cattle, cosmopolitan in nature with reported cases in all continents, except Antarctica. According to (Villa *et al.*, 2008) species within the *Megatrypanum* subgenus are defined by their host specificity, in that manner, *T. theileri* was defined as a cattle parasite. Even though *T. theileri* is regarded as non-pathogenic parasite, few cases have been reported in association with *T.*

theileri based on clinical symptoms as a severe regenerative anaemia in dairy cattle and calves (Davila and Silva, 2000; Villa *et al.,* 2008).

The subgenus *Herpetosoma* consists of two trypanosomes of interest, *T. rangeli* and *T. lewisi.* The *T. rangeli* is a pathogen of humans, wild and domestic animals in the central and South America. The distribution of *T. rangeli* overlaps with that of *T. cruzi* which has complicated infection; it could either be a single or mixed infections of these two pathogens. Furthermore, studies have reported cross-reactivity in serological assays due to similar surface antigens, nonetheless, there are few biological, immunological, biochemical and molecular methods that could distinguish between the two species. The animal reservoir hosts and the vectors (triatomine bug) are also shared between the two parasites (Grisard *et al.,* 1999; Guhl and Vellejo, 2003; Grisard *et al.*, 2010). In addition *T. lewisi* is a rat parasite transmitted by rat fleas, even though some reports observed the presence of this parasite in the oriental rat flea and dog fleas. In white rats, *T. lewisi* presence appeared to increase the susceptibility of the rats to *Toxoplasma gondii* infection (Linardi and Botelho, 2002; Rayat and Vanishta, 2014).

Lastly, of medical importance is *T. cruzi*, subgenus *Schizotrypanum* the leading cause of Chagas disease in humans. Chagas disease is a chronic infection of humans and often fatal. There are few transmission options associated with the infection, transmission could either be through a bite of the triatomine bug, transfusion which is responsible for approximately 10% of the cases or congenital transmission from a mother to child (Prata, 2001). This disease is known to affect people in the remote areas with poor sanitation, lacking proper facilities for diagnosis and organized proper health records. Urban migration has introduced Chagas disease in the non-endemic areas with reported cases in the USA, Europe and Asia (Tyler and Engman, 2001, Carrea and Diambra, 2016). Various strains of

T. cruzi differ in terms of the epidemiology, pathology, treatment response and immunogenic characters (Prata, 2001).

Briefly; the metacyclic trypomastigote in the hindgut of the vector, the reduviid bug, is the infectious stage of human or vertebrate host. When taking a blood meal, the bug defecates, and contaminate the wound with the faeces containing the metacyclic form. In the host, weak metacyclic trypomastigotes are likely to be phagocytosed by the host defence cells and killed but the surviving ones invade various cells in which they transform into the amastigotes. The amastigote multiply by binary fission in the host cells. Intracellular amastigotes then transform into the trypomastigotes and burst the cell and enter the bloodstream. The released trypomastigotes can either circulate in the blood or invade new cells. The bug will ingest the trypomastgotes during the bloodmeal which transforms into the epimastigotes in the midgut and finally differentiate into the metacyclic trypomastigotes in the hindgut (Fig. 3) (Tyler and Engman, 2001; Carrea and Diambra, 2016).

Epidemiology

The distribution of HAT and AAT is based on several factors, namely; the prevalence of the parasites, the presence of vector and the animal host. The distribution of the vector, correlates to that of the parasites. The prevalence zone of the tsetse fly and trypanosomes stretches for a distance of more or less 10 million km^2 marking a firm establishment of the disease based on the availability of the suitable habitat and conditions for tsetse survival (Yaro *et al.,* 2016). The presence of tsetse flies is directly affected by the climatic condition, ecological factors and the presence of the host. Population increase has resulted in the encroachment of people and livestock into the wild areas, and there by invading regions with tsetse and wild life. This circumstance has led to a gradual decrease in density of wild host

and tsetse, however, it has also increased a number of cattle exposed to the infection (Van den Bossche, 2001). Due to the climatic restriction of the tsetse fly, its prevalence is limited from 14° N to 29° S of Africa stretching for a distance of 8 to 11 million km^2 inhabited by approximately 260 to 300 million people and 50 million cattle (McDermott and Coleman, 2001; Van den Bossche *et al.*, 2010; Wamwiri and Changasi, 2016). Sleeping sickness and/or nagana affect approximately 37 African countries with 21 of the AAT endemic countries considered among the 25 poorest countries in Africa (Mitchell, 2017). Tsetse has infested the most fertile and arable land of Africa placing at risk more than 20 million cattle and 60 million people throughout the African continent.

T. evansi infection adaptation to mechanical transmission has introduced surra in regions outside the tsetse belt. Unlike nagana and sleeping sickness vectors that prevail only in good conditions, biting flies have the ability to strive even in dry zones (Eyob and Matios, 2013), with documented reports in dry areas of Africa (Sudan and Ethiopia), Asia and Latin America. Furthermore, wild animals act as the reservoirs of trypanosomosis therefore presenting an omnipresence of trypanosomosis, regardless the species, vector or the host.

Lastly, *T. equiperdum* which causes a disease called dourine is an infection of equids; horses, donkeys and mules. The disease is transmitted via coitus between infected and non-infected equids. Dourine was once a worldwide distributed infection, however, dourine has been eradicated from a number of countries, and for instance, Japan, Australia and the USA are currently considered free from dourine. As stated by the OIE, the infection is now only found in most of the Asian countries, southern Africa, Russia, parts of the Middle East, South America and Europe (Gizaw *et al.*, 2017).

Impact of trypanosomosis

Agriculture forms a backbone of the economy of most African countries, contributing 30 – 50% of the gross domestic product, with livestock production making up to 20 – 25% of the total 50% (Diao *et al.,* 2010). Ruminant livestock products such as the meat and milk are important components of the humans' diet, while wool plays a big role in human livelihood sustenance. Moreover, livestock provides the traditional role of draught power for cultivation, weed control, harvest as well as assisting the farmer in determining the arable area for cultivation. With the global population increase, livestock products demands are due to increase and the draught power expanded (Simukoko *et al.,* 2007; Van den Bossche *et al.,* 2010).

The presence of trypanosomosis in the fertile regions of the sub-Saharan Africa areas has tremendous and enormous impact on the agricultural production whereby the infection has rendered the most fertile parts of Africa unsuitable for human settlement and almost a quarter of animal rearing area useless due to mortalities associated with trypanosomosis. According to McDermott and Coleman (2001) total losses associated with trypanosomosis is estimated at 1.3 to 5 billion US$. Estimation depends however on the methodology utilized, assumptions made and type of losses estimated. The direct effects of the disease involve the well-being of the population through mortality, reduced productivity and costs of the control. Reduced livestock productivity due to animal trypanosomosis has resulted in loss of over 150 million cattle and 260 million small stock ruminants. In addition, the disease has an impact on crop production, human settlement and welfare where more than 7 million km^2 of the region was declared unsuitable for mixed crop-livestock farming system (Yaro *et al.,* 2016).

The indirect loss is mainly a result of the disease preventing agricultural development of potentially productive land, distorted draught oxen power and the expensive control management and prevention program scheme establishment and/or maintenance. The increasing introduction of livestock in the tsetse infested regions of Africa due to good climatic conditions and arable land, is likely to further increase the infection rate with 37% and 70% in sub humid and humid, respectively (Biryomumaisho *et al.,* 2013).

AAT caused by *T. congolense* is regarded as the most important disease of livestock in the African continent costing an estimated amount between 15 and 5 billion US$ annually on agricultural production losses (Coustou *et al.,* 2010).

Clinical symptoms

Trypanosoma infected animals can display a broad range of clinical symptoms, varying from acute to chronic signs. Nonetheless, persistent and common signs of animal trypanosomosis infected animals include, but not limited to, haematological changes, weight loss, anorexia, weakness and elevated body weight (Steverding, 2010; Jaiswal, 2015). Anaemia is a predominant symptom for AAT and is therefore used as an indicator for the severity of the infection, measured with the decrease of packed cell volume (PCV), red blood cells (RBC) and haemoglobin (HGB). Infected animals are at risk of secondary infection due to the immune-depressive effect associated with trypananosomosis (Gossense *et al.,* 1998). Late stage of animal trypanosomosis is characterized by parasites in the CNS which is marked by severe anaemia, cachexia and paralysis (Yaro *et al.,* 2016). In addition to the above mentioned, other symptoms reported include pica (the tendency of eating the non-food substances) and splenomegaly. The major hurdle to the economy is the pathological changes of the reproductive organs caused by the infection resulting in either temporary

and/or permanent anoestrus, abortions, still births, infertility, atrophy of the reproductive organs, neonatal deaths and ultimate death of the host (Goosens *et al.,* 1997; Goosens *et al.,* 1998). The mechanism through which the reproductive system is damaged depends solely on the trypanosome species. Moreover, it is estimated that approximately 20% of the losses are due to abortions while 30% is attributed to death of the animals (Okech *et al.,* 1996; Desquesnes *et al.,* 2013).

In humans, HAT or sleeping sickness results in an intermittent fever that is related and regulated by parasitaemia and lymphadenopathy, these are the symptoms predominant in the first stage of the infection. When the parasites have invaded the CNS, patients suffer headache, stiff neck, sleep disturbances, depression, progressive mental deterioration, focal seizures, tremors, and palsies. Untreated cases progress into coma and ultimate death (Kennedy, 2004).

Diagnosis

The diagnosis of trypanosomosis is primarily based on the detection of the parasites, antibody or antigen. Techniques capable of providing such are only few and limited, which are the observation of clinical signs, microscopy, serology and molecular techniques. Clinical signs have the ability to notify, determining and indicating an arising problem in the animals, however, they are not always reliable, provided that various disease share clinical signs manifestations (Kennedy, 2004). Thus far, the first line of detection is based on the detection of the parasites in the body fluids; lymph node aspirate, blood or the cerebrospinal fluid (CSF). Parasitological diagnosis provides the direct evidence of the infection therefore allowing the definite diagnosis (Chappuis *et al.,* 2005). There are an array of diagnosis based on the indirect evidence of the infection, this include the antibody

detection by the indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), card agglutination test (CATT), and a recently developed immunochromatographic test (ICT), and molecular detection of the parasite DNA/RNA by polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP).

Every diagnostic tool has disadvantages, which makes it difficult to select one over the other. Microscopic detection of the parasites is the easiest method, however, the parasites cannot be detected at low parasitaemia levels and species identification cannot be achieved (Bonnet *et al.*, 2015). Antibody detections can easily be conducted with high production of antigens but the technique requires adequate equipment and beside the newly developed ICT (Reyna-Bello *et al.*, 1998; Nguyen *et al.*, 2015), none of the antibody detecting techniques were applicable in the field, furthermore a once infected animal can retain the antibodies for long even after the clearance of the infection. Molecular techniques (PCR and LAMP) detect passive and active infections and they are highly sensitive and specific to the DNA sequence, but require equipment (Becker *et al.*, 2004; Thekisoe *et al.*, 2007; Njiru *et al.*, 2008; Wamwiri and Changasi, 2016). Researchers are trying to develop and optimise conditions for the improvement of the currently available diagnostic tools.

Control and treatment

Trypanosomes have developed a highly sophisticated and a complex phenomenon known as an antigenic variation utilized by the parasites to evade the immune response of the host against the infection. In the mammalian host there are about 10 million identical molecules of a glycoprotein, the variant surface glycoprotein (VSG) covering the whole trypanosome cell. VSGs have two main functions, to protect the parasite from humoral responses and to prolong the parasites viability by increasing the probability of transmission

to tsetse. The VSG is the main concept combatting the development of vaccine (La Greca and Magez, 2011; Tabel *et al.*, 2013). The infection can run from at least few months to years without a complete clearance from the host's system by immune response, even though trypanosomes are exposed to the immune response, as extracellular organism. The infection fluctuates with the immune response due to the variant antigenic types (VAT) expressed in different stages of infection. The VATs are acquired by the metacyclic (MVAT) form in the insect vector which is the infective stage to mammals. These VATs are shared by the parasites and are also expressed in the bloodstream form (BVAT). There are common VATs detectable at any early stage of infection and those that are detectable at a later stage. The parasites expressing detectable VATs are mostly cleared by the immune system of the host resulting in a decrease of parasitaemia, however, some parasites with different VATs survive and can produce a new wave of growth with new BVATs acquired in the bloodstream. Because of this phenomenon, vaccine has not yet been developed to combat trypanosomosis. Nonetheless, there is hope for vaccine in the near future given that there are only 27 MVATs expressed in *T. brucei* and 12 expressed in *T. congolense* (Turner and Vickerman, 1998; Maudlin *et al.*, 2004).

Meanwhile, in the absence of vaccine, there are three principal control strategies for the tsetse transmitted trypanosomosis:

Firstly; the vector control. The primary approach for the control of infections would involve attempts to halt transmission of the respective infection. There are numerous factors involved in halting the infection, mainly the control of the vector. Several attempts were conducted to eradicate the presence of the vector in Africa with the main focus on the tsetse fly. The initial and prompt response to the vector control was methods that included clearing of vegetation where the flies rested and/or shooting of the game animals on which the insects fed. Even though the clearance of the vegetation contributed in the reduction of the tsetse by

making the environment unsuitable for their survival, re-invasion is still possible especially when the running funds get depleted, that is why sustainability using the local resources is the key to success (Holmes, 1997; Van den Bossche, 2001). Such methods are however, no longer tolerated due to their obvious negative impact on the environment and the ecosystem as a whole.

From the 1940s, several insecticides for aerial spraying were introduced and their usage brought major positive changes towards the eradication of the vector. Tsetse are also highly susceptible to all known insecticides, therefore Schofield and Kabayo (2008) has mentioned that there has not been any insecticides resistance risen in the tsetse control. Regardless, criticism quickly arose due to the residual formulation from the sprays that ended up affecting the non-targeted organisms, therefore most of the insecticides got banned. With a number of insecticides been banned by the World Health Organization (WHO) aerial spraying became difficult and therefore, odour baited traps and targets were introduced. The obstacle of the odour baited traps and targets included the applicability in the small scale, community based participatory control operations (FAO, 1992), and the baits and are difficult to sustain for poor farmers in regions with poor socio-economic status (Holmes, 1997; McDermott and Coleman, 2001; Schofield and Maudlin, 2001). Odour baited traps and targets were the best option due to the slow reproduction. On the contrary, tsetse infest a vast areas and are highly mobile, not restricted to convenient areas for neither the insecticides nor the traps (Schofield and Maudlin, 2001). Nonetheless, the combination of aerial spraying with dichlorodiphenyltrichloroethane (DDT) and β-hexachlorohexane (HCH) together with the trap technique resulted in a successful eradication and control of *G. pallidipes* in South Africa and Zimbabwe, respectively (Holmes, 1997; De Beer *et al.*, 2016).

According to Holmes (1997), tsetse control techniques that do not result in environmental degradation, and are feasible can be applied in the wilderness area as a

measure to safe guard gains of the vector eradication in the adjacent settlements. A successful clearance of the vector needs the involvement of the neighbouring areas and/or countries, while taking into consideration factors such as the socio-economic status of a country, livestock and crop production and resource allocations.

The recently introduced strategy is the sterile insect technique (SIT). A successful eradication of *G. m. morsitans* in Zanzibar was achieved through the SIT (Vreysen *et al.*, 2014). In this technique, sterile male tsetse flies are bred in the laboratory settings and released into the wild, where they will compete with the wild tsetse males. This technique has been a success where executed well, provided that female tsetse mate only once in their lifetime. There are, however, even this technique has its own disadvantages responsible for failure of eradication of the tsetse in other areas. Thus far, not all the *Glossina* spp. can be bred under laboratory setting successfully, a large numbers of a greater magnitude than the natural population should be bred and released into the wild in order to obtain sufficient males to compete with the wild flies (Hursey, 1985; McDermott and Coleman, 2001; Vreysen, 2001; Abd-Alla *et al.*, 2013; Vreysen *et al.*, 2014).

Due to all the difficulties associated with the vector control, a different approach was established, breeding of trypanotolerant livestock. Trypanotolerance is the ability of livestock breeds to survive and remain productive under AAT challenge, with low mortality and with or without the use of trypanocidal drugs (Geerts *et al.*, 2009; Morrison *et al.*, 2016; Yaro *et al.*, 2016). Indigenous animals in the endemic areas develop tolerance to various infections including among others, trypanosomosis, babesiosis, dermatophilisis and other humid tropical diseases at an early stage of their lives. The genes conferring trypanotolerance in cattle or any other hosts have not yet been identified (La Greca and Magez, 2011). Trypanotolerant animals have the ability to control parasitaemia and anaemia, however, it is not absolute. Trypanotolerant cattle can still succumb to high challenges of

trypanosomosis with signs of anaemia, loss of weight, and to extreme cases death, therefore these animals also need treatment though not at frequent as the exotic breeds which are mainly susceptible to the trypanosomosis (Holmes, 1997; Moloo *et al.*, 1999; McDermott and Coleman, 2001). Geerts *et al.*, (2008) reported 32% sheep and 47% goats are trypanotolerant in the western and central Africa, respectively.

Trypanotolerant cattle are facing extinction in the west and central Africa with less than 10 million population remaining at the moment (ILCA, 1979; FAO, 1987). This is the result of indiscriminate slaughter, continuous extensive and uncontrolled breeding with exotic breeds, inappropriate husbandry techniques and neglect. According to Mwai *et al.* (2015), the livestock sector is facing serious challenges with high demands of livestock products, the long-term sustainability is questionable. Environmental factors such as climatic conditions are primary factors influencing the trypanotolerance of the cattle. Extinction of the cattle can be reversed and/or combatted by maintaining the cattle by in situ technique. Unfortunately, in situ preservation of the cattle is quite expensive (Mwai *et al.*, 2015; Gwaza and Momoh, 2016).

The last option for the treatment of trypanosomosis is the usage of the trypanocidal drugs. Trypanocides are the most widespread and well established method for the control of the persisting infection, trypanosomosis. There are only few compounds that could offer the chemotherapeutic and prophylactic effect against the infection in animals and humans. Currently on the market there is pentamidine, suramin, difluoromethylornithine (DFMO), nifurtimox-eflornithine combination therapy (NECT), and merlasoprol for the treatment of sleeping sickness, while homidium bromide, homidium chloride, diminazene aceturate (DA), isometamedium chloride (ISM), suramin, melarsomine and quanapyrimine sulphate are utilized for the treatment of nagana (Steverding, 2010). There are also a number of hurdles associated with these drugs. According to Bacchi (2009), pentamidine and suramin

discovered in 1930 and 1922, are only effective in the early stages of the Gambian and Rhodesians trypanosomosis, respectively. Until the 1990s, merlasoprol discovered in the 1940s was the only drug effective in the CNS stage of the infection, before the development of DFMO.

In animals ISM provides chemotherapeutic and prophylactic effects while homidium chloride and DA are primarily for chemotherapy. These drugs have been utilized, extensively since their discovery in the 1950s (Steverding, 2008; Kuriakose *et al.,* 2012; Sahin *et al.*, 2014). A study has shown the productivity increase in cattle treated with ISM while the non-treated cattle succumbed to the infection (Holmes, 1997). The extensive use of the drugs has rendered most, if not all less effective because of a well-established and globally recognized resistance. The drugs are widely spread recently, their sale is no longer centralized and obtained only through the veterinary channels, and instead they are now sold under generic brands, obtained through traders and shopkeepers. Estimation of their actual usage has become extremely difficult (McDermott and Coleman, 2001).

II. Characterization of the drugs

This study was initiated with the screening for 8 compounds in total, namely: artesunate, triclosan, azithromycin, curcumin, 2-hexadenocenoic acid, nerolidol, pepstatin A and melatonin. Based on the efficacy of the compounds trypanosome species, the cytotoxicity effects and the selectivity index, the best compounds were selected, which were azithromycin, triclosan and curcumin.

Azithromycin

Azithromycin (AZM) dihydrate, ($C_{38}H_{72}O_{12}\cdot 2H_2O$), is a semi-synthetic 15 membered azalide prototype with a lactone ring to which one or more sugars attached. AZM was synthesized in the 1980s as a second class derivative of erythromycin (ERY) that was discovered in 1952 (Parnham *et al.,* 2014; Adeli, 2017). Macrolide antibiotics (AZM, ERY and clindamycin (CLI)) were isolated from the products produced by *Streptomyces* spp.

The *Streptomyces* is a genus of a gram positive bacteria, growing in various environments. *Streptomyces* are the major producers of secondary metabolites and bioactive compounds such as the antifungal, antiviral, but mostly antibiotics and immunosuppressants for biotechnology industry (Procopio *et al.,* 2012; Al_husnan and Alkahtani, 2016).

AZM was derived in order to improve the pharmacokinetics of the first class of macrolides, ERY (Nahata, 1996; Yousef and Jaffe, 2010; Adeli, 2017). Macrolides antibiotics generally have a low solubility, low bioavailability, and poor availability after oral administration, they are also associated with a potential cardiac QT prolongation. The synthesis of AZM was to solve and/or rectify the above mentioned disadvantages. AZM differs from ERY by the addition of a methyl substituted nitrogen atom in the lactone ring. These changes were critical in making AZM stable in an acidic medium resulting in less gastrointestinal side effects and better oral availability as compared to the first class of macrolide. Moreover, AZM has a prolonged half-life of approximately 68 hours, allowing treatment once a day, readily absorbed orally with an increased bioavailability thereof, well tolerable and safe to high concentrations as 1.5 g/kg (Yousef and Jaffe, 2010; Zhang *et al.,* 2013; Parnham *et al.,* 2014; Adeli, 2017).

AZM is the most important member of the azalide class of antibiotics, with a wide spectrum of antimicrobial activity offering coverage to gram positive and gram negative

bacteria over other few macrolides (Jelic and Antolovic, 2016). Even though AZM is effective on both gram positive and gram negative organism, it is more potent on the gram negative. The effectivity of AZM covers various causative pathogens in the pelvic inflammatory diseases; *Neissera goorrhoeae, Chlamydia trachomatis*, mycoplasma, the intracellular pathogens and infections; *Legionella*, Lyme borreliosis, *Toxoplasma gondii*, *Helicobacter pylori*, the paediatric infection and opportunistic infections in acquired immunodeficiency syndrome (AIDS). In addition, AZM is used in the treatment of middle ear infection, tonsillitis, laryngitis, throat infections, bronchitis, pneumonia and typhoid (Mikamo *et al.,* 2014; Smith *et al.*, 2015; Sultana *et al.,* 2016; Adeli, 2017; Schwamesis *et al.,* 2017).

The mode of action of AZM, like the other macrolides, inhibit RNA-dependent protein synthesis by reversibly binding onto the 50 S ribosomal complex, therefore inhibiting the translation process of amino acid to proteins. AZM has an advantage over other macrolide antibiotics, which is the compounds ability to effectively accumulate in phagocytes and fibroblast, thus guaranteeing the delivery of the compound in high concentrations to the site of infection. Phagocytes transportation to the site of infection is triggered by their chemotactic activity, and are transported together with the compound (Yousef and Jaffe 2010; Muniz *et al.,* 2013; Parnham *et al.,* 2014). Macrolide drugs are usually associated with either the induction or the binding of the hepatic cytochrome P450 (CYP450) enzyme system. CYP450 system is responsible for the production of agents such as cholesterol and steroids, and on the other hand, this system is responsible for the detoxification of foreign materials or toxins and the metabolism of the drugs. This system could be induced and/or inhibited by certain drugs, when altered in any way, there will be developments of clinically significant drug-drug interaction which could result in unexpected therapeutic failures. Although AZM is metabolized in the liver, it does not

interact with CYP450 enzyme system making it less likely to interact with other drugs utilizing this system (Nahata, 1996; Yousef and Jaffe, 2010).

Triclosan

Triclosan (TCS), (5-chloro-2-(2,4-dichlorophenoxy)phenol), is a synthetic chlorinated aromatic compound that has an array of antimicrobial efficacy on gram positive and gram negative bacteria*; Eschericia coli, Klebsiella* spp. and *Salmonela* spp. amongst others, as well as moulds and viruses. The main functional groups of TCS are phenols and ether groups (Dhillon *et al.,* 2015; Kumar *et al.,* 2015). TCS was initially registered as a pesticide with the Environmental Protection Agency (EPA) in 1969. In 1996 TCS was approved by the Food and Drug Administration (FDA) for household products and for the first time in 1997 a toothpaste, Colgate, containing TCS was manufactured. Since then there has been a wide increase of TCS containing products such as the toothpaste, mouth wash, skin cleaners, hand soap and body lotion (Dhillon *et al.,* 2015). Recent studies have documented the effectiveness of TCS on *Plasmodium* spp., *Toxoplasma* sp. and *Babesia* spp. (McLeod *et al.,* 2001; Bork *et al.,* 2003; Singh *et al.,* 2009).

The pharmacokinetics of TCS include the ready absorption in the gastrointestinal tract and mucosa after oral administration (Paul *et al.,* 2010). The distribution and bioavailability is quite questionable due to the lipid solubility of the compound. Half-life of TCS after oral administration has been reported to range from 7 to 14 hours, nonetheless the major portion of the compound is excreted with urine within the first 24 hours of exposure (DeSalva *et al.,* 1989; Sandborgh-Englund *et al.,* 2006; Paul *et al.,* 2010).

TCS is an endocrine disruptor interfering with the hormones vital for the FAS II fatty acid synthesis, resulting the disruption of the normal development of cell membranes and

reproduction of all organisms possessing this complex. TCS blocks the active site of enoyl acyl carrier protein reductase (ENR) enzyme forming a tight complex with the reduced NAD^+ cofactor and therefore inhibiting the ENR activity. Fortunately, mammals do not have the ENR enzyme eliminating the activity of triclosan on normal pathways. Fatty acids play an important role in cells as metabolic precursors for biological membrane and represent a vital form of metabolic energy, making their biosynthesis pathway an excellent target for antimicrobial agents. The same route of action of TCS has been reported not only in bacteria but also organisms such as *P. falciparum in vitro, P. berghei in vivo,* bovine and equine *Babesia* spp., *T. gondii* and *Eimeria tenella in vitro* (El-Zawawy *et al.,* 2015; Kumar *et al.,* 2015; Gao *et al.,* 2017).

The major concern regarding TCS is toxicity effects, a number of studies were conducted to prove the safety and/or the toxicity of this compound. According to Bhargava and Leonard (1996) TCS is not an oral toxicant when administered acutely. The LD_{50} concentrations ranged from 3,750 to 5,000 mg/kg were observed in this study when TCS was administered into mice, dogs and rats. Toxicity differs from species to species tested and the route of administration. Intravenously, an LD_{50} of 19 to 29 mg/kg was recorded while intraperitoneal route resulted in an LD_{50} of 1,090 mg/kg.

Curcumin

Curcumin (CUR), ((1E, 6E)-1, 7-Bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione), is a natural polyphenolic compound isolated from *Curcuma longa.* The *C. longa* is an Indian spice rhizomatous plant belonging in the ginger family, Zingiberaceae. CUR is a principal curcuminoid of this plant in addition to desmethoxycurcumin and bis-desmethoxycurcumin. These three curcuminoids are

polyphenols responsible for the orange-yellow colour of turmeric (Mirzaei *et al.,* 2017; Shanbhag, 2017).

Tumeric has been used for years as a traditional medicine in Asia, particularly in the Southern Asian countries. The structure of CUR consists of numerous functional groups. The wide range of effects of CUR is attributed to these functional groups which are used by the molecule in order to bind and interact with a wide spectrum of pathogen targets pathways including proteins, nucleic acid and/or membrane. The binding of CUR to proteins and peptides results in the modulation of their conformation, dynamic and stability (Ahmad *et al.,* 2017). Several studies have confirmed that CUR possesses antioxidant, anti-inflammatory, immunomodulatory, antimicrobial, antiviral, antifungal, anti-carcinogenic, hepatoprotective, nephroprotective and antirhematic activities. In addition, CUR has shown therapeutic effects against diseases including; cancer, anxiety, depression, non-alcoholic fatty liver disease, cardiac disease and chronic lymphocytic leukaemia (Akram *et al.,* 2010; Moghadomtousi *et al.,* 2014; Jiang *et al.,* 2017; Lelli *et al.,* 2017; Mirzaei *et al.,* 2017; Shanbhag, 2017).

CUR is safe and tolerable even at very high concentrations. So far, no toxicity effects have been documented and/or reported in experimental animals and humans. The acute and subacute/sub-chronic tests conducted on CUR provided a reported LD_{50} greater than 5,000 mg/kg (Aggarwal *et al.,* 2016). Despite the wide range of effectiveness portrayed by curcumin, it is still not an independent drug but constitutes a main component of many Ayurvenic and Unami medicines (Ahmad *et al.,* 2017). Due to poor solubility, CUR has a low intestinal absorption, and bioavailability when given orally. Furthermore, CUR is rapidly cleared from the body, with an enormous 60% excreted in an unchanged form. According to Mirzaei *et al.,* (2017), absorption of CUR ranges between 60 – 66%, regardless the concentration. The active constituent of CUR are flavonoids which makes up a bigger

portion of the absorbed percentage of the compound, these flavonoids are metabolized in the liver and the intestinal mucosa. Elimination half-life in rats given CUR was 8.64 ± 2.31 and 32.70 ± 12.92 when administered by intravenous and orally (Akram *et al.,* 2010).

A number of studies are currently in progress, with an objective of modifying and improving the solubility, absorption and enhance a greater bioavailability of CUR in animals and humans. The studies includes the production of nanoparticles, formulation of enzymes such as bromelain, liposomes, adjuvants, micelles and phospholipid complexes (Akram *et al.,* 2010; Ahmad *et al.,* 2017; Mirzaei *et al.,* 2017).

Problem statement

Number of drugs available for the treatment of HAT and AAT is very limited. As the only widely used treatment and control measures of the infection these drugs are therefore faced with numerous obstacles. Affected regions in the African continents include the poorest countries in the world, with low socio-economic status which makes it very difficult for patients and farmers to afford the trypanocidal drugs.

The less toxic drugs, pentamidine and suramin are only effective in the early stage of the infection. Only merlasoprol and DFMO are available for the late stage of sleeping sickness. Merlasoprol toxicity is well documented, associated with severe side effects. Reports show that almost 10% of patients treated with merlasoprol develop arsenical-induced encephalopathy with pulmonary oedema and eventually die (Bacchi, 2009).

ISM, homidium chloride/bromide and DA, have been well documented for their toxicity effects in treated hosts. In Zebu cattle, the treatment of trypanosomosis with DA and ISM resulted in conspicuous dose dependent hepatotoxicity and nephrotoxic effects which are attributed to the wide distribution of the drug in the liver and the kidneys. Approximately

50% mortality was recorded in stressed zebu cattle treated with DA (Eisler *et al.,* 1997; Baldissera *et al.,* 2016). Goats treated with homidium bromide for the presence of *T. vivax* died and their death was preceded by signs of lacrimation, mucopurulent, bloody diarrhoea, amongst others (Youssif *et al.,* 2010).

The most important obstacle of the currently available trypanocidal drugs is the loss of effectivity due to a wide spread resistance of the pathogens towards the drugs due to the irrational and uncontrolled usage of the drugs. This is not the only factor contributing to the development of resistance. Due to high costs of the drugs, farmers improvise with little they possess, hoping for the best even though it is not for the best of the animals. The high cost of the trypanocidal drugs has resulted in the introduction of fake drugs in the market (Broussard, 1996; Holmes, 1997), some of which were reported not to even possessing a single effective compound but farmers buy them for their affordability and accessibility without acknowledging the possible implication/repercussions to the animals. Lack of skills to prepare correct dosages for the treatment of the infection, lack of knowledge and early detection of the parasites by the diagnostic tools has exacerbated the situation. In the remote areas the usage of trypanocidal drugs is not supervised by the veterinary offices and therefore, farmers are likely to be under dosing the animals. Frequently, when the farmers observe resistance of the parasites against the usage of drugs, they resort to increasing the recommended dosages for the treatment of the animals. This does not only worsen the resistance problem further but has resulted in death of the animals from wasting syndrome (Eisler *et al.,* 1997).

Furthermore, as shown in the picture below, the structural relatedness of the trypanocidal drugs has further exacerbated the issue of cross-resistance, where one drug possesses the same receptive side as the other (Fig. 4) (Mäser *et al.,* 2003). Therefore a structurally not related, effective, less expensive and safe drugs are in urgent need. And this

study proposes AZM, TCS and CUR. These are already produced compounds, AZM and TCS are a commercialized antimicrobial agents. This study proposes the usage of already available compounds, re-purposing of the compounds. According to Broussard (1996), pharmaceutical companies are reluctant to develop new drugs due to the fact that the countries ravaged by trypanosomosis are poor and most farmers cannot afford the drugs, hence re-purposing of the commercially available drugs.

So far, there are no orally administrable drugs for the treatment of trypanosomosis, neither in human nor animals. Trypanocidal drugs are either administered parenterally (i.v and/or i.m). Intravenous administration is fast but it can be painful with the ability of destroying the veins after several applications (Bacchi, 2009). Intramuscular injections have a good absorption associated with the rich blood supply to the muscles, however, the pressure exerted by a large quantity can be painful. In addition, parenterally administered drugs tend to remain at the injection site, exposing the injection site as a liability for secondary infections. Most importantly, parenteral administration require skilled technician and practicing operator to deliver the injection. Therefore, farmers cannot carry out administration themselves, but have to wait for technical assistance which could take long based on the accessibility of the place, farmers in remote areas wait longer than deems necessary until the animals are in bad shape. That is why the current study proposed the oral treatment of the compounds for trypanocidal effects (Irungu *et al.,* 2008). *T. congolense* is mostly a pathogenic parasite of ruminants, and the applicability of oral treatment will definitely be difficult but not impossible. Megazol ($C_6H_6N_6O_2S$) was tested and trialled for the oral effectivity on *T. congolense* infected sheep, unfortunately due to DNA damage toxicity observed during clinical testing, the drug was dropped from production (Enanga *et al.,* 2003).

Nonetheless, contrary to popular belief, AAT is not exclusively an infection of ruminants but other hosts such as dogs and pigs are implicated, in which oral treatment has never been difficult. Currently in the clinical testing for HAT, in the same group with megazol is fexinidazole ($C_{12}H_{13}N_3O_3S$) has demonstrated effectiveness when administered orally (Darsaud *et al.,* 2004; Torreele *et al.,* 2010; Kaiser *et al.,* 2011.

Therefore the current study seeks to evaluate and validate the usage of AZM, TCS and CUR for the treatment of AAT.

Aim and Objectives

Aim

To determine the trypanocidal effect of the compounds on animal trypanosomes.

Objectives

To determine the efficacy of AZM, CUR and TCS compounds *in vitro* on *T. congolense, T. b. brucei* and *T. evansi.*

To determine the cytotoxicity effects of AZM, CUR and TCS on the Madin-Darby bovine kidney cell (MDBK) and NIH 3T3 and determine the selectivity index thereof.

To determine a possible mode of action of trypanocidal compounds (AZM) using a transmission electron microscope analysis.

To establish an oral and/or intraperitoneal drug administration mode for the treatment of *T. congolense* and/or *T. b. brucei* in infected mice for AZM, CUR and TCS.

To improve the solubility, bioavailability and effectivity of CUR by using nanotechnology.

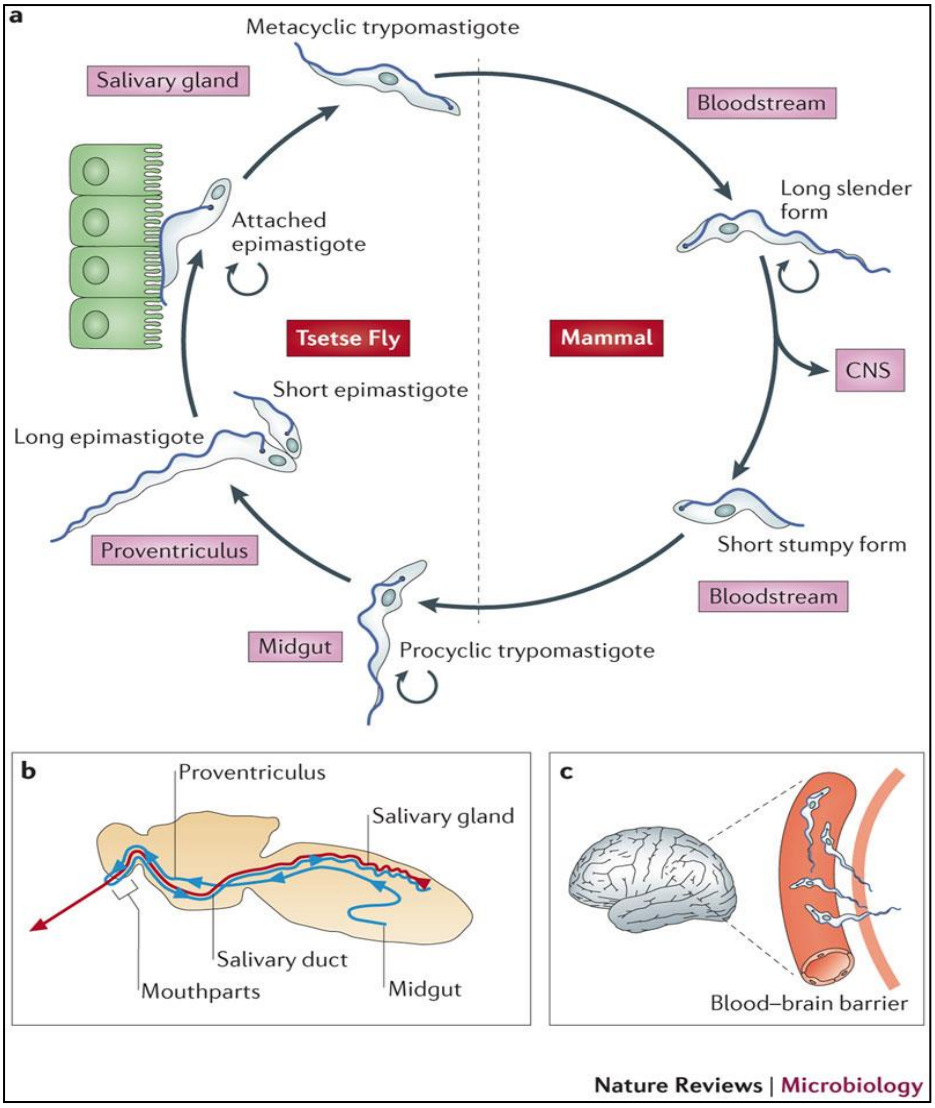


Figure 1: The general life cycle of *Trypanosoma brucei* – *Trypanozoon* (Langousis and Hill, 2014).

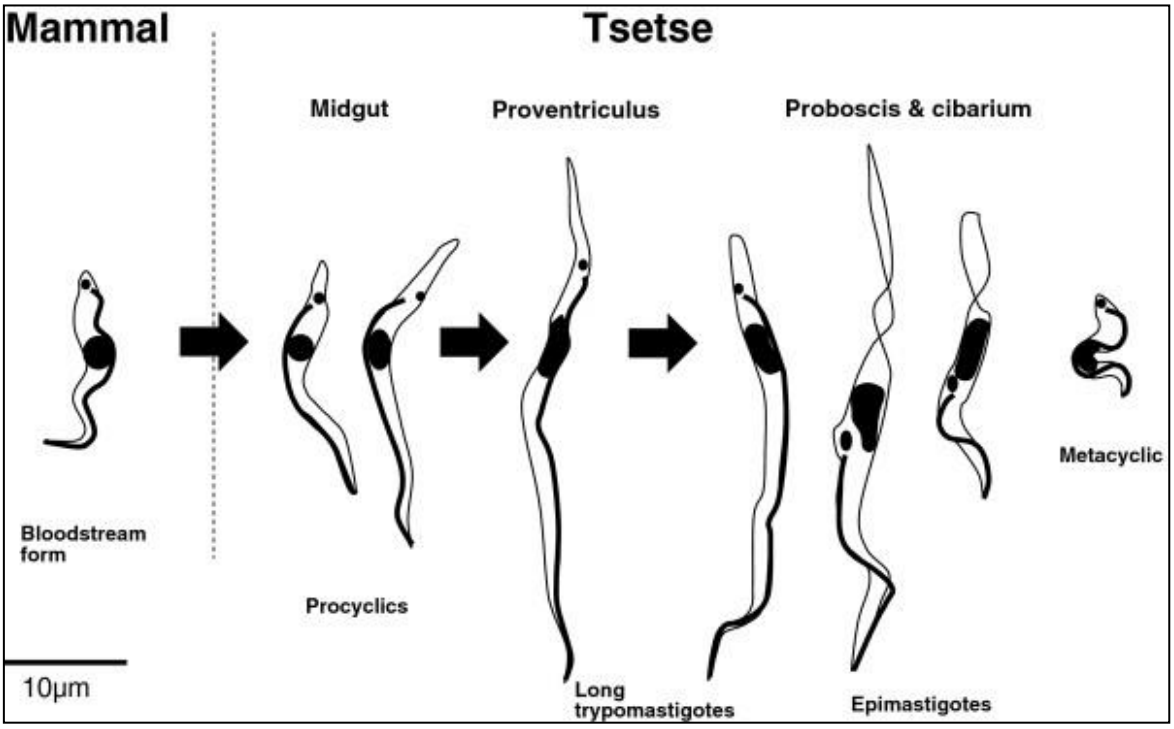


Figure 2: Life cycle of *Trypanosoma congolense* - *Nannomonas* (Peacock *et al.*, 2012).

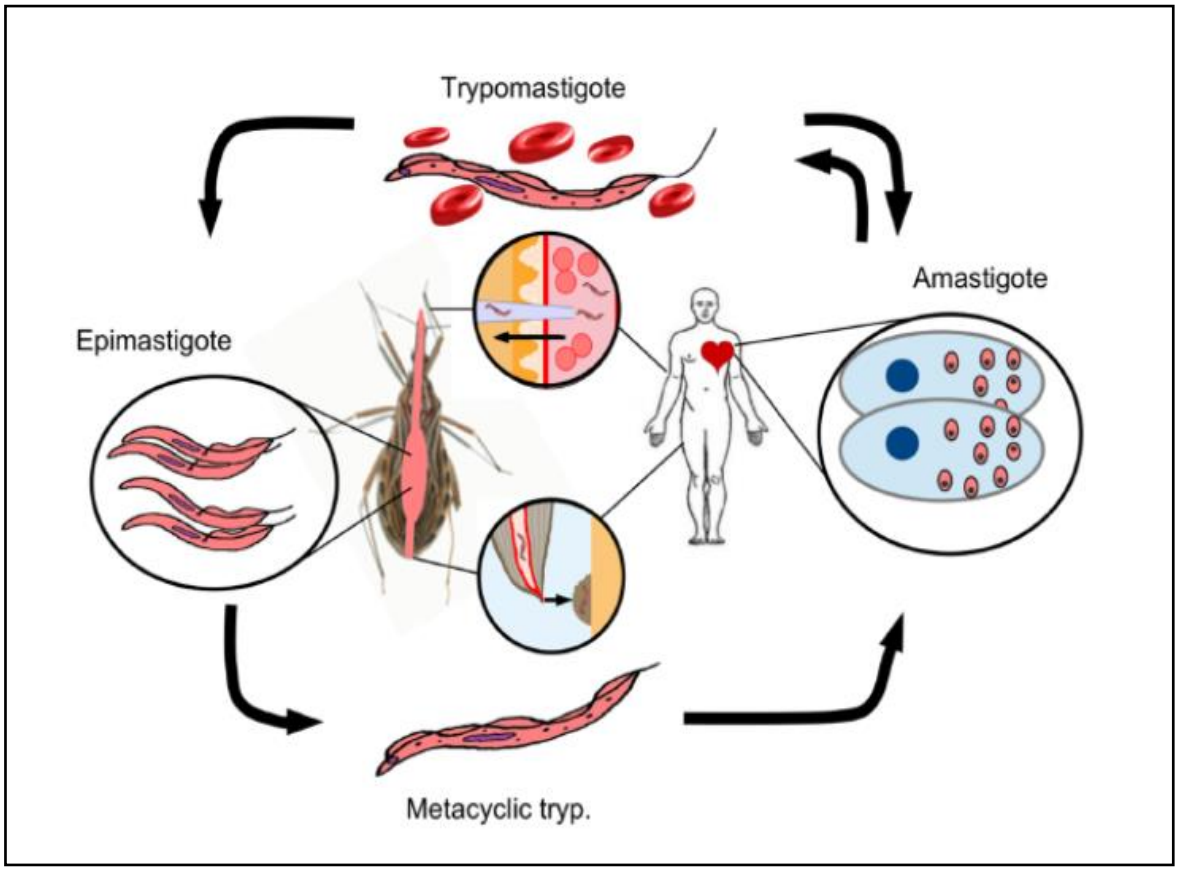


Figure 3: Life cycle of a typical stercorarian trypananosome (Carrea and Diambra, 2016).

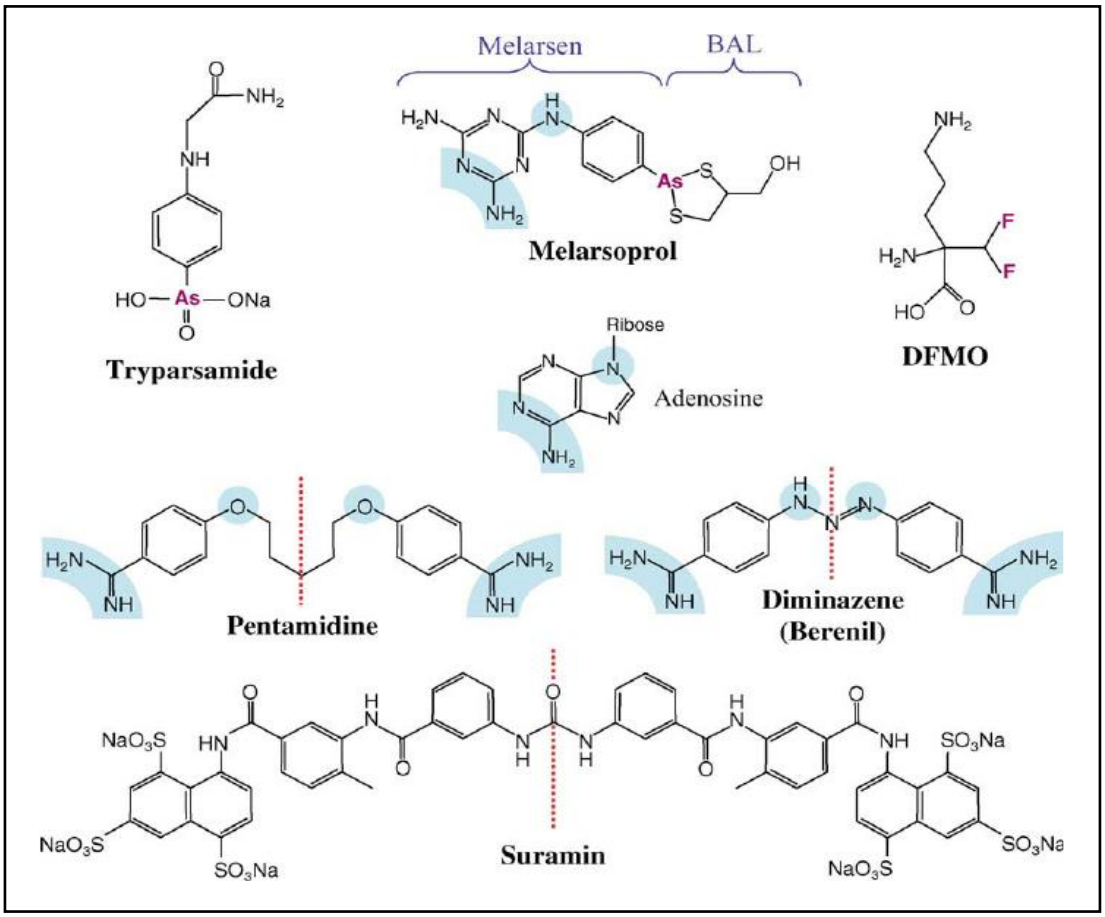


Figure 4: Structural relatedness of the trypanocidal drugs (Mäser *et al.*, 2003).

Chapter 1

Oral Administration of Azithromycin Ameliorates Trypanosomosis in *Trypanosoma congolense* and *T. brucei brucei* Infected Mice

Introduction

Azithromycin (AZM), $C_{38}H_{72}N_2O_{12} \cdot 2H_2O$, is a macrolide antibacterial agent that is used for the treatment of various bacterial infections (Parnham *et al.*, 2014). This compound has recently received a great deal of scientific attention due to its outstanding efficacy in the treatment of bacterial infections and other parasitic infections, including *Babesia* spp., *Toxoplasma gondii* and *Plasmodium* spp. (Blais, 1993; Birkenheuer *et al.*, 2004; Wilson *et al.*, 2015). As a widely used compound, it is generally considered safe for use by humans and is reported to be associated with fewer side effects in comparison to other macrolides. Most importantly, macrolide antibiotics are generally affordable (Wilson *et al.*, 2015). The main mechanism of action of macrolide antibacterial agents against bacteria is understood to be the inhibition of protein synthesis. AZM exhibits the same mechanism against other organisms, including *T. gondii* and *Plasmodium* spp. (Blais, 1993; Wilson *et al.*, 2015). In addition to the well-established long half-life, the compound accumulates in phagocytes, which enhances the delivery of high concentrations of the drug to the site of infection (Parnham *et al.*, 2014).

The first objective of the study was to determine the *in vitro* effects of AZM against animal trypanosome species (*T. congolense, T. b. brucei* and *T. evansi*), and to determine the efficacy of orally administered azithromycin in *T. congolense* and *T. b. brucei*-infected mice.

Materials and methods

Parasites and *in vitro* culture

T. congolense IL3000, a savannah type strain isolated near the Kenya/Tanzania border in 1966; *T. evansi* Tansui, akinetosplatic strain isolated in Taiwan, and *T. b. brucei* GUTat 3.1, Isolated in Uganda in 1966 (Hirumi *et al.,* 1997), were maintained in the bloodstream form (BSF), and propagated at 33°C and 37°C, respectively, in 5% CO_2 using HMI-9 medium (Hirumi and Hirumi, 1991). The medium is composed of Iscove's modified Dulbecco's medium (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 20% heat inactivated fetal bovine serum (HI-FBS), 60 mM HEPES (Sigma-Aldrich), 1 mM pyruvic acid sodium salt (Sigma-Aldrich), 0.1 mM bathocuproine (Sigma-Aldrich), 1 mM hypoxanthine and 16 μM thymidine (HT supplement: Thermo Fisher Scientific K.K., Yokohama, Japan), 10 μg/L insulin, 5.5 μg/L transferrin and 6.7 ng/L sodium selenite (ITS-X: Thermo Fisher Scientific), 0.0001% 2-β-mercaptoethanol (Sigma-Aldrich), 0.4 g/L (Sigma-Aldrich) and 2 mM L-cysteine (Sigma-Aldrich). *T. congolense* cultures were maintained by replacing the entire supernatant with fresh medium every other day, while those of *T. b. brucei* and *T. evansi* were maintained by replacing part of the supernatant (Suganuma *et al.,* 2014).

Cell line cultures

The Madin-Darby bovine kidney cell (MDBK NBL-1 strain: JCRB cell bank) and mouse embryonic fibroblast, NIH 3T3 (Courtesy of Prof. Makoto Igarashi, National Research Center for Protozoan Diseases, Obihiro, Japan) cell lines were revived from the liquid nitrogen-preserved stock of Obihiro University of Agriculture and Veterinary Medicine, Japan. The cells were cultured in Minimum Essential Medium Eagle (MEM)

suspended with 10% HI-FBS at 37˚C in an incubator under 5% CO_2. The cells were maintained by replacing the medium with fresh medium 2 days before the cells become confluent.

The *in vitro* evaluation of trypanocidal effects of AZM

T. congolense, T. b. brucei and *T. evansi* were seeded at 1×10^5; 1×10^4 and 2×10^4 cells/mL, respectively in a Nunc™ 96-well optical bottom plate (Thermo Fisher Scientific) and exposed to various concentration of AZM (0.32 to 25 µg/mL; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The plates were incubated at 33°C (*T. congolense*) and 37°C (*T. b. brucei* and *T. evansi*) for 72 h. Subsequently, 25 µL of CellTiter-Glo™ Luminescent Cell Viability Assay reagent (Promega Japan, Tokyo, Japan) was added to evaluate intracellular ATP concentration. The plate was shaken for 2 min (500 shakes/min) using a MS3 basic plate shaker (IKA® Japan K.K., Osaka, Japan) to facilitate cell lysis and the release of intracellular ATP. The plates were further incubated for 10 min at room temperature and were subsequently read using a GloMax® -Multi+ Detection System plate reader (Promega Japan) (Suganuma *et al.*, 2014). The experiments were conducted in triplicates.

The *in vitro* cytotoxicity tests

Mammalian cell lines (MDBK and NIH 3T3) were seeded separately at a concentration of 1×10^5 cells/mL in a 96 well microtiter plate (Thermo Fisher Scientific) and exposed to various concentrations of AZM (0.32 to 25 µg/mL). The surviving cell fraction was determined by a CCK-8 (Dojindo Laboratories., Kumamoto, Japan) assay by adding 10 µL of CCK-8 reagent onto the plates at 72 h after incubation. The plates were further incubated for an additional 2 hours at 37°C. Cell viability was determined using an

ELISA reader (MTP 500, Corona electric, Ibaraki, Japan), according to the amount of formazan that formed, which was determined based on the absorbance at 450 nm (Weyermann *et al.,* 2005; Lou *et al.,* 2010). The experiments were conducted in triplicates.

The selectivity index was calculated to allow for the possible identification of compounds with high efficacy and low cytotoxicity. This parameter reflects the quantity of the compound that is active against the pathogen but which is not toxic towards the host cells. The following formula was used:

Selectivity index = Minimum toxic concentration (μg/mL)/minimum inhibitory concentration (μg/mL)

where the minimum toxic concentration is the compound concentration that inhibited 50% of the cell growth, while the minimum inhibitory concentration was the concentration that inhibited the proliferation of the parasite by 50%.

Animal experiments

Healthy female BALB/c for *T. congolense* infection and C57BL/6 mice for *T. b. brucei* infection (CLEA Japan Inc., Tokyo, Japan) weighing 20–30 g and 16-25 g were used in the study, respectively. The mice were kept in the animal facility of Obihiro University of Agriculture and Veterinary Medicine, Japan. The animals acclimatized in plastic cages in an air conditioned environment and were maintained at 25 ± 2°C with 60 ± 10% relative humidity under a 12-h light and dark cycle for one week before commencing the experiments. All of the animals had *ad libitum* access to normal chow and water. The experiment was

approved by the animal ethics committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (Approval Nos. 28-129 and 28-169).

The trypanocidal effects of AZM on *Trypanosoma in vivo*

Short-term treatment

The virulent *T. congolense* IL3000 and *T. b. brucei* GuTat 3.1 strain were propagated in mice and used for infection. The parasites were passaged twice in mice before the experiment. The mice were intraperitoneally infected with 100 µL of *T. congolense* (5×10^3 parasites/mouse) and *T. b. brucei* (1×10^2 parasites/mouse) inoculated with Phosphate buffered saline with 1% glucose (PSG). The mice were randomly divided into 7 groups of 5 mice as follows: Group I (control group), the mice were infected but not treated; group II (positive control group), the mice were infected and treated with diminazene aceturate (DA - 3.5 mg/kg, intraperitoneally) (Sigma Aldrich, Japan); Groups III, IV, V, VI and VII (the test groups), the mice were infected and orally treated using a feeding needle with 50, 100, 200, 300 and 400 mg/kg AZM in a 200 µL inoculum, respectively. Treatment was initiated at 48 and 24 hours post-infection for *T. congolense* and *T. b. brucei*, respectively. The treatment was maintained for 7 consecutive days. The treatments were freshly prepared each day. Mice were observed for 90 days before terminating the experiment. Each day, the parasitaemia was evaluated and the effects of treatment were monitored using wet blood smears. Each slide was prepared with fresh blood collected from the tail vein (magnification: 400×). The experiments were conducted in duplicate.

Long term treatment

The long-term trypanocidal effects of AZM were further investigated in *T. congolense*- and *T. b. brucei*- infected mice. The mice were divided into 8 groups of 5 mice each, as follows: Group I (the healthy control group), the mice were not infected or treated; Group II (the vehicle control group), the mice were infected and treated with vehicle; and Group III (the positive control group), the mice were infected and treated with DA; Groups IV, V, VI, VII, and VIII (the treatment groups), the mice were orally treated with 50, 100, 200, 300 or 400 mg/kg in a 200 µL inoculum, respectively, of AZM using a feeding needle. Treatment was initiated at 48 hours and 24 hours post-infection for both *T. congolense* and *T. b. brucei*, respectively, and maintained for 28 consecutive days. The parasitaemia and the body weight of the mice were recorded twice a week, while the blood parameters were evaluated once a week. The objective of this particular treatment was to determine the duration for which the *Trypanosoma*-infected mice depended on AZM. These experiments were conducted in duplicate.

The hematological parameters

The blood samples that were collected were subjected to a blood count using an automatic hematology analyzer (Celltac α, Nihon Kohden, Tokyo, Japan). The red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular hemoglobin (MCH), white blood cell count (WBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and platelet (PLT) count were analyzed.

Ultrastructural analysis of AZM treated trypanosome by transmission electron microscope (TEM)

Trypanosoma congolense and *T. b. brucei* parasites were incubated with 25 μg/mL AZM for 7 and 24 hours. Subsequently, the samples were centrifuged twice with 0.1 M phosphate buffer (PB) to wash the medium. The samples were then resuspended in 2.5% glutaraldehyde (GA), kept for an hour at 4°C and centrifuge. For the analysis the samples were resuspended in 0.1 M PB and transported to the anatomy laboratory, a courtesy of Assistant Professor Daisuke Kondoh.

Statistical analysis

The results were expressed as the mean ± standard deviation (S.D) for the number of repeated trials indicated in each experiment. The statistical analyses were conducted in the acute phase of infection. The *t*-test was used for intergroup comparisons between the treated and non-treated groups. The survival curves were constructed using the Kaplan-Meier method and the curves were compared using a log-rank test. All of the data were compiled using GraphPad Prism Software program (version 5.0, GraphPad Software Inc., CA, USA). *P* values of < 0.05 were considered to indicate statistical significance.

Results

The *in vitro* trypanocidal activity of AZM

The activity of AZM was assessed *in vitro* after 72 h incubation of *T. congolense, T. b. brucei* or *T. evansi* BSF with the treatment regimens. The IC_{50} of AZM for *T. congolense, T. b. brucei* and *T. evansi* was 0.19 ± 0.17; 3.69 ± 2.26 and 1.81 ± 1.82 (mean [in μg/mL] ± S.D), respectively. AZM was less cytotoxic to MDBK and NIH 3T3 cells with an LC_{50} of >25 μg/mL in both cell types. Based on the IC_{50} and LC_{50} values that were obtained, the selectivity index values were calculated as >131.58, 6.78 and 13.81 for *T. congolense, T. b. brucei* and *T. evansi* respectively.

The *in vivo* trypanocidal effects of short-term treatment

Oral treatment of *T. congolense*-infected mice with AZM resulted in a moderate trypanocidal effect (determined based on parasitaemia) in comparison to the control in which parasitaemia increased from day 3 to day 8 (Fig. 1.1). All of the treated groups showed a dose-dependent trypanocidal effect with the initial clearance of the parasites from the peripheral circulation in all the groups; however, this was followed by a relapse, which resulted in the rapid growth of the parasites (Fig. 1.1). At 50 mg/kg, the parasite growth in the acute phase of the infection was slightly suppressed in comparison to the control group, with significant differences on days 4, 7, 5 and 8 ($p < 0.05$). In contrast, parasite growth in the AZM 100, 200, 300, 400 mg/kg groups and the DA group from day 3 to day 8 was significantly lower in comparison to the control group ($p < 0.05$; Fig. 1.1). There was a subsequent relapse in the 100, 200, 300 and 400 mg/kg groups on days 9, 10, 11 and 14, respectively (Fig. 1.1). The survival rate was significantly prolonged in all of the treated groups ($p < 0.001$). A number of mice survived in the AZM 200, 300 and 400 mg/kg groups,

while all of the mice in the control and the AZM 50 and 100 mg/kg groups died (Fig. 1.2). The mice that survived for 90 days were considered to have been cured. In all of the treated groups, survival was significantly prolonged in comparison to the control group ($p < 0.001$). The mice in the control group all died within 9 days of the experiment while those in the AZM 50 and 100 mg/kg groups died on days 15 and 16, respectively. In the AZM 200, 300 and 400 mg/kg groups, 30%, 30% and 20% of the mice survived, respectively, throughout the whole study period (Fig. 1.2).

In *T. b. brucei* infection, there was no significant difference between the AZM treated groups as compared to the non-treated group, except the DA treated group. AZM showed efficacy on *T. b. brucei* infected mice that led to different prepatent periods in the treated groups as compared to the non-treated group. The parasites could be observed in the peripheral circulation of non-treated group on day 2, while the other groups, 50, 100 mg/kg were 3 days, and 4 days for 200, 300 and 400 mg/kg (Fig. 1.3). The parasitaemia reached the first peak wave between days 6 and 8 in all the groups. Most of the mice that made it through the peak wave survived until the experiments were terminated, 90 days post infection (Fig. 1.3). There was no significant difference ($p > 0.05$) in the survival rate of the treated groups, except in the 200 mg/kg ($p < 0.05$) treated and DA treated groups, as compared to the control group. Nonetheless, there was an observed survival of the mice with records of 10, 10, 20 and 20% survival in 50, 100, 200 and 300 mg/kg, respectively. None of the mice in the 400 mg/kg survived (0%), while all the mice in the DA groups (100%) survived. The survival rate of the mice in the 400 mg/kg was up to 12 days as compared to 11 days of the control group (Fig. 1.4).

The long-term treatment test

Changes of body weight during *Trypanosoma* infection and AZM treatment

Trypanosoma congolense infected mice showed a significant body weight increase which was observed in the AZM 300 mg/kg group comparison to the non-treated control group ($p < 0.001$) while a decrease was observed in the AZM 200 mg/kg group ($p < 0.05$). The other groups showed no significant differences in comparison to the non-treated control group (Fig. 1.5).

There was no significant difference in the body weight of *T. b. brucei* infected mice at AZM 50 mg/kg as compared to the control group, while the other groups, 100, 300 and 400 mg/kg ($p < 0.001$) and 200 mg/kg ($p < 0.05$) resulted in a significant increase in body weight, in comparison to the non-treated control group (Fig. 1.6).

Parasitaemia

After the prepatent period of *T. congolense*, parasitaemia increased steadily in the control and AZM 50 mg/kg group, while parasitaemia was suppressed for an extended period (before eventually relapsing) in the other groups (Fig. 1.7). At a dose of 100 mg/kg, parasites were detected on day 8 even though the parasitaemia was kept at minimum levels until a rapid parasite growth observed between day 13 and 14, which led to the death of 80% of the mice (Fig. 1.8). The surviving mice relapsed and died before the next parasite count. At a dose of 200 mg/kg, relapse was observed on day 13 with rapid parasite growth that led to the death of 90% of the mice in this group (Fig. 1.8). A 10% of the mice in the 200 mg/kg group survived to the end of the experiment, even though the group showed a high level of parasitaemia. The mice in the AZM 300, 400 mg/kg and DA groups initially showed parasites in their peripheral circulation. Although the parasites were eliminated during the

treatment period in the AZM 300 mg/kg group, a relapse occurred at one week post treatment termination, which led to the death of 20% of the mice in this group (Fig. 1.8). The survival of the mice was significantly prolonged ($p < 0.001$) in all the treated groups. In contrast, the control mice all died on day 10, while the mice in the AZM 50 mg/kg and 100 mg/kg groups died on days 16 and day 21, respectively (Fig. 1.8). A 10% of the mice in the AZM 200 mg/kg group survived to day 31 while the rest died during the treatment period. All mice in the AZM 300 mg/kg, 400 mg/kg and DA groups survived until the last day of the treatment; however, 20% of the mice in the AZM 300 mg/kg group relapsed and died on day 38. The rest of the mice were considered cured as no trypanosomosis-related symptoms were observed and no parasites were observed microscopically for a period of 90 days.

The long term treatment of *T. b. brucei* with AZM resulted in a significant difference of the parasitaemia levels in groups 100, 200 and 300 ($p < 0.05$), and in the 400 mg/kg and DA ($p < 0.01$) treated groups in comparison to the control group (Fig. 1.9). The first parasitaemia peak was reached on the first week of the experiment. In the control, 50, 100 and 200 mg/kg groups, the peak was maintained at a steady increase of parasitaemia for a period of 2 weeks before the parasites could be cleared off the peripheral circulation. There was a decrease of parasitaemia in the 400 mg/kg even though the parasites were not yet cleared off, while 300 mg/kg resulted in a rapid clearance of the parasites on the second week (Fig. 1.9). Most of the mice that made it through the peak wave survived until the experiments were terminated, 90 dpi, without any relapse cases observed. There was a significant survival of the mice in the groups 200 ($p < 0.0001$), 300 and 400 ($p < 0.001$) mg/kg treated groups. The survival rates were recorded as 10, 20, 30, 40, 70 and 70% in the control, 50, 100, 200, 300 and 400 mg/kg groups, respectively (Fig. 1.10). All the mice in the DA group survived (Fig. 1.10).

Changes in blood parameters of the mice during *T. congolense* infection and AZM treatment

The blood parameters of mice infected with *T. congolense* related to anemia (specifically, the red blood cell [RBC] count; hemoglobin [HGB] level and the hematocrit [HCT] ratio) in the control group and treated groups were compared to those of healthy mice. A non-significant increase - which had been anticipated - was observed in the RBC of the control group. In contrast, a significant ($p < 0.05$) increase was observed in the RBC count of the AZM 50 mg/kg group within the first and the second week of infection. Significant increases were also observed in the HGB levels of the control, and the AZM 50, 100 and 300 mg/kg groups ($p < 0.05$; $p < 0.0001$) (Table 1.1). The parameters of the treated groups also fluctuated; however, they remained within the normal ranges throughout the study period (Table 1.1). In week 3, the HCT values of the AZM 300 and 400 mg/kg groups showed a significant decrease ($p < 0.05$); however, the values increased to within the normal range in the fourth week of the study (Table 1.1).

Ultrastructural analysis by TEM

There were minor but significant changes observed in the ultrastructure of the parasites. There was a development of a non-specific vacuole in *T. congolense* incubated with AZM for 7 hours (Fig. 1.11B) as compared to the control samples which showed all the organelles intact (Fig. 1.11A). Additionally, the trypanosomes treated with AZM for 24 hours showed the increase of a vacuole engulfing the cell (Fig. 1.11C).

Figure 1.12 shows the TEM imaging of *T. b. brucei,* control (Fig. 1.12A) and treated with AZM (Fig. 1.12B) for 7 hours. Glycosome-like structures were observed in both specimens, even though they were numerous in the AZM treated specimens. In addition, the treated

samples possessed the presence of the acidocalcisome-like structures, which were absent in the control specimens. Trypanosomes incubated with AZM for 24 hours showed the presence of a vacuole-like structure containing digested materials of the cells (Fig. 1.12C).

Discussion

AZM showed greater trypanocidal efficacy against *T. congolense* than *T. b. brucei* and *T. evansi in vitro*. AZM is a well-known protein synthesis inhibitor that has shown efficacy against *Toxoplasma gondii* (Blais, 1993) and *Plasmodium falciparum* (Wilson *et al.,* 2015). The heterogeneous efficacy against the tested *Trypanosoma* species may therefore be attributed to protein synthesis, since *T. congolense* (Subgenus *Nannomonas*) differs—both morphologically and taxonomically—from *T. b. brucei* and *T. evansi* (*Trypanozoon*). Furthermore, AZM uptake in trypanosomes has not yet been established. The establishment of the drug uptake of AZM in the trypanosomes would facilitate the characterization of AZM efficacy in different species of *Trypanosoma*. Therefore, further studies will be needed to confirm this hypothesis.

In vitro toxicity tests were conducted using MDBK and NIH 3T3 cells to determine the potential of AZM to induce cell death. No toxicity was observed in either of the cell types after 72 hours of AZM treatment. This resulted in an LC_{50} of >25 μg/mL. AZM is a well-studied compound that is used to treat a variety of infections and which is generally considered safe; thus, the results of the present study were in agreement with previous studies (Parnham *et al.,* 2014; Wilson *et al.,* 2015).

Mice infected with *T. congolense* savannah strain IL3000 were treated both orally and intraperitoneally (data not shown) in the current study, in order to determine the trypanocidal effect of AZM *in vivo*. AZM showed mild to moderate efficacy against *T. congolense* BSF when it was administered orally and short-term treatment cured some of the mice in the AZM 200, 300 and 400 mg/kg groups (Fig. 1.1 and 1.2). According to a previous report (Boonleang, 2007), AZM is readily and rapidly absorbed from the gastrointestinal tract and distributed to the body tissue with excellent tissue penetration,

which makes the oral route the preferred route of administration for this compound. In addition, AZM is generally stable in acidic media, such as the gastric environment (Lode, 1991; Sultana *et al.,* 2006; Parnham *et al.,* 2014). Another benefit is the greater oral bioavailability, which is associated with the prolonged elimination of the compound from the animal body due to the well-documented half-life (57 to 68 hours) (Yousef and Jaffe, 2010; Muniz *et al.,* 2013; Kong *et al.,* 2017). When AZM was administered intraperitoneally none of the mice were cured and all died (data not shown). Signs of toxicity also occurred in association with the intraperitoneal tests in the present study, which resulted in loss of body weight, loss of appetite, rough hair coat and hunched back in the AZM 400 mg/kg group (Parasuraman, 2011). Few studies have investigated the pharmacokinetics of intraperitoneally administered AZM.

There are numerous factors that are likely to influence the effectiveness of orally administered drugs in comparison to other routes of administrations. AZM has been shown to be effective when administered orally; however, not all of the tested concentrations were effective. This might be associated with factors such as drug-nutrient or food interaction(s) (Foulds, 1996; Bushra *et al.,* 2011). Bushra *et al.* (2011) stated that AZM absorption is negatively affected by the intake of food, which reduces the total bioavailability by 43%. This factor might be associated with the failure of other subliminal concentrations. Conversely, other studies have claimed that the intake of food does not affect the bioavailability of AZM in any way, unless antacids are consumed together with the compound (Foulds, 1996; Zuckerman, 2004). Under-dosing is another factor that could account for the treatment failure. The doses of AZM that are administered for babesiosis, toxoplasmosis and bacterial infections range from 500 and 1,000 mg/kg daily for a prescribed number of days (Chico *et al.,* 2008; Parnham *et al.,* 2014). In contrast, the highest dose that was administered in the present study was 400 mg/kg.

In the treatment groups, the parasite burden was relieved for a number of days. In contrast, patent parasites were observed in the control group throughout the study. However, although some of the mice did not relapse, the cessation of treatment was followed by relapse in all of the treatment groups. Relapse led to a rapid increase in parasitaemia; in all cases of relapse, this resulted in the death within 3 days of the relapse. The mechanism underlying the effect of AZM on bacteria is well documented: AZM inhibits protein synthesis in the bacteria; thus, AZM possesses a bacteriostatic effect rather than a bactericidal effect (Dorfman *et al.,* 2008). This mechanism has also been documented in *Toxoplasma* and *Plasmodium* studies (Blais, 1993; Wilson *et al.,* 2015). Thus, this mechanism was likely to have been responsible for the effects in the present study, given the fact that parasitaemia was initially suppressed (in all of the treated groups) and that relapse occurred when the treatment stopped (Fig. 1.1). The inability of the compound to clear the parasites from the peripheral blood circulation could be one of the reasons for the relapse. The second factor is that the compound is readily absorbed by the body tissues, which means that the concentration of the compound in the plasma drops rapidly as the concentration in the cells increases (Boonleang, 2007). AZM was not suitable as a prophylactic agent for the same reason (data not shown)—this was evidenced by the fact that all of the test mice died. Few mice were cured from infection when mice were treated with oral AZM for 7 days at doses of 200, 300 and 400 mg/kg.

Even though AZM is a bacteriostatic agent, several studies have claimed that it is likely to show bactericidal effects over longer treatment periods and at increased concentrations (Dorfman *et al.,* 2008). Thus, we also investigated the effects of long-term treatment in the mice infected with the *T. congolense* IL3000. Throughout the experiment, the body weight, parasitaemia, blood parameters and clinical signs were taken into consideration. In line with our hypothesis, the survival of the mice was significantly

prolonged in all of the treated groups, moreover, 80% and 100% of the mice in 300 and 400 mg/kg groups survived (Fig. 1.8). These mice were monitored for 90 days without showing any signs of parasitaemia in the peripheral circulation; they were therefore considered cured. The body weight of the surviving mice in these two groups was within the normal range (Fig. 1.5). Their blood parameters also fluctuated within the normal ranges (Table 1.1). No trypanosome-related symptoms were observed in these groups until the mice were sacrificed; the blood parameters also remained within the normal ranges, which might indicate that AZM ameliorated anemia, by minimizing the number of parasites, then eventually clearing the parasites from the peripheral circulation of the surviving mice. Thus, the hypothesis that AZM is trypanostatic and that the trypanocidal effects are concentration- and duration-dependent, was confirmed.

The *in vivo* tests between *T. congolense* and *T. b. brucei* confirmed the *in vitro* efficacy of AZM against these two causative agents. In comparison to efficacy of AZM on *T. congolense*, the compound was less effective on *T. b. brucei* where by no 100% survival was attained in neither the short nor the long term treatment (Figs. 1.4 and 1.10). The objective of conducting the *T. b. brucei* tests was to determine the efficacy of AZM on a pathogen closely related to the human pathogens, *T. b. gambiense and T. b. rhodensiense.* The *T. b. brucei* exists as a trypanolytic complex, and therefore can be lysed by the apolipoprotein L1 (APOL1) and the haptoglobulin in the human and most primate serum (Oli *et al.,* 2006; Thomson *et al.,* 2014). The *T. b. brucei* was selected and used for this study due to the fact that it is non-pathogenic to humans which is safe to handle for experimental purposes.

There are several factors that could be responsible for the observed differences in AZM efficacy on *T. congolense* and *T. b. brucei* which includes the target mode of action, VSG differences and the intra and extravascular traits of these two pathogens. Antiparasitic

drugs are developed in order to target and/or inhibit a specific intracellular signalling pathway that are vital to the pathogens, either being responsible for metabolism of the nutrients, survival of the pathogens in the hosts or for cell replication. The signalling pathways, however, are not universal but mostly species specific (Botero *et al.,* 2017). The similar phenomenon is also responsible for the efficacy of pentamidine and suramin on the early stages of *T. b. gambiense* and *T. b. rhodensiense*, respectively. Similar outcomes were reported by Sahin *et al.,* (2014) where *T. congolense* showed susceptibility towards isometamidium (ISM), and the commercial drugs, Veridium® and Samorin® in comparison to *T. b. brucei* that was 15 folds less susceptible. The difference in the efficacy was attributed to the variation in the mitochondrial energy metabolism existing in *Trypanosoma* species, a signalling pathway responsible for the uptake of ISM (Sahin *et al.*, 2014).

African trypanosomosis pathology correlates to the sites of accumulation of the parasite in the host. The susceptibility of *T. congolense* and the resistance of *T. b. brucei* observed in this study could be attributed to the tissue tropism of these two trypanosome species. Tissue tropism is the ability of a pathogen to invade one or more cells and/or tissues. *T. congolense* is a strictly intravascular parasite constrained in the blood vessels, which means that any effective compound well distributed in the blood is likely to affect and/or clear off this parasite. Furthermore, *T. congolense* is exposed continuously to the host circulatory factors (Kuriakose *et al.,* 2012). Whilst *T. b. brucei*, is broadly tropic with the potential to invade extracellular sites such as the tissues, adipose tissues and most importantly the CNS. The drug efficacy on *T. b. brucei* in the later stage is determined by the ability to penetrate tissues (McCall *et al.*, 2016). This is the leading cause of relapses in *T. b. brucei* infection post treatment, the parasites take refuge in the sites not accessible for treatment (Poltera *et al.,* 1981; Moulton, 1986; Ezeh *et al.,* 2016).

The infection takes on various stages as the pathogen moves from one site to the other in the host. Prior infection by *T. b. brucei,* the parasites multiply extracellularly for a period of weeks to months resulting in acute symptom manifestations. The highest parasitaemia attained in the blood is followed by a sudden clearance of the parasites from the peripheral circulation, resulting from two independent phenomena, either the immune response or most likely the penetration of the parasites into the extravascular sites (Bonnet *et al.,* 2017). The migration of parasites is through the blood vessel endothelium, spread through the connective tissues and eventually infiltrating the hosts' CNS. In the CNS, the parasites initiate a cascade of events leading to the characteristic neuropsychiatric symptoms expressed in the *T. b. brucei* hosts such as aggression, restlessness and sleeping disorders before eventually killing the host (Hill, 2003). Trindade *et al.* (2016), demonstrated the *T. b. brucei* potential to accumulate in the adipose tissue, whereby observed parasites made up to 100 to 800 fold more than in the brain related to the weight loss or the wasting disease of this infection. Research has shown that there are other sites which have not yet fully been explored for the presence of the parasites. Matzneller *et al.* (2013) study demonstrated the AZM concentration variability between the blood or plasma and the extracellular sites. According to this study, AZM concentration in the extracellular sites remains remarkably lower than the plasma, which could account for the distinct efficacy of AZM on *T. congolense* and *T. b. brucei* based on tissue tropism of these parasites. Nonetheless, regardless of the documented less concentrations of AZM in the extracellular sites, the compound still cured 70% of the *T. brucei brucei*- infected mice at 300 and 400 mg/kg. This factor was attributed to the immunomodulatory effects of AZM in mice as well as ability of the compound to influence the virulence of the pathogens (Krolewiecki *et al.,* 2002; Matzneller *et al*., 2013; Castro-Filice *et al.,* 2014).

BALB/c and C57BL/6 were used to conduct the *T. congolence* (IL3000) and *T. b. brucei* (GuTat 3.1) tests, respectively. According to Kemp and Teale (1998), variation in susceptibility or resistance to trypanosomosis do not only occur in humans or natural animal host but even in the laboratory rodents. The choice of mice was based on the susceptibility and/or resistance of each strain to the respective infections (Sima *et al.*, 2011). Pathogenic and virulent *Trypanosoma* strains were used in this study, mainly with an objective to determine the efficacy of the compounds in an acute setting. Virulence of the trypanosomes was measured by the clinical manifestations, such as the loss of appetite, rough hair, rapid respiration, lethargy, parasitaemia development and peak period as well as the survival period of mice. Even though there is no obvious correlation between the number of parasites and the severity of the disease, nonetheless, virulence is judged based on the parasitaemia levels attained in the host, the pre-patent periods, levels of pathology and the transmission efficiency by the vector. Parasites eventually become virulent due to the lack of self-limiting differentiation, allowing a continuous exponential growth resulting in an inevitable rapid death in the model mice (Gjini *et al.,* 2010; Morrison *et al.,* 2010).

In the *T. b. brucei* tests, no 100% survival was observed in any of the treated group, furthermore, 1/10 mice in the control group made it through the initial parasitaemia wave and further survived for 28 days post infection prior sacrifice (Fig. 1.10). Several studies demonstrated the resistance of C57BL/6 mice against various pathogens, including; *Leishmania* spp., *Plasmodium* spp., *Babesia* spp. and bacterial infections (Yap and Stevenson, 1994; Aguilar-Delfin *et al.,* 2001; Breitbach *et al.,* 2006; Colpitts and Scott, 2011). The survival of a non-treated mouse could be attributed to either of two options, the resistance of the mice strain and/or the chronic infection. Resistance of the host is based mainly on three components, the ability to resist parasite-induced immunosuppression, to resist the parasite induced pathological symptoms, anaemia and the ability to limit parasite

population expression (Black *et al.,* 1985). Resistant mice strain have the ability to clear the infection, develop low parasitaemia and show a prolonged survival rate without any treatment as compared to the susceptible strains, however, they eventually succumb to the infection (Morrison *et al.,* 2010). Even though not all individuals are capable of clearing the infection, survival rate of 1 out of 10 in non-treated hosts is acceptable. The surviving mouse is potentially clearing the parasite infection as was also the case in a study by Mitchell and Pearson (1986).

The C57BL/6 mice are relatively resistant to trypanosomosis with a reported 110 days following *T. congolense* while BALB/c and A/J strains survived for a period of 50 and 16 days, respectively (Kemp and Tealer, 1998). The exact analogous responsible for the resistance of the host towards trypanosomosis in either the cattle or the mice has not yet been determined, however, it is believed that several genes are involved in trypanotolerance. The surviving non-treated mouse was sacrificed on the 30th day post infection while the mice form other groups were left to survive for 90 days prior sacrifice. There were no trypanosomes observed in the CSF of neither the non-treated nor the treated group, however, it is suspected that the absence of the parasites in the control group was due to little time allocated to this mouse before the sacrifice, while other mice were left for an ample time to develop a chronic stage, provided that the infection is progressive and requires 3 weeks to 2 months' time for the parasites to invade the CSF (Mogk *et al.,* 2014).

AZM is a well-known protein synthesis inhibitor as has been documented in several studies (Parnham *et al.,* 2014). During the parasitaemia count, morphological alteration was observed microscopically. The alterations were confirmed through TEM analysis on *T. congolense* which demonstrated the presence and/or the development of a non-specific vacuole. As depicted in the microscopic analysis, there was a transformation of the long slender into short stumpy form, justified with the observed decrease in area, the perimeter

as well as the shortening of the major axis. Numerous authors have associated the vacuole development with either apoptosis and/or autophagy (Uzcategui *et al.,* 2007; Veiga-Santos *et al.,* 2012; Romano *et al.,* 2014).

Numerous cell deaths exist in the unicellular parasites, even though apoptosis and autophagy are the commonest of all. Often, apoptosis and autophagy occur almost concurrently, with reports on *Leishmania* spp., *T. b. brucei* and *T. cruzi*. These two phenomena are triggered as a response to various and diverse stimuli such as the Reactive Oxygen Species (ROS), starvation, antimicrobial peptides, antibodies, mutations in the cell cycle and the antiparasitic drugs (Smirlis *et al.,* 2010).

In *T. b. brucei* factors attributed to apoptosis includes dysfunction of the mitochondrion, associated with unstable changes of the mitochondrial membrane changes ($\Delta\Psi m$). $\Delta\Psi m$ functions as a key indicator for possible apoptosis of the mitochondrion. Most often, antiparasitc drugs induce physiological stress, nutrient deprivation, heat stress, prolonged endoplasmic reticulum (ER) stress in the parasites, of all which are likely the conditions associated with the presence of apoptosis. Therefore, antiparasitic drugs activity could be mediated by the loss of $\Delta\Psi m$. Mitochondrial dysfunction therefore serves as the initial stage of apoptosis in the trypanosomatids. Another factor responsible for apoptosis is the ROS in association with imbalance calcium homeostasis. ROS is triggered by lipid peroxidation, a process where free radicals access electrons from lipids making up the cell membrane and therefore affecting the membrane fluidity and eventually disturbing the calcium channel function and homeostasis (Smirlis *et al.,* 2010). Besides the above mentioned, there is still dearth of knowledge regarding the cause and/or source of apoptosis in trypanosomatids due to the fact that these pathogens do not possess most of the documented genes or enzymes responsible either for apoptosis or combating apoptosis in mammals (Matsuo *et al.,* 2010; Smirlis *et al.,* 2010).

Even though apoptosis is mostly associated with autophagy, there are still controversy issues as to whether they occur concurrently or independently of each other. Autophagy reportedly plays a fundamental role in the life cycle of the trypanosomatids as the parasite differentiates from one form to the other by eliminating unnecessary components for the new stage (Vickerman and Tetley, 1970). Reported by Brennand *et al.* (2012), *T. cruzi* treated with edelfosine or ketoconazole expressed morphological alterations in plasma membranes and in reservosomes of the epimastigotes when used independently. Whilst, severe mitochondrial damage, formation of autophagic structures and multinucleation were induced in combination of the two processes. The authors further stated that the trypanocidal agents such as dinitroaniline herbicide trifluralin and its intermediate chloralin induced the appearance of vacuoles with damaged membranes, these effects were associated with autophagy. In *T. b. brucei*, the induction of autophagy has been observed from the addition of dihydroxyacetone (DHA) (Uzcátegui *et al.,* 2014). Unlike most cells, trypanosomes cannot process the toxic DHA due to lack of DHA kinase used by cells to phosphorylate the DHA utilized by the cells to feed it in the glycolysis pathways, but they rather take up the molecule via aquaglyceroporins. As a result of the non-metabolised DHA, morphological alterations such as the vesicular structures in the cytosol, multivesicular bodies and autophagy like vacuoles were observed. Generally, the trypanosomatids resort to autophagy during unfavourable conditions, attributed to vacuole development observed in *T. congolense* in the current study, possibly induced stress by the treatment with AZM (Michels *et al.,* 2006; Besteiro *et al.,* 2007; Brennand *et al.,* 2012). This is according to my knowledge the first report in *T. congolense.*

In comparison to changes observed in *T. congolense* incubated with AZM for 7 hours, no significant changes were observed in the *T. b. brucei* treated samples. Glycosome-like structures and acidocalcisome-like structures were observed, which are vital for the survival

of the parasites in different environmemntal conditions. Glycosomes and acidocalcisomes are perculiar to trypanosomatids. Glycosomes contains the majority of the enzymes of the glycolytic pathway and are therefore used by *T. b. brucei* bloodstream form for glycolysis since the this form depends entirely on glucose metabolism for ATP supply (Haanstra *et al.,* 2016) . Acidocalcisomes are a major storage compartmet for phosphorus compounds and cations such as calcium in different species belonging to the group of lysosome related organelles. These organelles play a role in osmoregulation and interact with the contractile vacuole complex. Their acidification is driven by either vacuolar H^+-ATPase or a vacuolar H^+- pyrophosphatase in *T. brucei* (Huang and Docampo, 2015).

Studies have shown that glycosomes and acidocalcisomes are affected by the enviromental stress such as starvation, hyper and hyposmotic stress and play a major role in the parasite response to evironmental stress (Brennand *et al.,* 2012; Moreno and Docampo, 2009). External stress have an effect on the glycosome composition, enzymatic composition, cellular metabolism and therefore contribute greatly to the response of the parasites to variable conditions. The result of external stress include in rapid hydrolysis leading to the sythesis of acidocalcisomes. Furthermore, acidocalcisomes are associated with autophagy of the trypanosomes, especially *T. brucei* (Li and He, 2014). According to (Docampo *et al.,* 2010), starvation and chemical-induced autophagy is accompanied by acidocalcisome acidification. It was conlcuded that blocking of acidocalcisomes blocks autophagy due to the fact that lysosomes are needed for the autophagy process initiation. In the current study, acidocalcisome-like structures were only observed in the AZM treated *T. b. brucei* samples, which suggests that autophagy could have taken place and indicated by the IC_{50} values calculated at the beginning of the study.

In conclusion, AZM possessed *in vitro* trypanocidal effects against *T. congolense*, *T. b brucei* and *T. evansi* parasites *in vitro* without any cytotoxic effects in MDBK and NIH

3T3 cells. The mode of action exhibited by AZM on trypanosomes is not yet known, however, the development of a vacuole was observed in the current study on AZM treated *T. congolense* parasites as shown in figures 1.11 and 1.12, therefore indicating the need for further studies in order to seek understanding of this observation. Oral treatment with AZM showed a marked trypanocidal effect against *T. congolense*. Animal trypanosomosis does not only affect ruminants, but the infection is actually life-threatening in indigenous African dogs. Moreover, there has been a misidentification of trypanosome species pathogenic to pigs, *T. suis* and *T. simiae*. Several studies have reported the pathogenicity of *T. congolesnse* in pigs (Hoare, 1936; Katunguka-Rwakishaya, 1996). Thus, oral AZM treatment might be a better solution for the treatment of dogs and/or pigs. This is the first study to demonstrate the efficacy of oral AZM against *T. congolense* and *T. b. brucei* in mice. Subsequent studies should be aimed at determining the efficacy of oral AZM against animal trypanosomes and to investigate whether it is a suitable treatment for the definitive hosts of *T. congolense.*

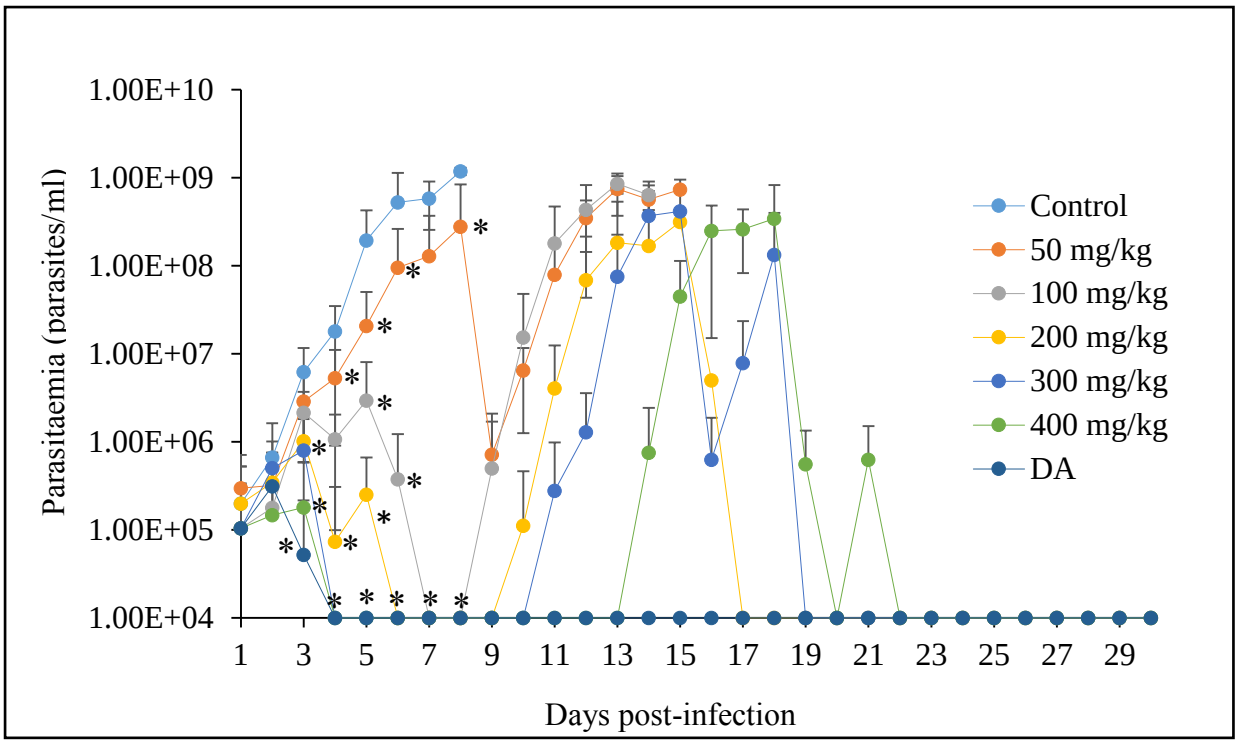


Figure 1.1: The evaluation of parasitaemia in mice infected with *T. congolense* and orally treated with different concentrations of AZM for 7 days. * $p < 0.05$ at 100 mg/kg on days 4, 5, 7 and 8, while the parasitaemia of all of the other groups showed significant differences in comparison to the control group from days 3 to 8. The data are expressed as the mean ± S.D. 1×10^4 represents parasitaemia below the detection levels.

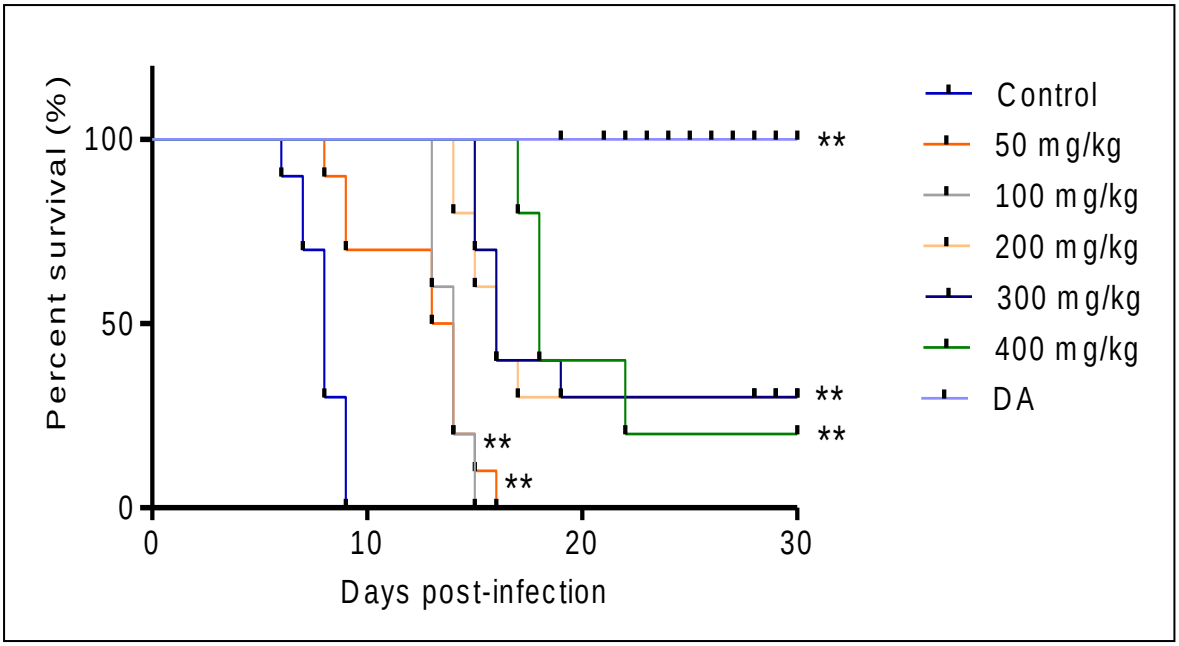


Figure 1.2: Survival curves of mice infected with *T. congolense* and orally treated with different concentrations of AZM. The survival rate was significantly different from that of the control group (n = 10), ** $p < 0.001$ (Log-rank test).

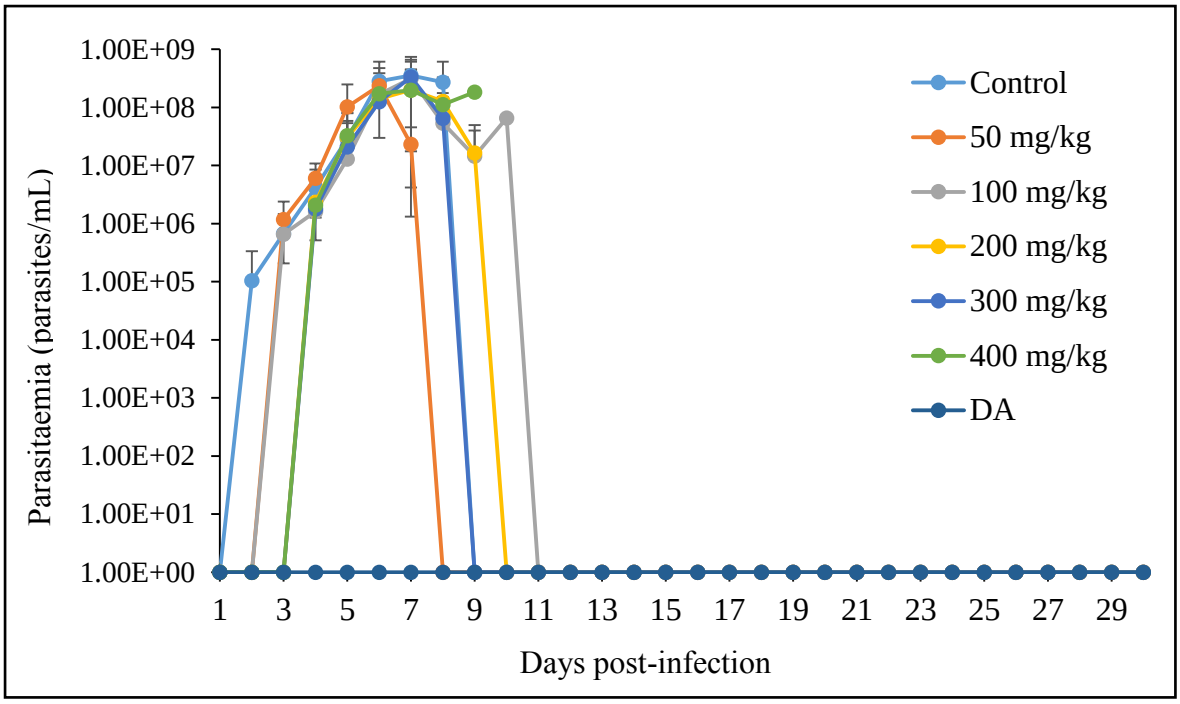


Figure 1.3: The evaluation of parasitaemia in mice infected with *T. b. brucei* and orally treated with different concentrations of AZM for 7 days. There was no significant difference in the treated groups in comparison to the control group ($p > 0.05$). The data are expressed as the mean ± S.D. 1×10^0 represents parasitaemia below the detection levels.

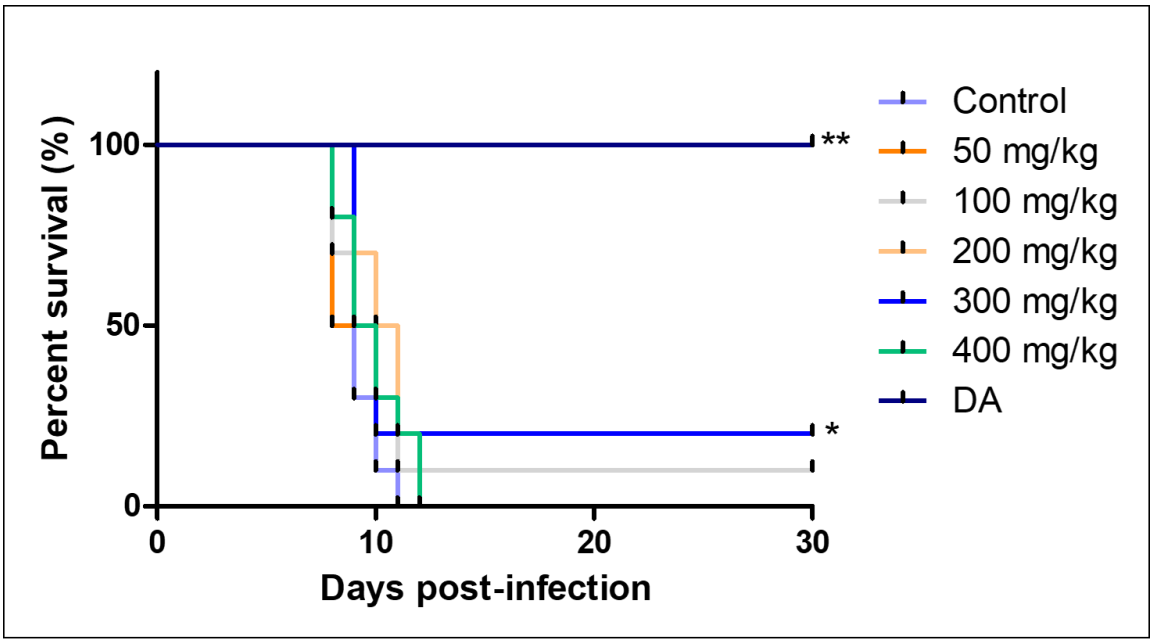


Figure 1.4: Survival curves of mice infected with *T. b. brucei* and orally treated with different concentrations of AZM. The survival rate was not significantly different from that of the control group (n = 10), ** $p < 0.001$ in DA group and * $p < 0.05$ at 200 mg/kg (Log-rank test).

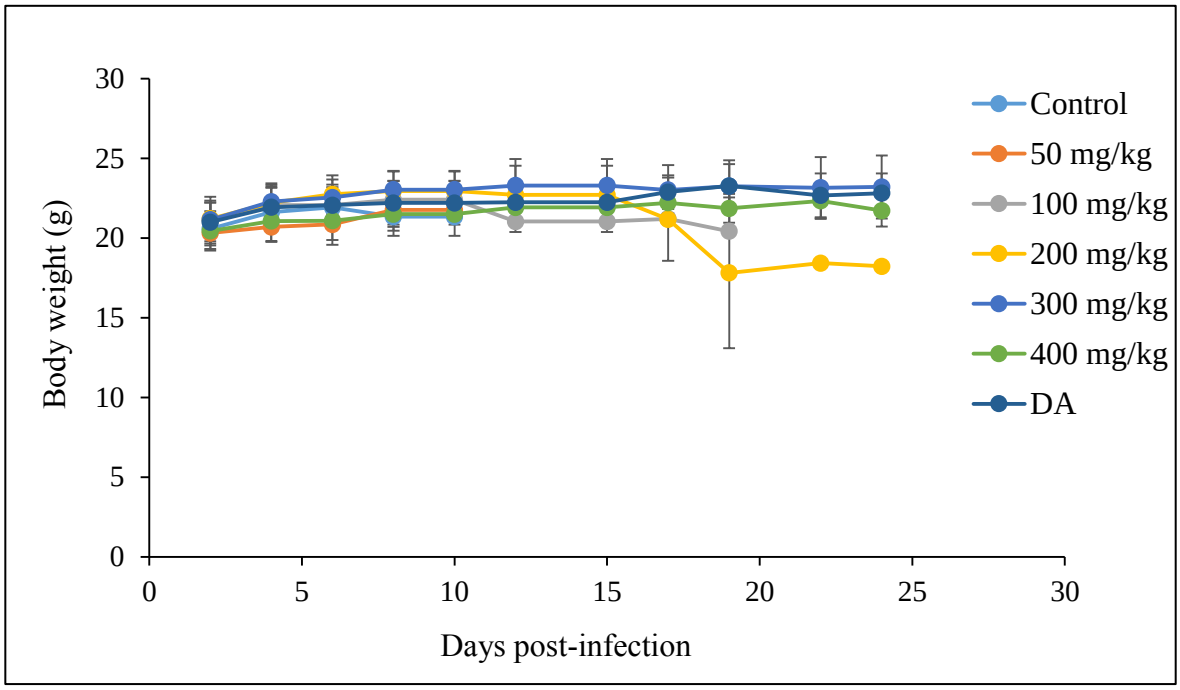


Figure 1.5: The effect of 28 days of oral treatment with AZM on the body weight of *T. congolense*-infected mice (n = 10). There was a significant difference in the 300 mg/kg treated group ($p > 0.05$) in comparison to the control group. The data are presented as the mean ± S.D.

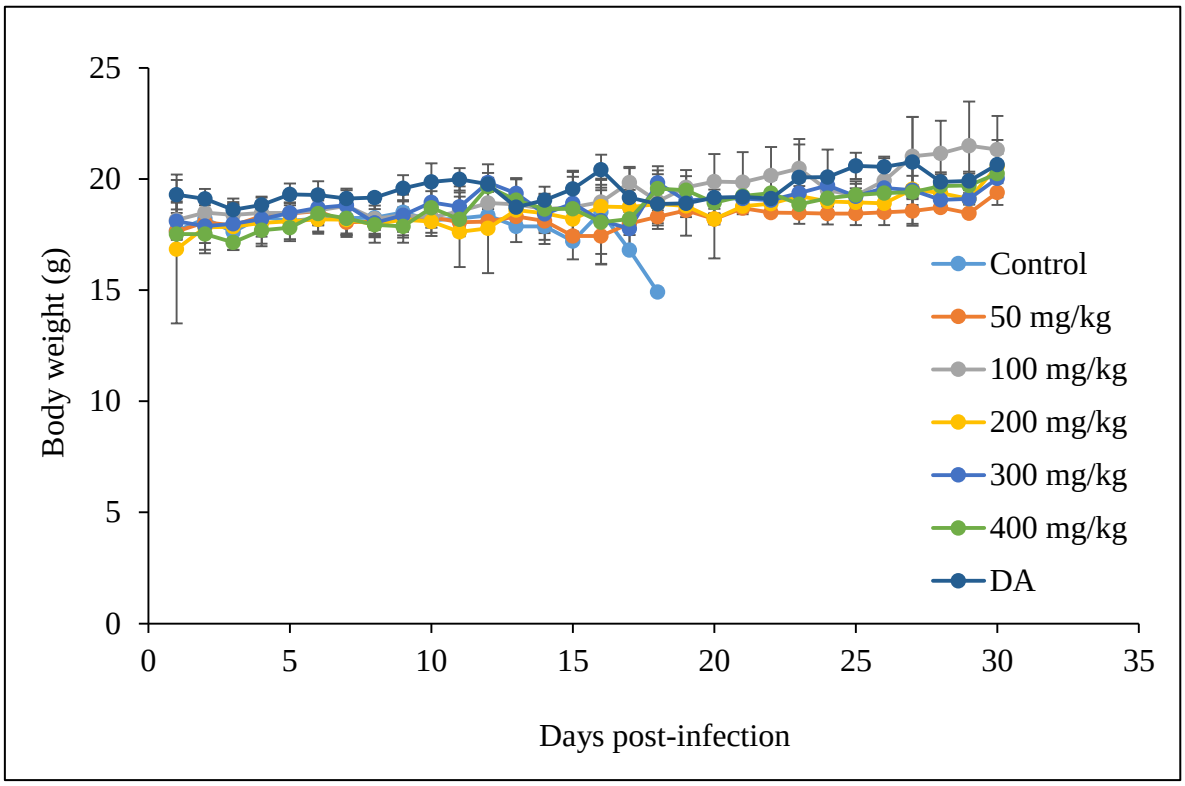


Figure 1.6: The effect of 28 days of oral treatment with AZM on the body weight of *T. b. brucei*-infected mice (n = 10). The data are presented as the mean ± S.D**.**

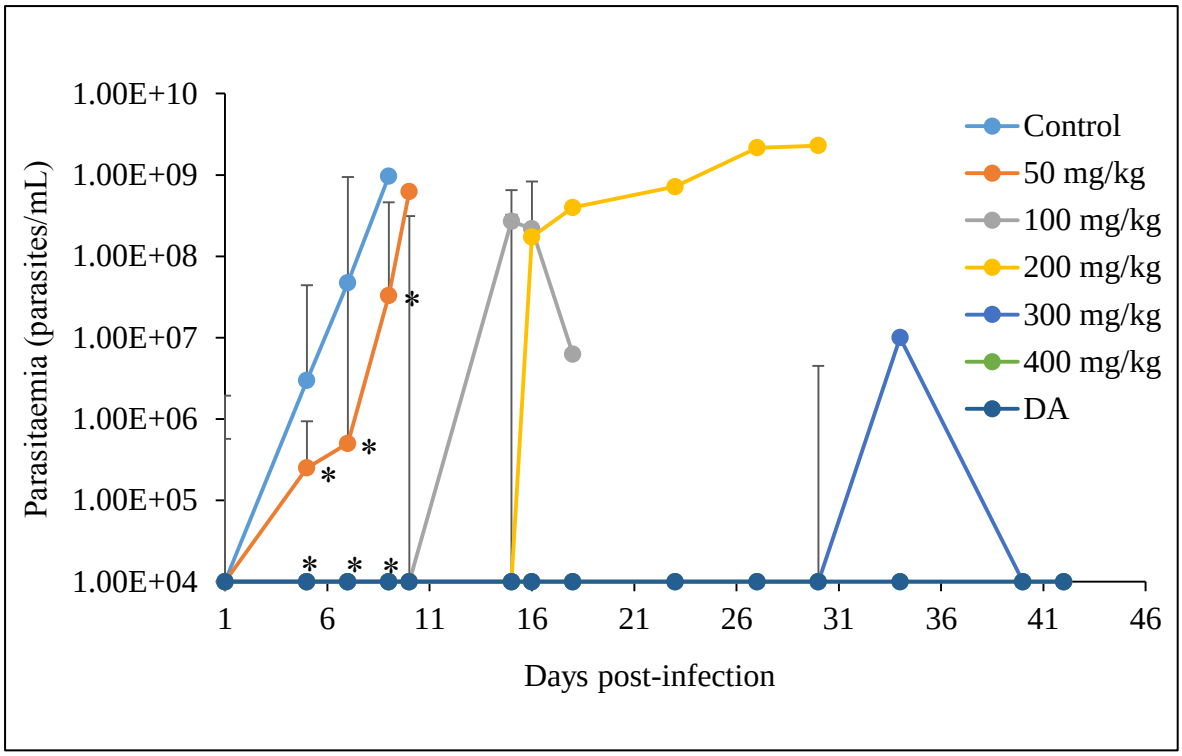


Figure 1.7: The evaluation of parasitaemia in mice infected with *T. congolense* and orally treated with different concentrations of AZM for 28 days. * $p < 0.05$ at 100 mg/kg on days 5, 7 and 9 while the parasitaemia of all of the other groups showed significant differences in comparison to the control group from days 3 to 9. The data expressed as the mean ± S.D. 1×10^4 represents parasitaemia below the detection levels.

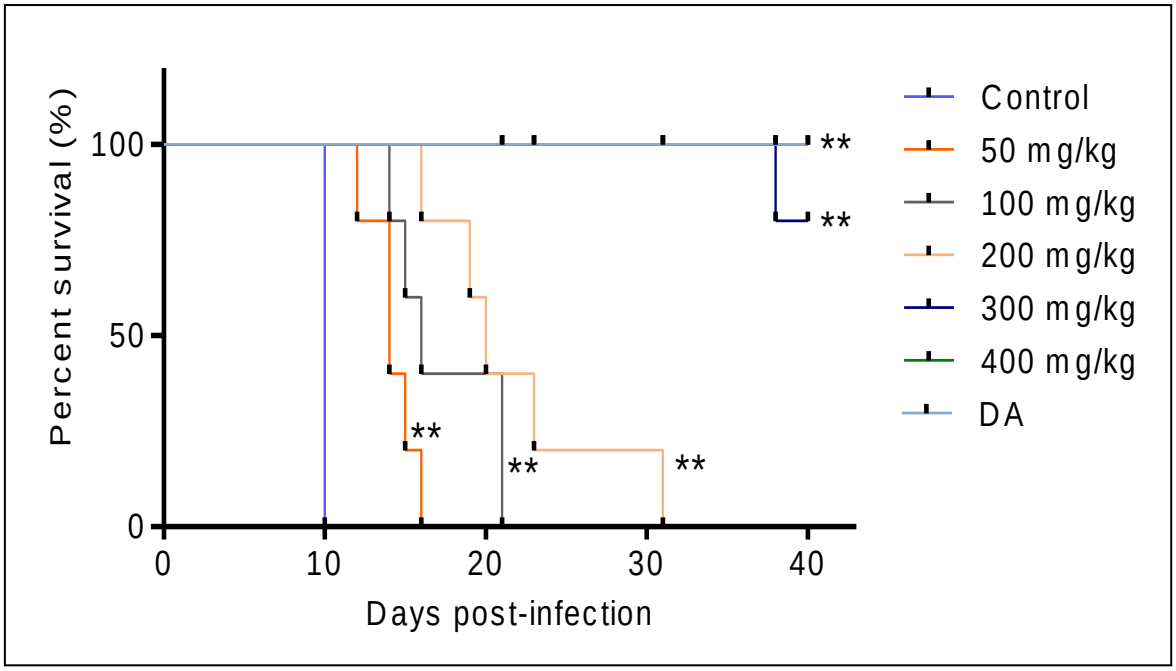


Figure 1.8: The survival curves of mice infected with *T. congolense* and orally treated with different concentrations of AZM for 28 days. The survival rate was significantly different from the control group (n = 10), ** $p < 0.001$ (Log-rank test).

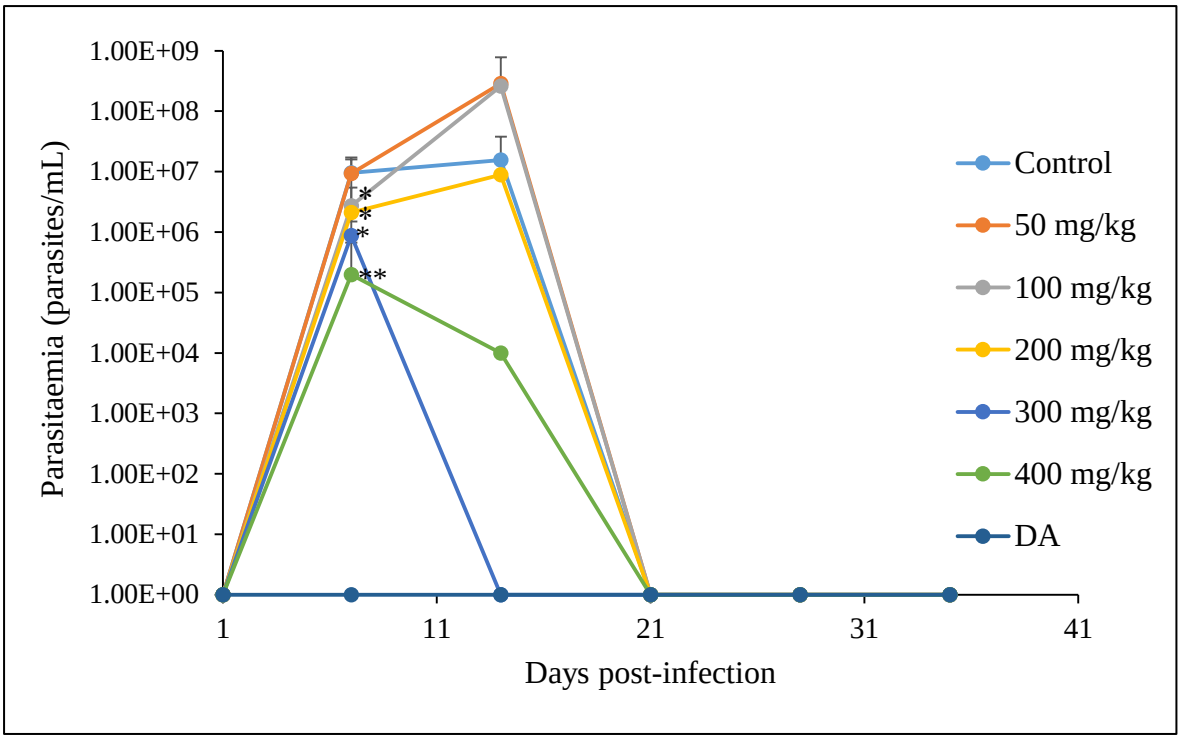


Figure 1.9: The evaluation of parasitaemia in mice infected with *T. b. brucei* and orally treated with different concentrations of AZM for 28 days. * $p < 0.05$ at 100, 200 and 300 mg/kg; ** $p < 0.01$ at 400 mg/kg on week one. The data expressed as the mean ± S.D. 1×10^0 represents parasitaemia below the detection levels.

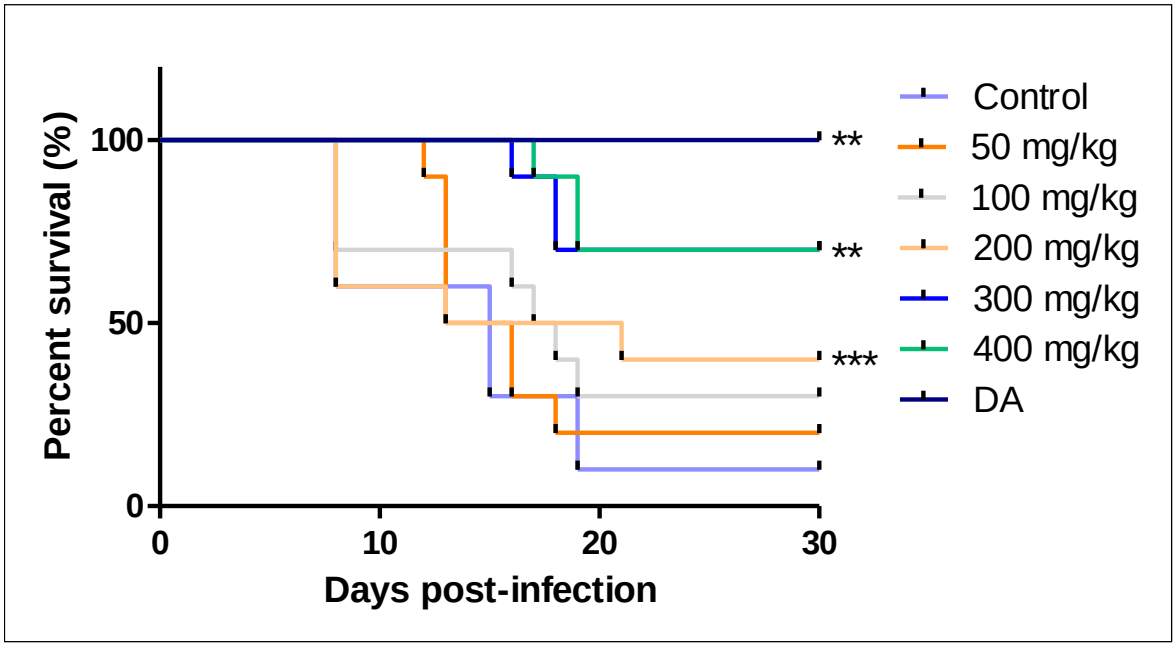


Figure 1.10: The survival curves of mice infected with *T. b. brucei* and orally treated with different concentrations of AZM for 28 days. The survival rate was significantly different from the control group (n = 10), ** $p < 0.001$; *** $p < 0.0001$ (Log-rank test).

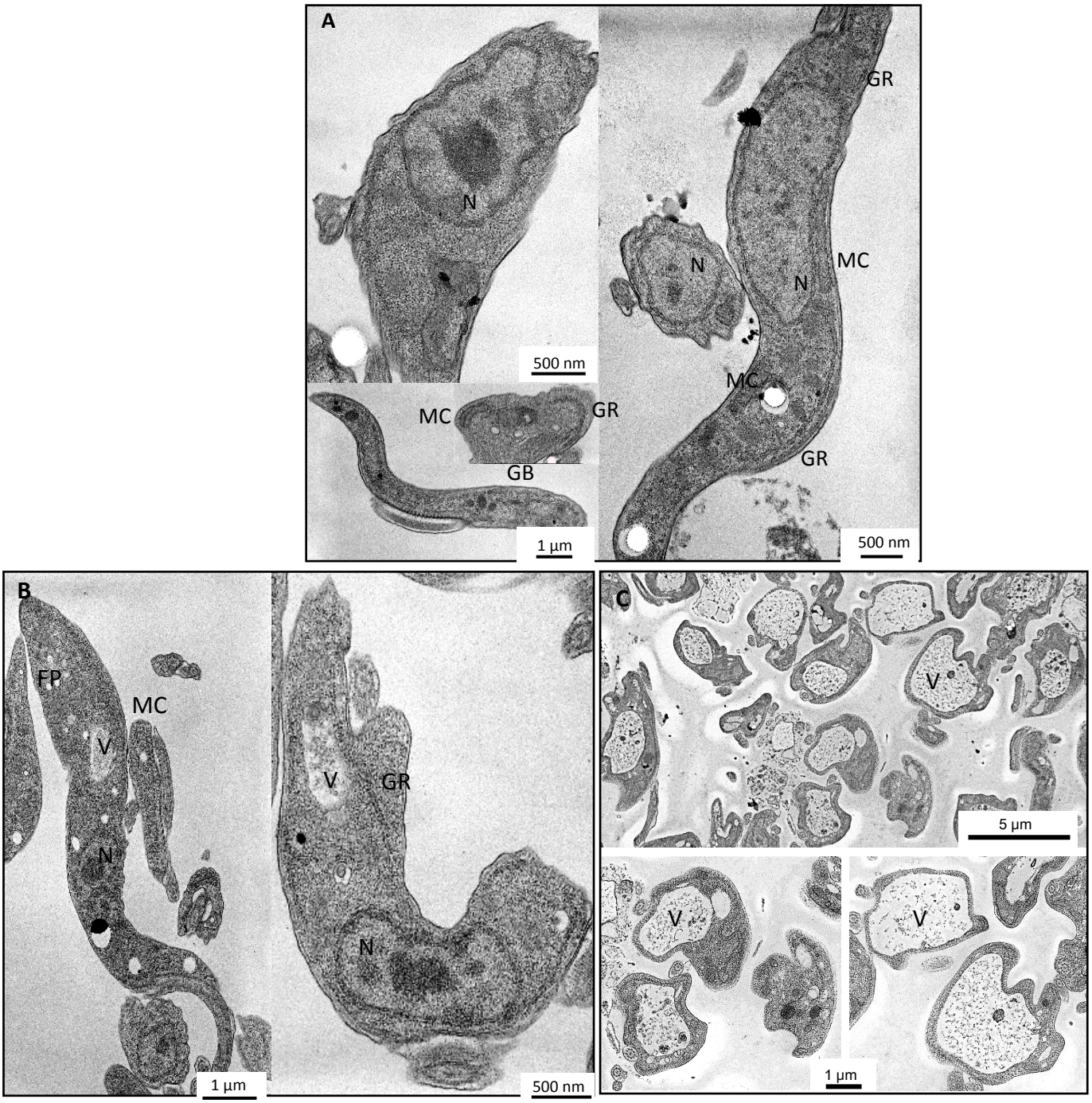


Figure 1.11: The effects of AZM on the cellular morphology of *T. congolense* cells grown in HMI-9 in the A) absence (control) and in the B) presence of 25 µg/ml AZM for 7 hours and C) 24 hours monitored by using transmission electron microscopy (TEM). N: Nucleus, MC: Mitochondrial canal; GB: Golgi bodies; GR: Granular reticulum, FP: Flagellar pocket, V: Vacuole-like structure. The treated group showed the development or presence of a vacuole, degradation of the granular reticulum in comparison with the control group.

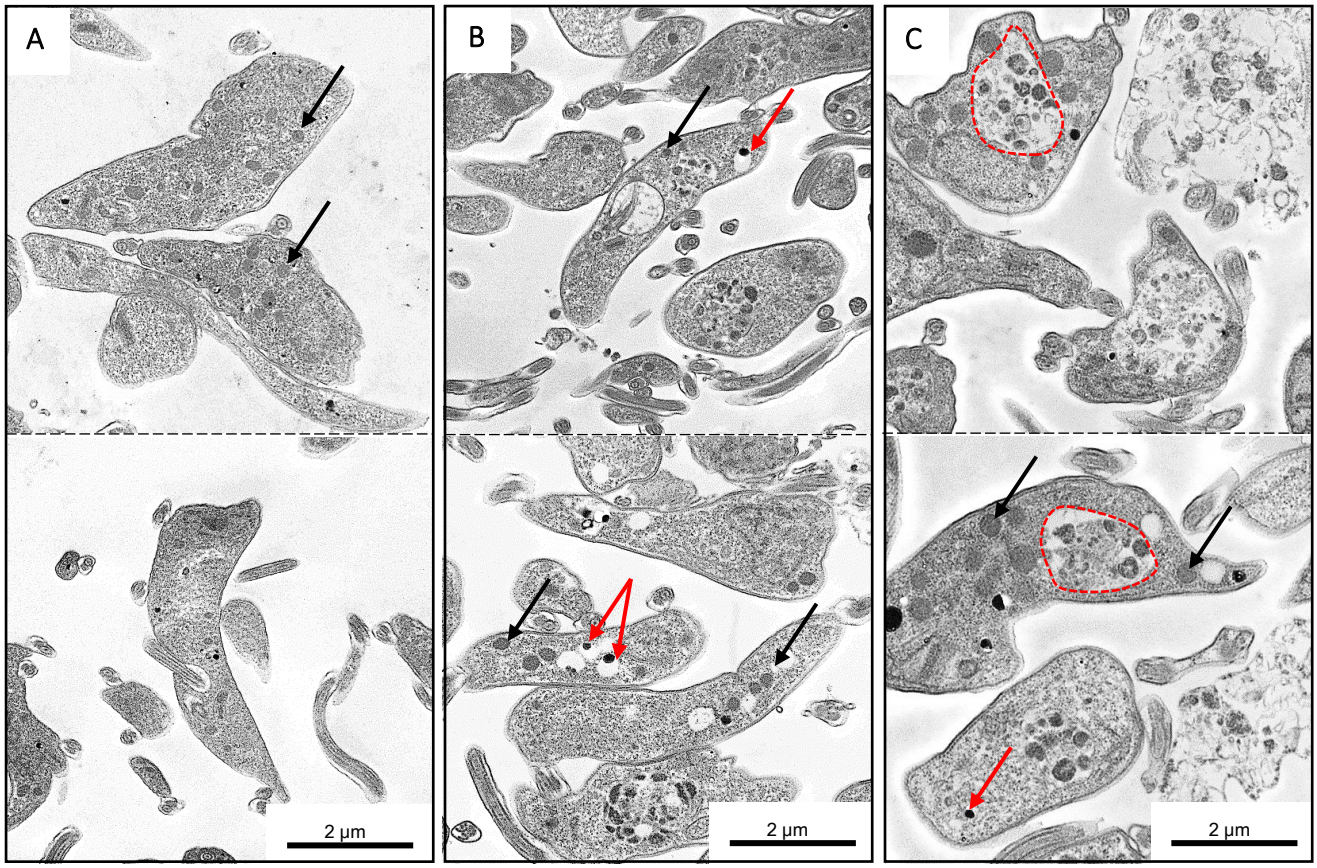


Figure 1.12: The effects of AZM on the cellular morphology of *T. b. brucei* cells grown in HMI-9 in the A) absence (control) and in the B) presence of 25 µg/ml AZM for 7 hours C) 24 hours monitored by using transmission electron microscopy (TEM). Black arrows: Glycosomes like; Red arrows: Acidocalcisomes like structures; Red circle: Vacuole containing digested materials.

Table 1.1 The effects of 28 consecutive days of oral treatment with AZM on the hematological parameters of *T. congolense*-infected mice.

RBC (10^4/µl)								
Groups	**Healthy**	**Control**	**50 mg/kg**	**100 mg/kg**	**200 mg/kg**	**300 mg/kg**	**400 mg/kg**	**DA**
Week 1	878.60 ± 52.27	931.80 ± 44.57	992.40 ± 37.19*	934.80 ± 22.65	878.40 ± 57.82	956.80 ± 46.92	901.6 ± 115.89	915.80 ± 85.32
Week 2	983.00 ± 202.32	-	764.50 ± 16.82*	918.20 ± 120.43	862.60 ± 67.77	941.80 ± 22.25	844.80 ± 82.43	872.60 ± 29.18
Week 3	936.00 ± 33.64	-	-	-	-	846.80 ± 54.32	831.6 ± 68.41	865.20 ± 865.2
Week 4	888.80 ± 108.91	-	-	-	-	934.60 ± 86.81	832,6 ± 84,76	831.00 ± 68.57
Week 5	922.80 ± 43.15	-	-	-	-	911.75 ± 63.85	850.4 ± 94.07	877.00 ± 29.97
HGB (g/dl)								
Groups	**Healthy**	**Control**	**50 mg/kg**	**100 mg/kg**	**200 mg/kg**	**300 mg/kg**	**400 mg/kg**	**DA**
Week 1	12.86 ± 0.75	14.70 ± 0.54*	15.62 ± 0.50***	14.56 ± 0.40*	13.78 ± 0.89	14.80 ± 0.73**	14.12 ± 1.60	14.00 ± 1.13
Week 2	13.16 ± 1.57	-	12.18 ± 2.36	14.40 ± 1.88	13.64 ± 1.24	14.82 ± 0.39	13.48 ± 1.26	13.52 ± 0.53
Week 3	13.68 ± 0.41	-	-	-	-	13.14 ± 0.81	12.94 ± 0.96	13.32 ± 13.32
Week 4	13.44 ± 1.51	-	-	-	-	14.54 ± 1.31	12.70 ± 1,24	12.70 ± 0.89
Week 5	13.98 ± 0.72	-	-	-	-	13.70 ± 13.70	12.76 ± 1.36	13.28 ± 0.40
HCT (%)								
Groups	**Healthy**	**Control**	**50 mg/kg**	**100 mg/kg**	**200 mg/kg**	**300 mg/kg**	**400 mg/kg**	**DA**
Week 1	42.08 ± 2.67	43.14 ± 1.95	45.78 ± 1.86	43.44 ± 0.70	40.60 ± 3.00	43.90 ± 2.46	41.40 ± 4.83	42.30 ± 3.89
Week 2	42.72 ± 4.15	-	35.63 ± 7.21	42.66 ± 5.21	39.62 ± 3.16	43.54 ± 0.47	39.10 ± 3.56	40.46 ± 1.53
Week 3	44.74 ± 2.12	-	-	-	-	39.02 ± 2.35*	38.28 ± 2.98**	40.06 ± 40.06
Week 4	42.22 ± 4.96	-	-	-	-	43,20 ± 3,53	37.98 ± 3.71	38.18 ± 2.84
Week 5	43.34 ± 2.36	-	-	-	-	42.15 ± 42.15	39.04 ± 4.06	40.24 ± 1.34

The values are presented as the mean ± S.D. Asterisks indicate a statistically significant difference between the healthy and the *T. congolense* infected groups, non-treated and AZM-treated group * $p < 0.05$; ** $p <$ 0,001; *** $p < 0.0001$. – Dead group

Chapter 2

Evaluation of Safety Status of Triclosan in BALB/c Mice and Its Efficacy on *Trypanosoma congolense*- Infected Mice

Introduction

Triclosan (TCS), 2'-hydroxy-2,4,4,-triclorodiphenyl ether, is a broad spectrum antimicrobial agent that has been used as an active ingredient in a number of cosmetics, personal health care and house hold products for decades (Dhillon *et al.,* 2015). TCS is considered safe for humans as they possess no enoyl-acyl protein reductase (ENR), a key enzyme in the type II fatty acid synthesis (FAS II) system and has been tolerated and accepted for use by the US Food and Drug Administration (U.S. FDA). Mammalian ENR domain has a different fold from its functional analogues of the bacterial type II FAS (Massengo-Tiasse and Cronan, 2009). Bhargava and Leonard (1996) reported LD_{50} values of TCS as 3,700 to 5,000 mg/kg orally and 10,190 mg/kg intraperitoneally in mature mice, rabbits and dogs, indicating the safety status of the compound.

However, according to Weiss *et al.*, (2015) there has been a number of inconsistent studies and unreliable data regarding the safety of the compound. Additionally, several other studies have disputed the safety claims of TCS by documenting the androgenic and thyroid effects, endocrine disruption and skin irritation effects to TCS exposure (Kumar *et al.*, 2015; Gao *et al.*, 2017; Wang *et al.*, 2017). Moreover, TCS is metabolised in the liver therefore its long-term treatment is suspected to likely induce liver tumour in the host (Yueh, 2015). This matter therefore has led to uncertainty of the usage of TCS. The approval of TCS containing toothpaste was based on the ability to fight gingivitis reported from 70 clinical testing conducted on over 10,000 patients as stated by the manufacturing company. Nonetheless, in 2010 a concerned letter was sent to the department of health and human services of the FAO

raising suspicions and controversies on the compound toxicity effects, therefore challenging the safety concentration recorded prior that. The letter focused on the possible health impacts of TCS due to the repetitive daily human exposure (Halden *et al.,* 20017). On the 2nd of September 2016 the U.S. FDA issued a final rule establishing that over the counter consumer antiseptic wash products containing TCS can no longer be marketed. The ruling to remove TCS from generally recognized as a safe and effective (GRASE) compound is based on the fact that the manufactures did not provide sufficient safety information and/or demonstrate the safety of TCS for a long term daily use. The final ruling therefore calls for further investigations for the development and submission of new safety and reliable data for the compound.

Studies have shown that TCS is active against different types of fungi, bacteria and even parasites by blocking the ENR necessary for fatty acid biosynthesis of these pathogens (Yang *et al.,* 2015; Gao *et al.,* 2017). Amongst others, TCS possesses an effect against protozoan pathogens such as, Toxoplasma gondii, bovine and equine Babesia and malaria causing Plasmodium parasites (McLeod *et al.,* 2001; Bork *et al.,* 2003; Singh *et al.,* 2009; El-Zawawy *et al.,* 2015a; El-Zawawy *et al.,* 2015b).

Animal trypanosomiasis is devastating spectrum of diseases; nagana, surra and dourine. These infections are widely distributed and infect a wide range of species of both wild and domesticated animals (Baldissera *et al.,* 2016). The major problem of these infections is treatment related, available treatments are either no longer effective due to the emerging resistance or they are toxic. Therefore; alternative drugs are urgently in need. For the fact that there are controversies regarding the safety measures of TCS, the current study has was formulated to determine safe concentrations and doses *in vitro* using mammalian cell-lines and *in vivo* using BALB/c mice model.

Thus the aim of this study was to demonstrate the trypanocidal effect of TCS *in vitro* on *T. congolense, T. b. brucei* and *T. evansi,* cytotoxicity on the MDBK and NIH 3T3 host cells, determine the safe concentration safe for BALB/c mice tests and finally to determine *in vivo* its efficacy on *T. congolense* infected mice.

Materials and methods

TCS compound

TCS [5-chloro-2-(2,4-dichlorophenoxy)phenol] was purchased from Sigma-Aldrich, Japan.

Parasites and *in vitro* culture

The trypanosome parasites (*T. b. brucei, T. congolense, T. evansi*) *in vitro* cultures were prepared as described in Chapter 1.

Cell line cultures

The mammalian cell line cultures were prepared as described in Chapter 1.

The *in vitro* evaluation of trypanocidal effects of TCS

The *in vitro* trypanocidal tests were conducted as described in Chapter 1 only that in this occasion the compound tested was TCS.

The *in vitro* cytotoxicity tests

The *in vitro* cytotoxicity tests were conducted as described in Chapter 1 only that in this occasion the compound tested was TCS.

Experimental animals

Animal experiments were conducted as described in Chapter 1, only that they were treated with TCS.

Dose determination tests

The dose determination test was divided into two parts, the single dose and the repeated dose test.

Single dose test

A single dose determination was evaluated as per Organization for Economic Cooperation and Development (OECD) guideline 423 (OECD, 2001). The animals were randomly divided into 6 groups comprising each of 3 animals. Group I served as the healthy group which received water, group II received a vehicle (10% DMSO-Corn oil) as the control group while group III, IV, V and VI test groups which received 100, 300, 1,000 and 2,000 mg/kg of TCS orally, with vehicle per body weight, respectively. Mice were monitored closely for any behavioural changes and other parameters such as body weight, convulsion, tremor, changes in eyes and skin colours from the point of administration up to 14 days.

Repeated dose tests

This test was performed following the OECD test guideline 407 (OECD, 2008). Ten healthy female BALB/c mice were randomly divided into 4 groups. Group I served as a vehicle control group and received 10% DMSO-Corn oil. Groups II, III and IV received TCS dissolved

in Corn oil orally at doses of 100, 300 and 500 mg/kg per body weight, respectively, for 28 consecutive days. The test regimens were freshly prepared every morning.

During this time, all the animals were observed for abnormalities, changes, signs of toxicity, body weight fluctuation and mortality. On 29th day, all the mice were sacrificed with an overdose of isoflurane, blood was collected by heart puncture for clinical pathology (haematology and biochemistry), while the vital organs (heart, liver, spleen and kidneys) were collected, weighed to calculate the relative body weight of the organs then sectioned and preserved for histopathological examination.

Haematological analysis

The collected blood samples were subjected to a blood count using an automatic haematology analyser (Celltac α, Nihon Kohden, Tokyo, Japan). The red blood cell (RBC), haematocrit (HCT), mean corpuscular haemoglobin (MCH), haemoglobin (HGB), white blood cell count (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and platelet (PLT) count were analysed. Additionally, red cell width (RDW), procalcitonin (PCT), mean platelet volume (MPV) and platelet distribution width (PDW) were also analysed.

Biochemical analysis

Blood samples were allowed to clot, centrifuged at 1500 r/min for 10 minutes to obtain serum which was separated and stored at -80°C until the biochemical analyses test were conducted at Wako Pure Cheminal Industries, Ltd., Osaka, Japan. Serum was analysed for total protein (TP), albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase

(ALT), gamma glytamyltranferase (rGT), total bilirumin (T-BIL), and lactate dehydrogenase (LDH).

Histopathological analysis

The heart, liver, kidneys, spleen tissues were collected for histological studies. The tissues were washed in normal saline and fixed immediately in 10% formalin overnight, dehydrated with isopropanol, embedded in paraffin, cut into 4-5 µm thick sections, and stained with haematoxylin-eosin (H & E) dye for photomicroscopic observation. The microscopic features of treated groups were compared with the control group. The histopathological analyses were performed by Assistant Professor Kenichi Watanabe, Research Center for Global Agromedicine, Obihiro University of Agriculture and Veterinary Medicine.

The *in vivo* trypanocidal effects of TCS on *Trypanosoma congolense*

Oral treatment

The virulent *T. congolense* IL3000 strain was propagated in mice and used for experimental infection. The parasites were passaged twice in mice before the experiment. The mice were intraperitoneally infected with 100 µL of *T. congolense* (5×10^3 parasites/mouse) inoculated with PSG. The mice were randomly divided into 6 groups of 5 mice as follows: Group I (control group), the mice were infected but not treated; group II (positive control group), the mice were infected and treated with diminazene aceturate (DA) (3.5 mg/kg, intraperitoneally); Groups III, IV, V and VI (the test groups), the mice were infected and orally treated using a feeding needle with 50, 100, 200 and 300 mg/kg TCS in a 200 µL inoculum, respectively. Treatment was initiated at 48 hours post-infection and was maintained for 7

consecutive days. The treatments were freshly prepared each day. The surviving mice were observed for 90 days post infection (dpi). Each day, the parasitaemia was evaluated and the effects of treatment were monitored using wet blood smears. Each slide was prepared with fresh blood collected from the tail vein (magnification: 400×). The experiments were conducted in duplicate.

Intraperitoneal treatment

The virulent *T. congolense* IL3000 strain was propagated in mice and used for infection. The parasites were passaged twice in mice before the experiment. The mice were intraperitoneally infected with 100 µL of *T. congolense* (5×10^3 parasites/mouse) inoculated with PSG. The mice were randomly divided into 9 groups of 5 mice as follows: Group I (control group), the mice were infected but not treated; group II (positive control group), the mice were infected and treated with diminazene aceturate (DA) (3.5 mg/kg); Groups III, IV, V, VI, VII, VII, IX (the test groups), the mice were infected and intraperitoneally treated with 25, 50, 75, 100, 150, 200 and 300 mg/kg TCS in a 200 µL inoculum, respectively. Treatment was initiated at 48 hours post-infection and was maintained for 7 consecutive days. The treatments were freshly prepared each day. The surviving mice were observed for 90 days while the others were observed until death. Each day, the parasitaemia was evaluated and the effects of treatment were monitored using wet blood smears. Each slide was prepared with fresh blood collected from the tail vein (magnification: 400×). The experiments were conducted in duplicate.

Statistical analysis

The results were expressed as the mean ± standard deviation (S.D) for the number of repeated trials indicated in each experiment. The statistical analyses were conducted for the acute phase of infection. The *t*-test was used for intergroup comparisons between the treated and non-treated groups. The survival curves were constructed using the Kaplan-Meier method and the curves were compared using a log-rank test. All of the data were compiled using GraphPad Prism Software program (version 5.0, GraphPad Software Inc., CA, USA). The means and standard deviations were calculated for measurement data in each group, which included body weight, relative organ weight, haematological parameters and biochemical parameters. One-way ANOVA was conducted to determine the significant differences, a Dunnett *t* test was performed for inter-group comparisons. *P* values of < 0.05 were considered to indicate statistical significance.

Results

The *in vitro* trypanocidal activity of TCS

The activity of TCS was assessed *in vitro* after 72 h of incubation with *T. congolense, T. b. brucei* and *T. evansi* bloodstream forms. The IC_{50} (Inhibitory concentration) observed were 1.93 ± 0.86; 1.82 ± 0.21 and 1.06 ± 0.07 (mean in µg/ml ± S.D) for *T. congolense, T. b. brucei* and *T. evansi*, respectively. The cytotoxicity of TCS was measured on the MDBK and NIH 3T3 cells and LC_{50} values were calculated to be 15.15 ± 6.83 and 2.20 ± 1.01 (mean in µg/ml ± S.D), respectively. Based on the IC_{50} and LC_{50} values obtained, selectivity index (SI) was determined for all trypanosome species studied. This parameter reflects the quantity of compound that is active against the pathogen but is not toxic towards the host cell and was calculated as $SI = IC_{50}/LC_{50}$. Obtained average selectivity index values were calculated and obtained as 4.50, 4.80 and 2.43 for *T. congolense, T. b. brucei* and *T. evansi*, respectively.

Dose determination

Single dose tests

No treatment related toxicity symptoms, abnormalities and/or mortality was observed at 100, 300, and 1,000 mg/kg, while all the mice in the 2,000 mg/kg group died. There were no major behavioural changes observed on the animals in the other groups for 4 h after administration. The mice were further monitored for 14 days without any behavioural changes observed. The tested compounds appeared to be safe at the dose level of 1,000 mg/kg with an LD_{50} greater than 1,414.2 mg/kg, due to the fact that none of the animals in 2,000 mg/kg group survived. Sluggishness that lasted for a few minutes after administration was observed in all groups receiving TCS, regardless of the concentration.

Repeated dose test

General observations

Generally, the animals that received TCS for 28 days showed no major toxicity symptoms or abnormalities but sluggishness. The animals in 500 mg/kg, developed bloody paws from which they recovered from in a period of 2 to 3 days. In the 500 mg/kg group, 20% of mice died after 21 days of administration, this mouse expressed signs of diarrhoea, irritation with raised fur and red paws and a drastic body weight loss prior to death. The 100 and 300 mg/kg groups showed no major symptoms until the day of the termination of the study.

Body weight

The administration of TCS for 28 days resulted in a significant difference in the body weight in mice treated with 300 mg/kg ($p < 0.0001$) as compared to the control group. There was a progressive body weight increase in the 500 mg/kg group throughout the study though not statistically significant due to the death recorded in this group. Additionally, no statistical difference was observed in the body weight body of the 100 mg/kg treated mice (Fig. 2.1).

Relative organ weight

A dose dependent hepatomegaly was observed visually in all the treated groups of 100, 300 and 500 mg/kg, in comparison to the control group, even though the increase was significant in 300 and 500 mg/kg ($p < 0.0001$). There was no significant difference between relative organ weight of the other organs, namely; heart, kidneys, spleen and pancreas as compared to their respective control groups (Table 2.1).

Haematological analysis

At 100 mg/kg, a significant decrease of MCH ($p < 0.05$) was observed together with an increase in PLT ($p < 0.001$) and RDW ($p < 0.0001$) in comparison to the respective control group. At 300 mg/kg a significant decrease in HGB ($p < 0.01$); MCH and MCHC ($p < 0.0001$) with a significant increase in PLT ($p < 0.001$); RDW ($p < 0.0001$) and PCT ($p < 0.05$). At 500 mg/kg, there was a decrease in HGB even though it was not significant ($p > 0.05$), a significant decrease in MCHC and MCHC ($p < 0.0001$), a significant increase of PLT ($p < 0.001$); RDW ($p < 0.0001$) and PCT ($p < 0.05$) were noted in comparison to their respective control groups. The other parameters fluctuated in normal ranges (Table 2.2).

Biochemical analysis

The biochemical analysis was conducted on as many as possible parameters, however, the liver enzymes were given more attention. These parameters of interest included TP, ALB. AST, ALT, Ɣ – GT, T- BIL and LDH. There was an increase in almost all the parameters, even though the difference was not significant. In cases of ALT, AST and LDH, the observed increase in levels were dose dependent (Fig. 2.2).

Histopathological analysis

The histological changes observed were dose dependent, ranging from mild to severe. The initial signs of hepatotoxicity were observed visually during the sacrifice of the mice, fatty patches resembling necrosis were spotted on the abnormally bigger liver in the treated groups. Even though not specific, a dose dependent hydropic degeneration also known as vacuolar degeneration was observed in all the treated groups, 100, 200 and 300 mg/kg as compared to

the non-treated control group. Additionally, further changes were also observed in the vital organs, nonetheless, the liver possessed most of the significant changes, which included the presence of megakaryocytes and extramedullary haematopoiesis (EH) (Fig. 2.3). At the 500 mg/kg group, the EH was observed in the liver, kidneys, lungs and the spleen.

The *in vivo* trypanocidal effects of TCS

Oral treatment

The parasitaemia was significantly reduced in the treated groups identifying the acute phase of the infection as compared to the control group. The parasites in the control group proliferated well followed by a steady and gradual increase of parasitaemia, that prevailed until the parasitaemia peak was reached in 9 days, while treated groups reached lower peaks. At 50 mg/kg, the parasitaemia levels were significantly reduced from day 4 to 9, without the clearance of the parasites from the circulation. No parasites were detected in the peripheral circulation of the mice in the 100, 200 and 300 mg/kg on days 5, 5 - 6 and 4 - 7, respectively, with a significant suppression of parasitaemia between day 3 and 9 in these groups. Nonetheless, all the mice eventually relapsed followed by a gradual increase of parasitaemia in all the groups. No relapse was observed in the DA group. A 10% of the mice was declared cured from the infection in the 100 mg/kg treated group (Fig. 2.4). The survival rate of the treated mice was significantly prolonged ($p < 0.0001$) in comparison to the non-treated control group. All the mice in the control group died within 10 days while those in the 50, 100, 200 and 300 mg/kg died on days 23, 17, 26 and 18, respectively. Ten percent of the mice in the 100 mg/kg along with all the DA treated group survived (Fig. 2.5).

Intraperitoneal treatment

There was a significant inhibition of parasitaemia growth in the treated groups 50, 75, 100, 200 and 300 mg/kg ($p < 0.05$) in comparison to the 25 mg/kg ($p > 0.05$) and control group in the acute phase of the infection. All the mice eventually showed the presence of the parasites, regardless of a prolonged prepatent period in the 300 mg/kg group by a day. At 100 mg/kg, no parasites were detected on days 9 and 10 followed by a rapid increase in parasitaemia on day 11 until all the mice died. All the treated groups reached a lower parasitaemia peak than that of the control, except the insignificant difference in 25, 50 and 75 mg/kg (Fig. 2.6). The mice in the control group, 25, 50 and 75 mg/kg ($p > 0.05$) died within 8 days, while in the other groups, the survival was prolonged significantly in 50, 75, 100, 300 mg/kg ($p < 0.001$) and 200 mg/kg ($p < 0.0001$). Death of the mice in the treated groups were recorded on days 16, 14, 16 and 17 in 100, 150, 200 and 300 mg/kg, respectively (Fig. 2.7).

Discussion

The *in vitro* tests of TCS resulted in a uniform efficacy between *T. congolense, T. b. brucei* and *T. evansi* infections even though the efficacy against *T. evansi* infection was slightly better than the others. TCS showed a slight cytotoxicity on the NIH 3T3 cells as compared to the MDBK cells. Nonetheless, the selectivity index value obtained allowed further studies. Mice embryonic cells were documented by several studies to be more susceptible to many compounds as compared to the other cells, including the MDBK cells.

The effects of a single dose of TCS in mice resulted in no adverse effects for 14 days of experimentation at the concentrations as high as 1,000 mg/kg, while all the mice in 2,000 mg/kg group died and therefore resulting in calculated LD_{50} of 1,414.12 mg/kg. The obtained value of acute toxicity of TCS is in agreement with a study recorded by (Dann and Hontela, 2011) where TCS was documented to possess no acute toxicity at 500 mg/kg on terrestrial animals. Contrary to the study by Bhargava and Leonard (1996), who recorded the acute LD_{50} of TCS in mature mice, dogs and rats ranging between 3,750 to 5,000 mg/kg orally and 1,090 mg/kg intraperitoneally, this study demonstrated lower LD_{50}. Any pharmaceutical compound with an oral LD_{50} of greater or equal to 1,000 mg/kg could be considered safe and less toxic (Kifayatullah *et al.,* 2015). There were, however, minor symptoms right after the administration which disappeared within an hour, these symptoms included rough fur coat around the neck and sluggishness in groups receiving TCS. In the repeated dose exposure of the mice to TCS, various parameters such as the body weight, food consumption, haematology, biochemical, relative organ weight and histopathology were monitored either during the commencement of the study or at the end of the experiments. Test concentrations of 100, 300 and 500 mg/kg were administered with one recorded death on day 21 in the 500 mg/kg group. According to Liu *et al.* (2016) the change in body weight functions as an indicator for damage resulting from the test substance. In the current study at 100 and 500 mg/kg, there was an increase in body weight

even though it was not significantly ($p > 0.05$) different as compared to the negative control. On the other hand, 300 mg/kg body weight increase was significant ($p < 0.05$) as compared to the control group which signalled the first complication. The mice in the 300 mg/kg group were conspicuously bigger than the rest of the other groups.

Relative organ weight calculation showed an increase in the liver weight, though only 300 mg/kg was statistically significantly different. Increase in liver weight can be associated with a number of factors and differing histological appearances, which could either be used to depict toxicity, overt adverse changes such as inflammation, necrosis, and degeneration or even completely distinct aspects such as hyperplasia and hypertrophy resulting in no visual changes (Hall *et al.*, 2012). The fatty spots observed on the livers in this study were associated with necrosis due to the obvious hepatomegaly element. The above mentioned drastic body weight of the mice receiving TCS is relatively related to the hepatomegaly. Ting *et al.* (2015) reported that tangeretin toxicity effects with an increase in the liver weight. The authors hypothesised that toxic components of tangeretin lead to increased metabolism demand by the CYP450 prompted by liver to increase its capacity for the purpose of responding to this higher stress level. There was no significant difference in other organs, heart, spleen, kidney and pancreas which were weighed.

Evaluation of haematological parameters was used to determine the extent of deleterious effects of compounds on an animal's blood. Treated groups of mice showed significant different decrease in HGB, MCV and MCHC and increase in PLT, RDW and PCT. The observed decrease in RBC and HGB signals a decrease in oxygen content in the blood and a potential decrease in metabolism and the immune response of the mice. While the increased PLT counts suggests the possibility of blood coagulation and/or reduced oxygen carrying capacity (Duan *et al.*, 2010).

Biochemical analysis showed the insignificant fluctuations of the liver functioning enzymes. Recorded increase in TP, ALB, AST, ALT, r-GT, T-BIL, and LDH was observed. The elevation of these parameters were dose dependent, even though not significant. Nonetheless, their elevation supports the hypothesis of TCS hepatic alteration and/or liver toxicity. As depicted in the figure 2.2, at 500 mg/kg, there was increase in the AST, ALT and the LDH. AST and ALT, liver functioning enzymes, found in the liver, kidney, heart, however, greater proportion was observed in the liver. Elevated levels of these enzymes suggests possible liver cell injury, necrosis, tissue damage, or toxin induced liver damage. Peak levels are not directly proportional to the severity of the disease, however, could be used to guide the urgency and the necessity of further investigation. In addition to these points, the elevated levels of LDH confirmed the presence of a non-specific cell damage. LDH release into the circulation can only prove the cell breakage that occurred during the treatment period (Giannini *et al.*, 2005; Sahu, 2016).

Histopathology analysis showed a dose dependent hydropic degeneration in groups that received TCS as compared to the normal liver observed in the control group. This condition confirmed the presence of necrosis of the hepatocytes, the mice body weight increase, hepatomegaly and the elevated liver enzyme levels. Histological alteration ranged from less to mild. Studies have shown that hydropic degeneration, hepatocyte necrosis and the presence of the megakaryocytes could be due to oxidative stress, cellular degeneration and/or inflammation. Hepatocytes necrosis observed might indicate oxidative stress due to the action brought about by the action of the metabolic pathways generated by the system against toxic compounds, which is why reactive oxygen species are known as important mediators for cellular degeneration (Vaucher *et al.,* 2011; Jarrar and Taib, 2012; Ozen *et al.,* 2014). Hydropic degeneration also results from the accumulation of water or fluids in the liver cells which is likely to be the other factor contributing to increased weight of the liver (Hall *et al.*, 2012).

Mice receiving 500 mg/kg TCS displayed the presence of EH and megakaryocytes. EH is the process of blood cell production outside the medulla of the bone marrows. Under normal circumstances, EH takes place due to foetal development and normal immune response, their presence are displayed in the spleen and kidney. Besides these conditions, EH occurs as a result of pathological circumstances observed in almost all the vital organs, kidney, spleen, liver, lungs and rarely in the heart. In the current study the presence of EH was observed in the kidneys and lungs and was therefore associated with toxicity of TCS. It has previously been documented that EH can occur as the result of fatty acid synthesis (FAS) deficiency in mice, whereby haematopoietic cells are resistant to apoptosis therefore allowing these cells to grow in other organs which are labelled as not conducive for the development of cells (Kim, 2010). Finally, significant number of megakaryotes were observed in the spleen and this serves as an indicator for the possibility of inflammation (Vaucher *et al.,* 2011).

Obtained TEM results on *T. congolense* imitate and support the study by Paul *et al.* (2004). The *in vitro* efficacy of TCS on animal trypanosomes was not reciprocated *in vivo* where an insignificant percentage of mice treated with TCS was cured, regardless of the survival rate elongation and significant inhibition of parasitaemia. Additionally, there was a clear difference in efficacy between orally and intraperitoneally treated mice. Oral route in the mice did not cure the infection but possessed a significant efficacy as compared to the intraperitoneal route and the control group. Studies have confirmed that most of the lipophilic compounds administered orally have better pharmacokinetics, including the ready absorption and the bioavailability of the compound from the gastrointestinal tract. Lipophilic compounds such as TCS have low solubility and most likely, low oral bioavailability resulting in a limited absorption, high inter and intra subject variability and lack of dose proportionality. This phenomenon was attributed to the observed oral efficacy (Kalepu *et al.*, 2013).

According to Sandborgh-Englund *et al.* (2006), pharmacokinetics of TCS given orally includes ready absorption from the gastrointestinal tract with a great turnover in humans, levels of TCS in plasma increase rapidly reaching the maximum concentration within 1 to 3 hours post treatment, fair rapid elimination associated with the terminal half-life of 21 hours after a single dose. Furthermore, reports showed that the urinary excretion of TCS differs per individual, attributed this factor to the variation in bioavailability, distribution and renal clearance in each individual. The bioavailability could either be influenced or affected by the lipophilic nature of the compound (Queckenberg *et al.*, 2010; Kumar *et al.*, 2015).

The inefficacy of TCS *in vivo* as was also the case in the study by Paul *et al.* (2004), who showed that the well-known mode of action of TCS could be refractory in trypanosomes. Their study revealed that TCS had no efficacy on *T. brucei.* This was accounted for by the possibility of structural difference between the *T. brucei* ENR and the conventional type II ENR enzyme, FabI in bacteria. The efficacy of TCS *in vitro* and the inefficacy *in vivo* suggests different mode of action in these two distinct settings.

There are a number of factors influencing the effectiveness of the treatment such as food, initial treatment time and the period of treatment. Contrary to popular belief that the higher the concentration, the better efficacy, high concentration could also alter the immune function and result in its decreased efficiency of the host defence to multiple pathogens while low concentrations are likely not sufficient for the treatment. Intraperitoneal treatment of the mice did not relieve the mice from the infection even though parasitaemia was significantly suppressed. Two factors are suspected for the ineffectiveness of TCS intraperitoneally, toxicity and the insolubility of the compound in the abdominal cavity.

Parenteral route of administration are widely used due to the certain advantages such as great bioavailability, quick absorption, free from hepatic metabolism and high concentration

are achieved in the blood. However, the route has its own disadvantages including the increase of toxicity in the host and affecting the pharmacokinetics of the compound (Amlacher and Hoffmann, 1984). TCS administered orally altered some morphological functioning of the mice, as demonstrated in the above section, which indicates the possibility of high toxicity effects intraperitoneally. Judging from several toxicology studies on other compounds and/or drugs, intraperitoneal route often possesses a lower LD_{50} than the oral route (Wang *et al.,* 2015). A significant efficacy was observed at 100 mg/kg, as compared to all the other tested concentrations, this means it is likely to be an optimum concentration in both oral and intraperitoneal settings. This situation projected a possible hypothesis that would require further studies to prove, the lower concentrations were simply infective, optimum concentrations were effective without a curative effective while higher concentration altered the immune response of the mice due to possible toxicity effects. Additionally, few studies have reported the potential oxidative stress induction of TCS as well as the gut microbiome composition change due to high concentration and prolonged period of treatment (Gao *et al.*, 2017; Wang *et al.,* 2017). TCS is a lipophilic compound and does not easily dissolved in polar solvents preferred for parenteral administration. The compound was not easy to dissolve which resulted in the usage of polyethylene glycol as a solvent. This action has in one way or the other played a role in the ineffectiveness of the compound as the solvent affects the safety and the efficacy as well as the pharmacokinetics in general.

Lastly, the treatment was commenced 48 hours post infection, given with food. According to (Büscher *et al.,* 2017) the efficacy of any drug is influenced by the initial time of treatment, period of treatment, tolerance of the host and the establishment period of the pathogen.

In conclusion, the current study demonstrated the *in vitro* efficacy of TCS on *T. congolense*, *T. b. brucei* and *T. evansi* as well as the cytotoxicity effect. However, due to

conspiracy theories behind the cancellation of TCS by FDA over the counter drug, dose findings were conducted to determine safe concentrations for experimental mouse tests. Based on the toxicity effects observed in this study, long tern treatment with TCS was not conducted. The safe concentrations in mice showed prolonged survival without curative effects against the trypanosome infections. Therefore, further studies are necessary to determine the factors responsible for the failure of the compound in relieving the mice from the infection.

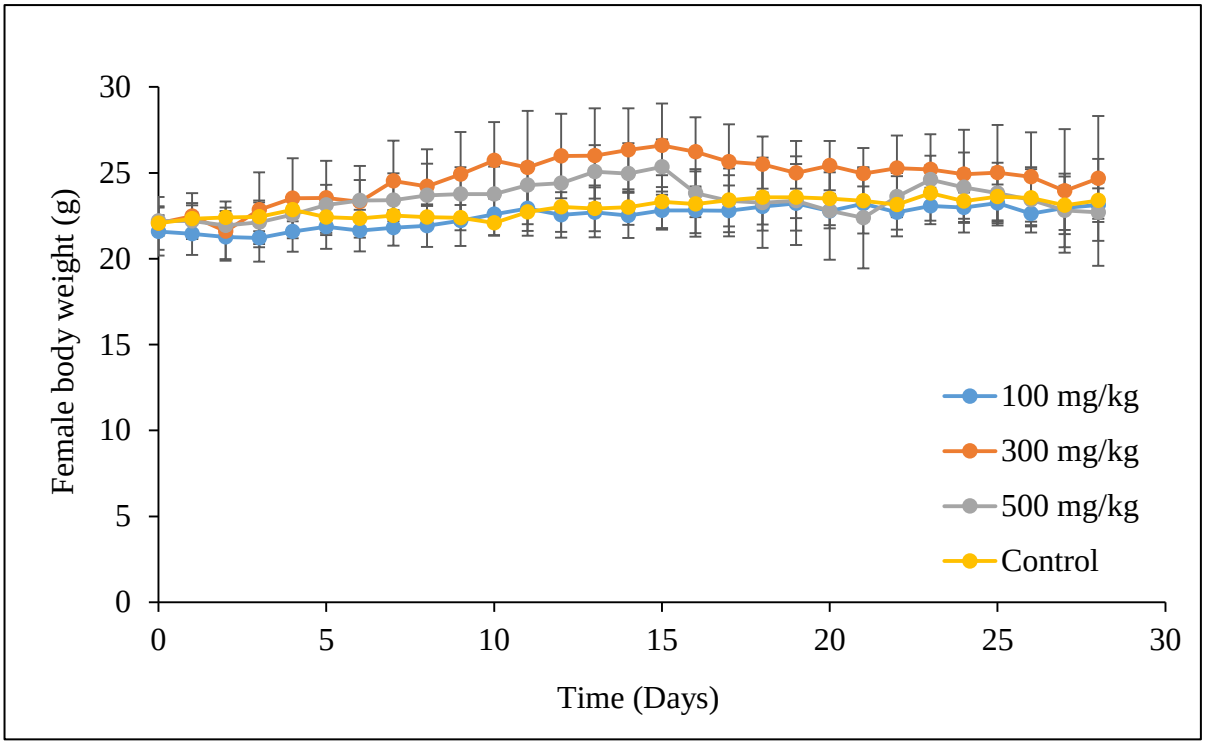


Figure 2.1: The effect of 28 days of oral treatment with TCS on the body weight of *T. congolense*-infected mice (n = 10). There was no significant difference in the treated groups of 100 and 500 mg/kg, while 300 mg/kg ($p < 0.0001$) in comparison to the control group ($p > 0.05$). The data are presented as the mean ± S.D.

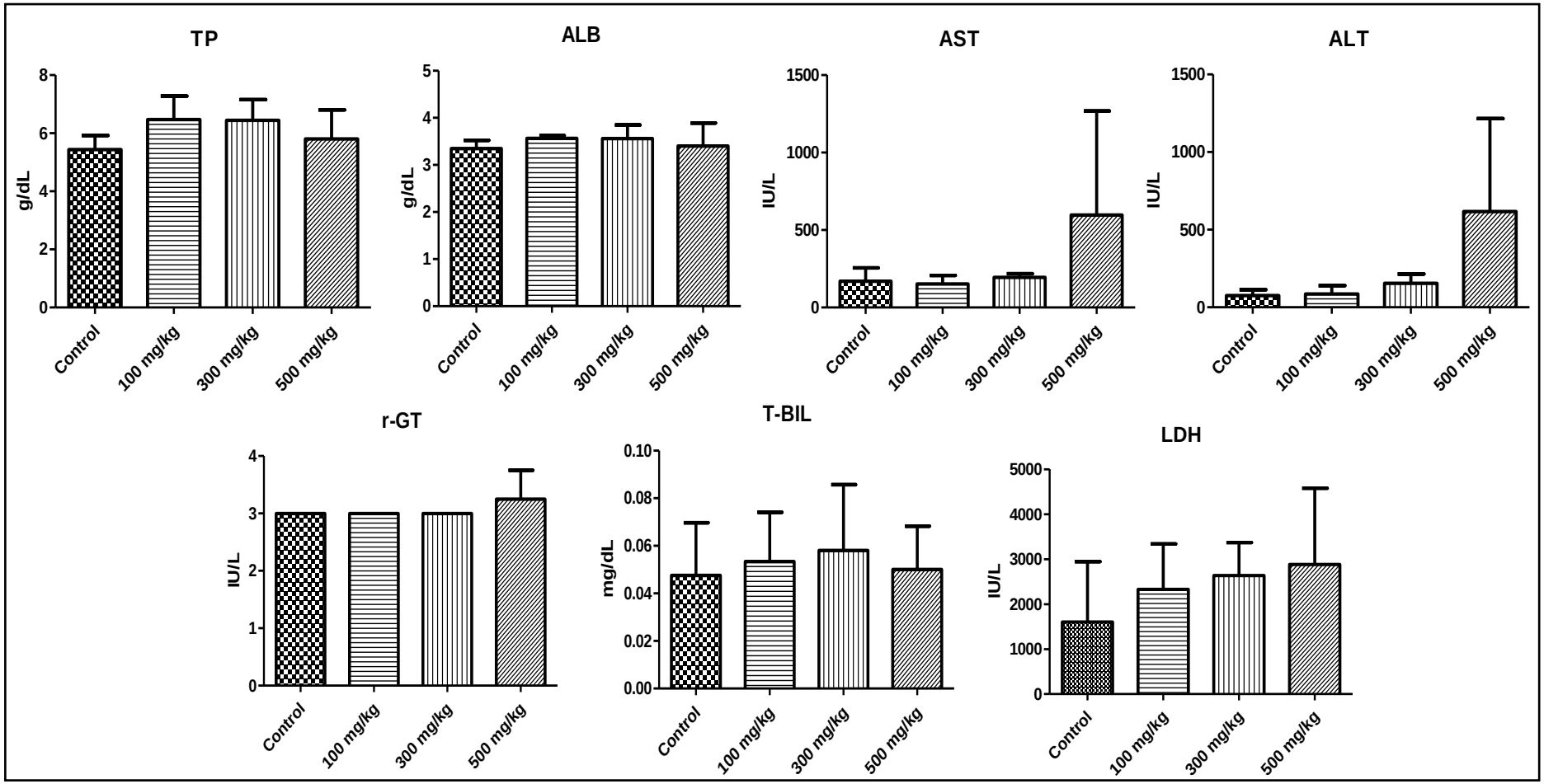


Figure 2.2: The effects of TCS on the biochemical parameters (liver functioning enzymes) on the mice that received TCS orally for 28 days. There was no significant difference in the biochemical parameters of the treated groups ($p > 0.05$) as compared to that of the respective control groups.

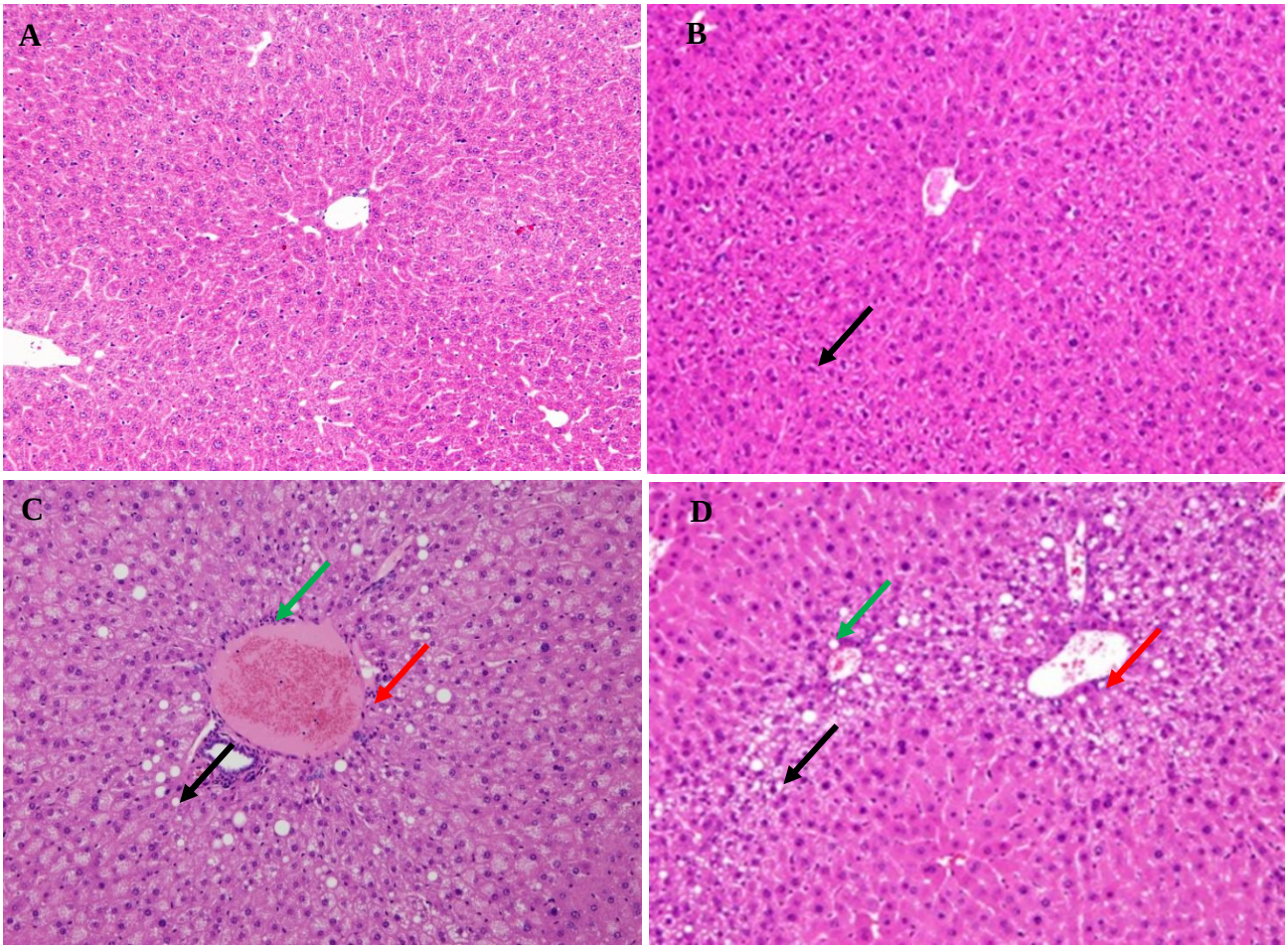


Figure 2.3: Liver histology of TCS treated mice A) control group B) 100 mg/kg C) 300 mg/kg and D) 500 mg/kg showing dose dependent hydropic degeneration of the hepatocytes (black arrow); megakaryocytes (green arrow) and extramedullary haematopoiesis (red arrow). Magnification: 400×.

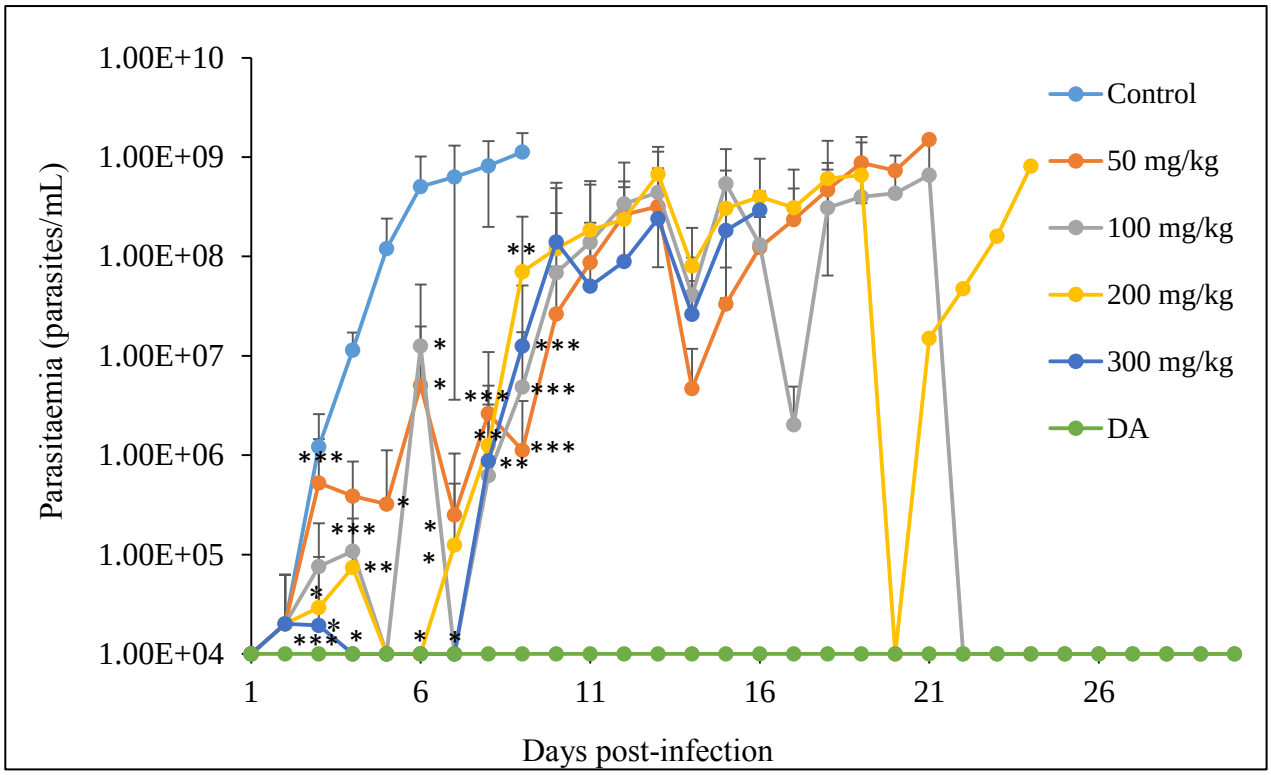


Figure 2.4: The evaluation of parasitaemia in mice infected with *T. congolense* and orally treated with different concentrations of TCS. The parasitaemia at 50 mg/kg was significantly different from days 4 to 9 while the parasitaemia of all of the other groups showed significant differences in comparison to the control group from days 3 to 9. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$. The data are expressed as the mean ± S.D. 1×10^4 represents parasitaemia below the detection levels.

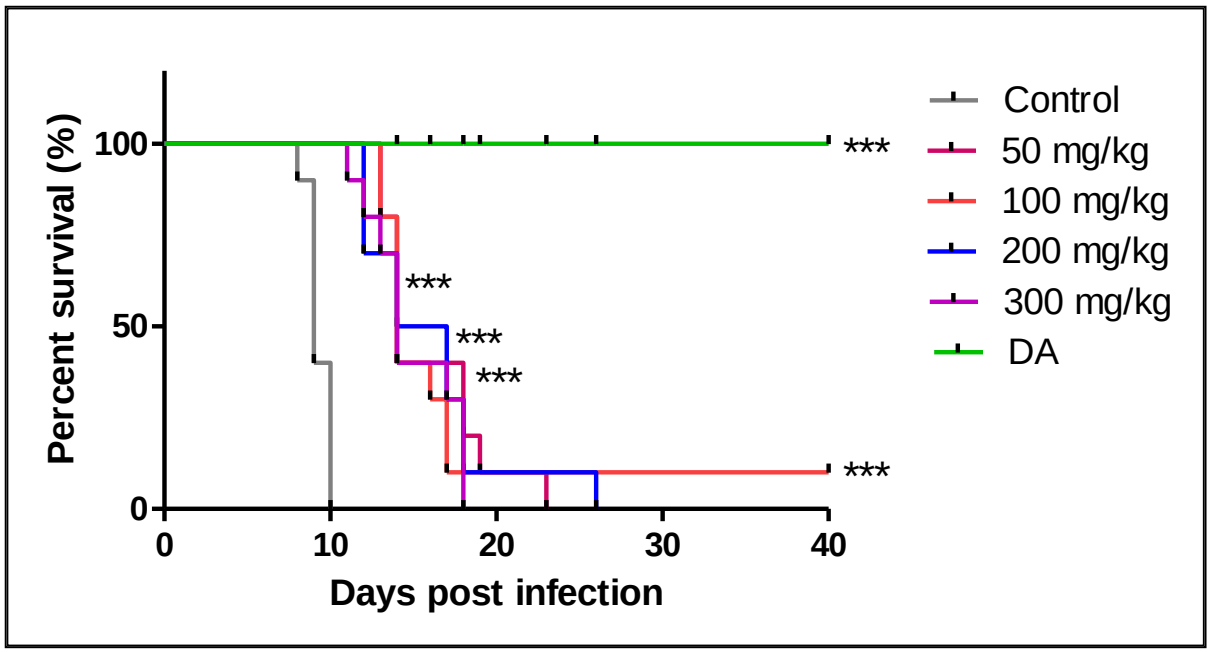


Figure 2.5: Survival curves of mice infected with *T. congolense* and orally treated with different concentrations of TCS. The survival rate was significantly different from that of the control group (n = 10), *** $p < 0.0001$ (Log-rank test).

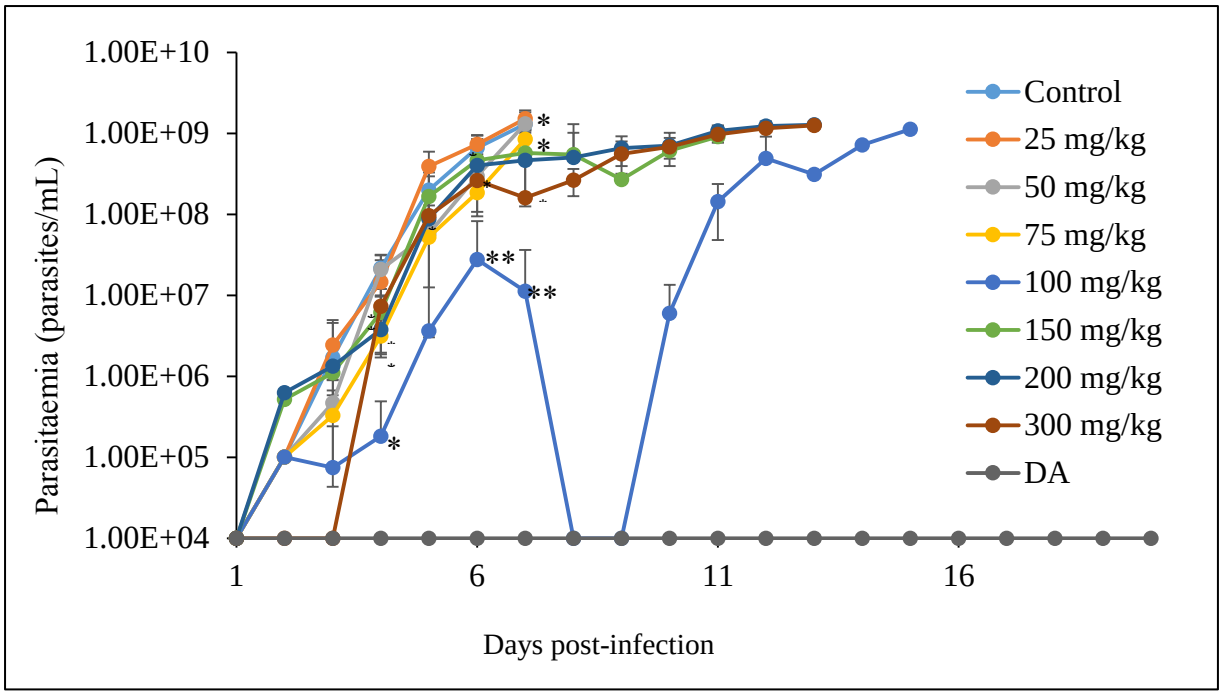


Figure 2.6: The evaluation of parasitaemia in mice infected with *T. congolense* and intraperitoneally treated with different concentrations of TCS. * $p < 0.05$ at 100 mg/kg on days 4, 5, 7 and 8 while the parasitaemia of all of the other groups showed significant differences in comparison to the control group from days 3 to 8. * $p < 0.05$; ** $p < 0.001$. The data are expressed as the mean ± S.D. 1×10^4 represents parasitaemia below the detection levels.

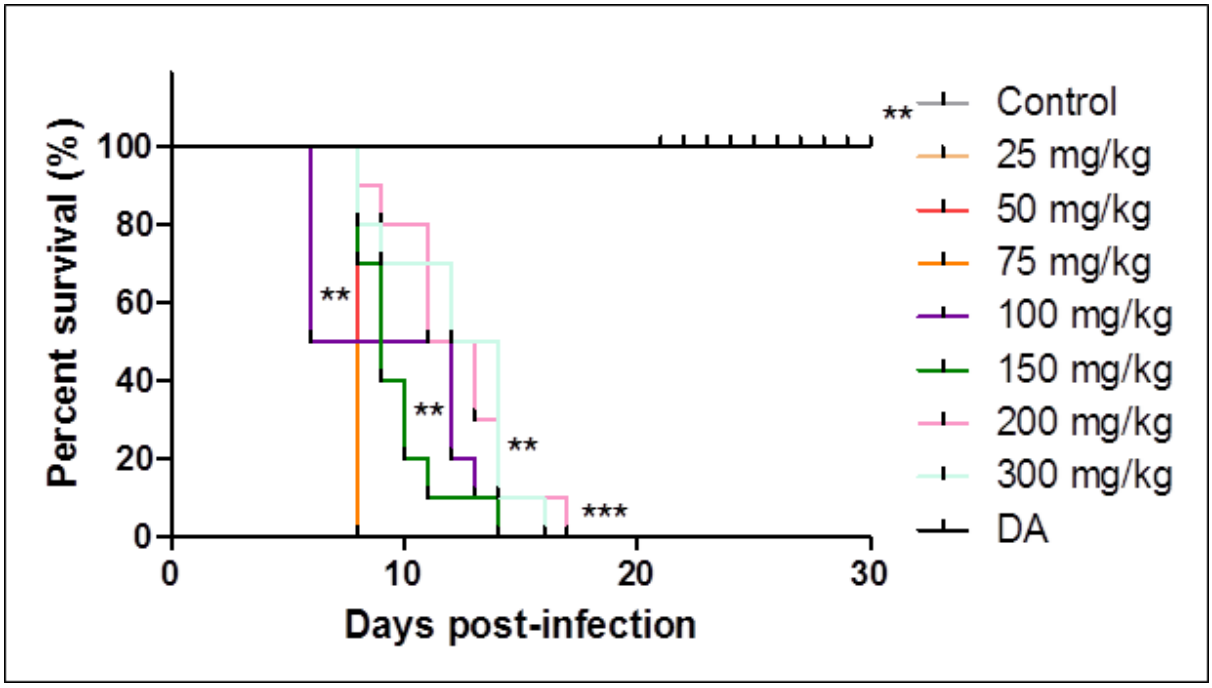


Figure 2.7: Survival curves of mice infected with *T. congolense* and intraperitoneally treated with different concentrations of TCS. The survival rate was significantly different from that of the control group (n = 10), ** $p < 0.001$; *** $p < 0.0001$ (Log-rank test).

Table 2.1: Effects on the relative organ weight of the orally treated mice with TCS.

Organs	Negative control	100 mg/kg	300 mg/kg	500 mg/kg
Heart	0.56 ± 0.09	0.51 ± 0.06	0.45 ± 0.07	0.49 ± 0.38
Liver	4.85 ± 0.58	6.53 ± 1.55	9.73 ± 1.34***	11.63 ± 0.73***
Kidney	1.53 ± 0.16	1.35 ± 0.32	1.78 ± 0.57	1.03 ± 0.65
Spleen	0.27 ± 0.16	0.50 ± 0.08	0.35 ± 0.04	0.35 ± 0.23
Pancreas	0.23 ± 0.10	0.25 ± 0.15	0.54 ± 0.02	0.23 ± 0.33

The values are represented as mean (g) ± standard deviation of mean. Statistical difference between control and TCS treated group were given as *** $p < 0.0001$.

Table 2.2: Effects on haematological parameters of mice orally treated with TCS for 28 days

	Control	**100 mg/kg**	**300 mg/kg**	**500 mg/kg**
WBC (10^2/ µl)	144.00 ± 22.73	196.40 ± 30.29	186.40 ± 46.22	149.50 ± 34.68
RBC (10^4/ µl)	932.80 ± 97.32	901.40 ± 57.98	793.00 ± 161.65	841.00 ± 71.12
HGB (g/ dL)	14.04 ± 1.45	12.96 ± 0.87	10.82 ± 2.20**	11.65 ± 0.69
HCT (%)	43.42 ± 4.00	41.48 ± 2.66	36.90 ± 6.45	39.05 ± 2.24
MCV (fL)	46.60 ± 1.11	46.02 ± 0.23	46.82 ± 1.85	46.56 ± 1.65
MCH (pg)	15.06 ± 0.29	14.38 ± 0.26*	13.64 ± 0.29***	13.90 ± 0.58***
MCHC (g/dL)	32.32 ± 0.78	31.24 ± 0.59	29.20 ± 1.16***	29.83 ± 0.30***
PLT (10^4/ µL)	96.48 ± 11.61	128.75 ± 2.22**	140.50 ± 0.71**	131.00 ± 16.37**
RDW (%)	12.58 ± 0.34	13.96 ± 0.23**	15.22 ± 0.92***	15.23 ± 0.44***
PCT (%)	0.32 ± 0.06	0.39 ± 0.02	0.45 ± 0.01*	0.41 ± 0.04*
MPV (fL)	3.32 ± 0.25	3.05 ± 0.13	3.20 ± 0.14	3.10 ± 0.10
PDW (%)	17.90 ± 0.84	17.20 ± 0.41	17.24 ± 0.70	17.15 ± 1.12

The values are represented as mean ± standard deviation of mean. Statistical difference between control and TCS treated group were given as *$p < 0.05$; **$p < 0.001$ and *** $p < 0.0001$.

Chapter 3

In vitro and *in vivo* trypanocidal efficacy of curcumin and curcumin nanoparticle on animal trypanosomes

Introduction

Curcumin, (CUR) 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione is a natural yellow polyphenol compound extracted from the rhizome of *Curcuma longa* plant. It is commonly used as a food additive, a principal ingredient in foodstuff. CUR possesses an array of biological and pharmacological effects such as anti-inflammatory, anti-malaria, antimicrobial, anticancer, anti-diabetic effects and the potential treatment of hypertension, liver injury and Alzheimer's disease (Mishra *et al.,* 2009; Witkin and Li, 2013; Altıntoprak *et al.,* 2016; Duan *et al.,* 2016; Kukongviriyapan *et al.,* 2016).

According to Witkin and Li (2013) CUR expresses its diverse biological action on the cells through three major systems, namely; inflammatory, cell death pathways linked to nuclear factor κB (NF-κB) and the oxidative stress system. Additionally, CUR possesses a beneficial action of scavenging the superoxide radical, the ROS, and therefore combating oxidative stress caused by either pathogens or metabolic exercises induced stress. Moreover, the efficacy of CUR is due to its ability to impact cell adhesion and neurogenesis which has thus far resulted in the documented cell arrest, apoptosis by caspase induction, and inhibition of cell proliferation and metastasis of the cancer cells (Prasad *et al.,* 2014; Duan *et al.,* 2016).

CUR and turmeric products have been characterized as safe by the Food and Drug Administration (FDA) in the United States of America, the Natural Health Products Directorate of Canada and the joint FAO/WHO Expert Committee on Food Additives of the Food as well as Agriculture Organization/WHO (Kukongviriyapan *et al.,* 2016). CUR is relatively an inert drug and thus far has not appeared toxic in either animals or humans. Supporting studies of

Witkin and Li (2013) and Moghadamtosi *et al.* (2014) have stated that CUR is tolerable at concentrations of 8 g/day and 12 g/day, respectively.

Regardless of all the documented efficacy, CUR is still not yet a pharmaceutically available drug due to its poor pharmacokinetics and/or physicochemical properties. The hydrophobic nature (solubility: 20 mg/mL in water) of CUR constitutes a primary constriction, resulting in a low bioavailability from the gastrointestinal tract, poor absorption and metabolism. Furthermore, the compound lacks stability in an alkaline medium even though it is quite stable in an acidic medium, with a short plasma life. Lastly, undergoes a rapid metabolism in a form of degradation of CUR by alkaline hydrolysis, followed by the molecular fragmentation (Huynh *et al.,* 2013; Duan *et al.,* 2016; Kumar *et al.,* 2016).

In a venture to improve the pharmacokinetics and/or physicochemical properties of CUR, researchers have established the usages of nanotechnology. This initiative has positively affected the pharmacokinetics of CUR, with reported improvement of the aqueous solubility, bioavailability, alkaline stability, prolonged circulation, better permeability and tolerance of the compound by the hosts (Nguyen, 2011; Desai, 2012).

Nanotechnology is a branch of medicine utilizing particles smaller than 100 nm in size for the treatment of various diseases and infections. These particles are useful in a diverse range of applications. In the pharmaceutical branch, nanoparticles are seen as a vital means of overcoming challenges facing hydrophobic agents, utilized in a form of a drug delivery system. So far the nanoparticles can be classified into liposomes, micelles and encapsuled nanoparticles, phospholipid complexes (Prasad *et al.,* 2014).

Nanoparticles improve the bioavailability of drugs by enhancing the brain blood vessel membrane fluidity, with the ability of accumulating higher concentrations in the experimental animals, thereby improving the permeability of the compound. A tremendous improvement of the bioavailability of several compounds has been achieved through nanotechnology, which

amongst others are the trypanocidal compounds, diminazene aceturate (DA), pentamidine and suramin (Gonzales-Martin *et al.,* 1998; Olbrich *et al.,* 2004; Arias *et al.,* 2015). Above all, the utilization of nanoparticles has enhanced the efficacy of the drugs as compared to the free compounds. The better efficacy of a nanoparticle drug is attributed to the ability of the nanoparticle to withstand physiological stress due to their stability in various mediums (Huang *et al.,* 2012; Date *et al.,* 2016). A concern was raised by Burgos-Moron *et al.* (2009), who reported the possible toxicity of the free curcumin. Among some toxicity symptoms recorded was diarrhoea which raised an interest based on the fact that several studies have documented a significant percent of CUR excreted rapidly in either urine or faeces and therefore likely to induce diarrhoea (Anand *et al.,* 2007).

Several studies were conducted on the trypanocidal effect of curcumin on *Trypanosoma evansi, T. b. brucei* and *T. cruzi* (Nose *et al.,* 1998; Gressler *et al.,* 2015; Novaes *et al.,* 2016). The compound was tested both as a free compound and loaded in the nanoparticle. According to my knowledge this is the first study with an objective of determining the trypanocidal effect of CUR and CUR-nanoparticles conducted *in vitro* against *T. congolense, T. b. brucei* and *T. evansi* bloodstream forms simultaneously. Furthermore, to determine the cytotoxicity on the host cells, and to demonstrate *in vivo* CUR and CUR-nanoparticles efficacy against *T. congolense.* Additionally, histopathological analysis was also conducted to determine the long-term effects of CUR on the small intestine, as a way of monitoring the extent of CUR induced diarrhoea in healthy mice.

Materials and methods

Curcumin compound

CUR [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-hep-tadiene-3,5-dione] compound was purchased from Sigma Aldrich, Japan.

Preparation of nanoparticles

Antisolvent precipitation with a syringe pump (ASPS)

The solution of original CUR were prepared in ethanol at the predetermined concentration of 20 mg/mL. The syringe was filled with 10 mL of the prepared solution and secured onto a syringe pump. The drug solution was quickly injected at a fixed flow rate of 2 - 10 mL/min into a deionized water that acted as the antisolvent of a definite volume under magnetic stirring at 1,000 rpm. Ratio of ethanol to water used was 1:10 (v/v) corresponding to a volume of 100 mL of water to 10 mL of a drug solution. The CUR-nanoparticles formed were filtered and vacuum dried (Taitec VD-500R freeze dryer, Japan) (Kakran *et al.*, 2012).

Evaporative precipitation of nanosuspension (EPN)

The solution of an original CUR was prepared in ethanol (Sigma-Aldrich, Japan) and then a nanosuspension was formed by quickly adding into hexane (Wako Pure Chemical LTD., Japan) that acted as an antisolvent. Drug particles in the nanosuspension were obtained by quick evaporation of the solvent and antisolvent under vacuum evaporator to obtain dry particles (Kakran *et al.*, 2012).

Wet-milling method (WN)

Stock of 20 mg/mL CUR solution was prepared by dissolving CUR powder in 10 mL dichloromethane (Sigma-Aldrich, Japan). The solution was injected into a syringe and secured onto a syringe pump. The solution was added drop-wise into the deionized boiling water under ultrasonication condition with ultrasonic power (Branson, 3510 Yamato, Japan) at a frequency of 50 kHz. The solution was sonicated for 30 minutes then the mixture was stirred at 800 rpm for 20 minutes until the orange colored precipitate was obtained. Thereafter, the solution was vacuum dried to obtain the powdered nanoparticles (Pandit *et al.,* 2015).

Preliminary detection of CUR-nanoparticle by UV-visible spectroscopy

The preliminary detection of synthesized CUR-nanoparticle was carried out by UV-visible spectrophotometer (Thermo Fischer Scientific, Japan), scanning the absorbance spectra in the range of 190 - 500 nm wavelength.

Particle size and shape analysis

The synthesized CUR-nanoparticles were subjected to TEM analysis to determine their size and shape. The nanoparticles were dissolved in a deionized water to prepare a 10 mg/mL solution. The solution was added onto the carbon grid and left overnight at room temperature. Once dried, the samples were then analyzed (Hanaichi Ultrastucture Research Institute, Aichi, Japan). Additionally, NIH ImageJ software was used to calculate the average particle size of the nanoparticles.

Parasites and *in vitro* culture

The trypanosome parasites (*T. b. brucei*, *T. congolense*, *T. evansi*) *in vitro* cultures were prepared as described in Chapter 1.

Cell line cultures

The mammalian cell line cultures were prepared as described in Chapter 1.

The CUR *in vitro* cytotoxicity tests

The *in vitro* cytotoxicity tests were conducted as described in Chapter 1 only that in this occasion the compound tested was CUR.

Animal experiments

Animal experiments were conducted as described in Chapter 1, only that they were treated with CUR and CUR-nanoparticles. Additionally, the HFF cells were used to determine the cytotoxicity of the CUR-nanoparticles.

Histopathological analysis

The heart, liver, kidneys, spleen and small intestine tissues were collected for histological studies from healthy mice that received CUR in corn oil for 28 consecutive days. The tissues were washed in normal saline and fixed immediately in 10% formalin overnight, dehydrated with isopropanol alcohol, embedded in paraffin, cut into 4 - 5 µm thick sections, and stained with haematoxylin-eosin (H & E) dye for photomicroscopic observation by Dr.

Kenichi Watanabe at the Research Center for Global Agromedicine, Obihiro University of Agriculture and Veterinary Medicine. The microscopic features of treated groups were compared with those of the control group.

The trypanocidal effects of CUR and CUR-nanoparticles on *Trypanosoma in vivo*

Oral administration

The virulent *T. congolense* IL3000 strain were propagated in mice and used for infection. The parasites were passaged twice in mice before the experiment. The mice were intraperitoneally infected with 100 µL of *T. congolense* (5×10^3 parasites/mouse) inoculated with phosphate buffered saline with 1% glucose (PSG). The mice were randomly divided into 7 groups of 5 mice as follows: Group I (control group), the mice were infected but not treated; group II (positive control group), the mice were infected and treated with diminazene aceturate (DA - 3.5 mg/kg, intraperitoneally) (Sigma Aldrich, Japan); Groups III, IV, V, VI and VII (the test groups), the mice were infected and orally treated using a feeding needle with 50, 100, 200, 300 and 400 mg/kg CUR and nanoparticles, in a 200 µL inoculum, respectively. Treatment was initiated at 48 hours post-infection and was maintained for 7 consecutive days. The treatments were freshly prepared each day. The surviving mice were observed for 90 days while the others were observed until death. Each day, the parasitaemia was evaluated and the effects of treatment were monitored using wet blood smears. Each slide was prepared with fresh blood collected from the tail vein (magnification: 400×). The experiments were conducted in duplicate.

Intraperitoneal administration

The virulent *T. congolense* IL3000 strain was propagated in mice and used for infection. The parasites were passaged twice in mice before the experiment. The mice were intraperitoneally infected with 100 µL of *T. congolense* (5×10^3 parasites/mouse) inoculated with PSG. The mice were randomly divided into 9 groups of 5 mice as follows: Group I (control group), the mice were infected but not treated; group II (positive control group), the mice were infected and treated with diminazene aceturate (DA) (3.5 mg/kg, intraperitoneally); Groups III, IV, V, VI, VII, VII, IX (the test groups), the mice were infected and intraperitoneally treated with 50, 75, 100, 150 and 200 mg/kg CUR and nanoparticles, respectively. Treatment was initiated at 48 hours post-infection and was maintained for 7 consecutive days. The treatments were freshly prepared each day. The surviving mice were observed for 90 days while the others were observed until death. Each day, the parasitaemia was evaluated and the effects of treatment were monitored using wet blood smears. Each slide was prepared with fresh blood collected from the tail vein (magnification: 400×). The experiments were conducted in duplicate.

Statistical analysis

The results were expressed as the mean ± standard deviation (S.D) for the number of repeated trials indicated in each experiment. The statistical analyses were conducted in the acute phase of infection. The *t*-test was used for intergroup comparisons between the treated and non-treated groups. The survival curves were constructed using the Kaplan-Meier method and the curves were compared using a log-rank test. All of the data were compiled using GraphPad Prism Software program (version 5.0, GraphPad Software Inc., CA, USA). *P* values of < 0.05 were considered to indicate statistical significance.

Results

The nano detection was conducted using the UV-visible spectroscopy and was scanned in the range of 200 to 800 nm. The typical CUR absorption was obtained with an observed peak at 419 nm (Fig. 3.1A). TEM imaging of aqueous dispersion showed the average particle size of the CUR-nanoparticle was 120 nm (Fig. 3.1B), furthermore, the particle shape varied between oval and a spherical shape. The CUR-nanoparticles, both filtered and non-filtered, dissolved better in water than the pure CUR compound (Fig. 3.2).

In vitro trypanocidal effects

The CUR-nanoparticles were 2 folds more effective than the free curcumin. The CUR and CUR-nanoparticle showed more efficacy against *T. congolense* than *T. b. brucei* and *T. evansi*, regardless of the preparation method used. Nonetheless, the ASPS prepared nanoparticles showed a remarkable efficacy on all the trypanosome strains, with less cytotoxicity recorded on the host cells, even though the selectivity index was not higher than that of the WM prepared nanoparticles (Table 3.1). The cytotoxicity effects of CUR was determined on the MDBK and NIH 3T3 cells while those of nanoparticles were tested on the MDBK and HFF cells.

Histopathological analysis

Histological analyses of the duodenum and ileum tissues in female mice exposed to CUR treatment showed a dose dependent atrophy of the villi and the thinning of the mucosa propria (Fig. 3.3 and Fig. 3.4) as compared to the control group.

***In vivo* tests**

Oral administration

CUR

Parasitaemia levels were significantly reduced in all the treated groups in days 4, 5, 6 and 7 as compared to the control group. A low parasitaemia was obtained and maintained for some days in the treated groups, whist that of the control group was observed on day 8 resulting in the death of the mice. Parasites were not cleared completely from the blood circulation, but significantly suppressed and allowed a longer survival of the mice in all the treated groups. None of the mice was cleared cured in all the groups, except those in the DA treated group. At 400 mg/kg, on day 14, the mice showed the ability to withstand high infection illustrated as the highest peak wave of the whole study (Fig. 3.5).

There was a recorded significant difference in the survival of the mice in the treated groups ($p < 0.0001$) even though none of the mice survived in the CUR treated groups. The survival prolongation of the mice resulted in death of the mice in the treated groups on days 16, 17 and 20 while those in the control group died on day 9 (Fig. 3.6).

CUR-nanoparticle

The oral treatment of the mice with CUR-nanoparticle resulted in a significant decrease and suppression of the parasitaemia in the infected mice between days 5 and 7, in comparison to the control group. As could be depicted in the figure, the parasitaemia in the control group increased steadily until all the mice had died. The treated group displayed the first parasitaemia wave on day 6 followed by a decrease in parasitaemia. The period between day 7 and 10 showed a suppressed parasitaemia in all the treated group. The resurface of the parasites in the circulation was followed by a rapid increase in parasitaemia of the treated group which led to

a total death of the mice in groups 100 and 300 mg/kg while few in the 50 and 400 mg/kg groups were declared cured from the infection (Fig. 3.7).

There was a significant survival rate of the mice of $p < 0.05$ at 100, 200 and 300 mg/kg, $p < 0.01$ at 400 mg/kg and $p < 0.001$ at 50 mg/kg as compared to the control group. On day 9, all the mice in the control group died while those in 100, 200 and 300 mg/kg died within days 20, 16 and 18, respectively. A 10 % of mice in the 50 and 400 mg/kg survived until the termination of the study, 90 days post infection. None of the mice in the DA group died (Fig. 3.8).

Intraperitoneal administration

The intraperitoneally treatment of *T. congolense* infected mice possessed no efficacy against the infection. Neither the mice treated with free curcumin nor the CUR-nanoparticles were declared cured (Fig. 3.9A and Fig. 3.10A). Mice in both the treatment groups died within 8 days. There was no significant difference in the survival of the mice in the treated group in comparison to the non-treated control group (Fig. 3.9B and Fig. 3.10B).

Discussion

CUR is a natural compound extracted from a plant, which makes it an important part of drug discovery. Due to the poor pharmacokinetics of CUR, this study synthesized CUR-nanoparticles as a mean of improving the efficacy of the compound on trypanosomes. Synthesized CUR-nanoparticle average size was obtained to be 120 nm, which was higher than 2-40 nm and 92-110 nm range reported by Bhawana *et al.* (2011) and Pandit *et al.* (2015), however, comparable to the average size of 330 nm reported by Kakran *et al.* (2012).

In the current study the trypanocidal effect of CUR and CUR-nanoparticle was tested on three animal trypanosome species. Using the *in vitro* trypanocidal activity evaluation assay, CUR and CUR-nanoparticle were more effective on *T. congolense* as compared to *T. b. brucei* and *T. evansi*. Phylogenetically it has been proven that *T. b. brucei* and *T. evansi* are closely related to each other than *T. congolense*, which forms a distinct clade all together (Maudlin *et al.,* 2004), therefore, the observed distinct efficacy is likely a phylogenetically based object of these three species of animal trypanosomes. The efficacy record in the current study was better with lower IC_{50} of 2.82 ± 1.22 values of on *T. b. brucei* as compared to 4.77 ± 0.91μM (17.57 μg/mL) observed in the study by Nose *et al.* (1998).

Cytotoxicity assays are widely used as indicators of the ability of the test compounds to induce cell death as a consequence of damage to one or more cellular function (Weyermann *et al.,* 2005). Herbal derived medicine are gaining importance globally, it is therefore necessary to establish safety, toxicity, efficacy and quality data in accordance with regulatory guideline (Khan *et al.,* 2016). This study demonstrated the cytotoxicity effects of CUR and CUR-nanoparticles on the MDBK, NIH 3T3 and HFF cells, with NIH 3T3 cells being more susceptible to the compound as compared to the MDBK and HFF cells. The study by Xia *et al.* (2008) reported and confirmed the susceptibility of mouse derived cell lines, however, it has also been documented that cells from the same species of origin and tissue susceptibility can

differ. The cytotoxicity of free CUR compound on the NIH 3T3 cells was in agreement with the Jiang *et al.* (1996) which stated the ability of the compound to induce apoptosis of the host cells, for this reason NIH 3T3 cells were used only for cytotoxicity determination of CUR and not that of the CUR-nanoparticles.

Even though CUR is considerably safe; there has been reports of mild diarrhoea, nausea and skin irritation (Witkin and Li, 2013; Xiao *et al.,* 2015) as side effects, for this reason, the current study sought to monitor the extent of diarrhoea induced by CUR in healthy mice by providing CUR orally for 28 days in the healthy mice. From the histopathology images, there were no adverse changes on the morphology of the small intestines except the atrophy of the villi and the thinning of the mucosa propria (Fig. 3.3 and 3.4). During the experiments diarrhoea was observed visually with CUR coloured faeces and urine. The presence of dose dependent atrophy of the villi and the thinning of the mucosa propria demonstrated a dose dependent induction of diarrhoea in mice. Higher concentrations were tested as a measure of follow up studies in either lower or similar concentrations. The mechanism of diarrhoea induced by compounds is not yet understood, however, Kamar *et al.* (2004) reported severe diarrhoea that revealed duodenal villous atrophy in patients treated with mycophenolate motifel. Nonetheless, due to high concentrations trialled for the histopathological analysis, the histological changes in the ileum and duodenum were not significant to prevent further animal experiments.

In vivo, in *T. congolense* infected mice, a mild CUR efficacy was observed in the orally treated mice as compared to the intraperitoneal administration. The oral administration effectivity of CUR and its nanoparticle was not significantly different from each other as was anticipated from the onset of the study. Both compounds were affected by various factors, such as the solvent, drug given with food and the initial period of the treatment. The nanoparticle regimens dissolved in corn oil possessed a better efficacy than the samples dissolved in water. Nanoparticle size affects the solubility of a compound in water. Previous reports have shown

that bigger sized nanoparticles have the tendency to remain hydrophobic. Furthermore, the bigger sized nanoparticles are likely to coalesce within the cell membrane bound vesicle which means that they are at risk of being phagocytized (Chunbai *et al.,* 2010). Based on this hypothesis, the nanoparticles produced in this study could have been insignificantly bigger, for they visually dissolved in water. Or perhaps, the modification of the pharmacokinetics required CUR loaded in lipid core nanocapsule instead (Gressler *et al.,* 2015).

Several studies have illustrated good absorption of compounds administered in the absence of food due to the food-drug interaction phenomenon. Fasting the mice before drug administration enhances the oral absorption of the active ingredient from the gastrointestinal tract but similarly alters the physiological function of the intestines and possibly the metabolic enzymes (Bushra *et al.,* 2011; Arinkar *et al.,* 2012). Few of the compounds effective in the absence of food include tetracycline, penicillin and tobramycin (Kung *et al.,* 1995; De Leo *et al.,* 2010). Food taken simultaneously with drugs have the proclivity to reduce and delay the absorption and the bioavailability of the active ingredient by increasing the drugs metabolic clearance rate. Nonetheless, some drugs contain irritants and should be taken with food (Winstanley and Orme, 1989). However, currently, mice treated in the absence of food succumbed faster to the infection than mice receiving food. As mentioned before, CUR is a food additive, so far there has not been any food-drug interaction reported.

Mice treated 48 hours post infection were not quite relieved from the infection as the ones treated within the first 24 hours. None of the mice were cured from trypanosomosis but the ones treated earlier survived longer regardless of the relapsed cases suggesting the quick establishment of *T. congolense* in BALB/c mice. Treatment efficacy relies on the period of establishment of the infection in mice. The frozen stock parasites were passaged in mice by syringe for pre-infection and then eventual infection. According to Cressler *et al.* (2016) syringe passage of the trypanosomes induces higher virulence in the hosts. Nonetheless, in the

actual setting, the sooner the treatment the better treatment options and efficacy, hence early diagnostics remains important. The firm establishment of infection requires a compound with long half-life to treat, which is why the free CUR was not much effective, without any mice relieved from the infection, not even on a single day. A compound with short half-life, less bioavailability, low permeability like CUR is likely to result relapse of parasitaemia when used as treatment based on the possible varying concentrations absorbed into the circulation even if the compound has some efficacy. The nanoparticle pharmacokinetics were better than that of the free curcumin, judging from few cured mice in this group and the solubility in water (Pandit *et al.,* 2015).

CUR-nanoparticle was more effective *in vitro* as compared to the free curcumin, which illustrated the perfect absorption of the compound in the parasites. The *in vivo* tests were conducted on *T. congolense* based on the preliminary *in vitro* tests as well as the selectivity of the compound. However, in the *in vivo* experiments, the efficacy of CUR loaded with nanoparticles was not satisfying, with observed efficacy which was short lived with observed relapses. Moreno *et al.* (2010) stated that the *in vitro* efficacy does not allow the prediction of therapeutic outcome in the hosts. This concept is challenging the scientific world, and it is associated with different mode of action of the active compound *in vitro* and in the host system (Feyera *et al.,* 2014). Similar cases were observed in previously documented studies, the difference between *in vitro* and *in vivo* efficacy of the drug might be due to different mode of action of the drug *in vitro* as compared to the animals (Moreno *et al.,* 2010). Additionally, the host-pathogen factors influence the efficacy of the compound *in vivo* as compared to the medium cultured parasites tested *in vitro.* Parasites encounter different environments in the medium culture and the blood containing all the blood cells and other cell types, which is potentially responsible for the efficiency of the drugs (Kisalu *et al.,* 2014). There was no efficacy whatsoever on the intraperitoneally treated mice, whereby all mice died within 8 days

post infection. The solubility of the compound in organic solvents such as DMSO, ethanol and acetone, poses an important hurdle for parenteral administration, there are therefore few solvents that are completely safe for parenteral administration. DMSO has widely been used as a powerful solubility tool of lipophilic compounds (Bhawana *et al.,* 2011). DMSO is preferred over the others because of its ability of increasing the membrane solubility, however, according to previous reports, it is not completely safe when administered directly into the system (Cavas *et al.,* 2005; Turner *et al.,* 2011), which places the orally effective compounds and solvents in demand.

According to nanoparticle studies, there are various factors affecting the effectiveness of nanoparticles. Nanoparticles produced at the same time tend to vary in shape and size hence, average sizing of the nanoparticles is vital. As mentioned above the size of the nanoparticle has the potential to determine the uptake of the compound by macrophages or they coalesce in the cell membrane where they will be exposed to the immune defence of the host. Particle size and surface charge are also responsible for the efficiency of the compound as well as determining the pathway of the cellular uptake of the particles. The smaller the size of the particle the better the absorption (Chunbai *et al.,* 2010; Gressler *et al.,* 2015). Regarding the shape of the particle, each scenario is independent. According to (Ernsting *et al.,* 2013) the most important factor is the uptake and internalization of the particles than necessarily a particular shape. The spherical and non-spherical particles alike showed good uptake and internalization at one point or the other, depending on the pathogen in question.

The last important subject related to the nanoparticles is their toxicity. The nanoparticle concept has enhanced the permeability and absorption of active ingredients in the circulation, which exacerbates the toxicity effects due to higher accumulation of the compound in the host cells and the pathogens. Toxicity effects of liposomes was documented in monkeys while pentamidine encapsulated in polyhexylcyanoacrylate carriers was toxic to mice (Fusai *et al.,*

1994). The toxicity of nanoparticles can be modulated and regulated based on the type of the study. Nonetheless, the current study only demonstrated the safety of nanoparticles *in vitro*. The efficacy of CUR-nanoparticle could be improved by changing the carrier of the compound, perhaps lipid based carriers could be more effective.

In conclusion, there is still much to do to demonstrate the efficacy of CUR and CUR loaded nanoparticles against *T. congolense.* Whereby, thorough toxicity tests are required to further demonstrate the safety of these particles. Furthermore, there are various factors that could be monitored in order to improve the moderate trypanocidal effect observed in the current study, such as considering the long term treatment. Other factors include the particle size, the shape and the solubility in an array of solvents or nanocapsulation of the particle. This was according to my knowledge the first attempt to treat *T. congolense* infected mice with CUR and nanoparticles.

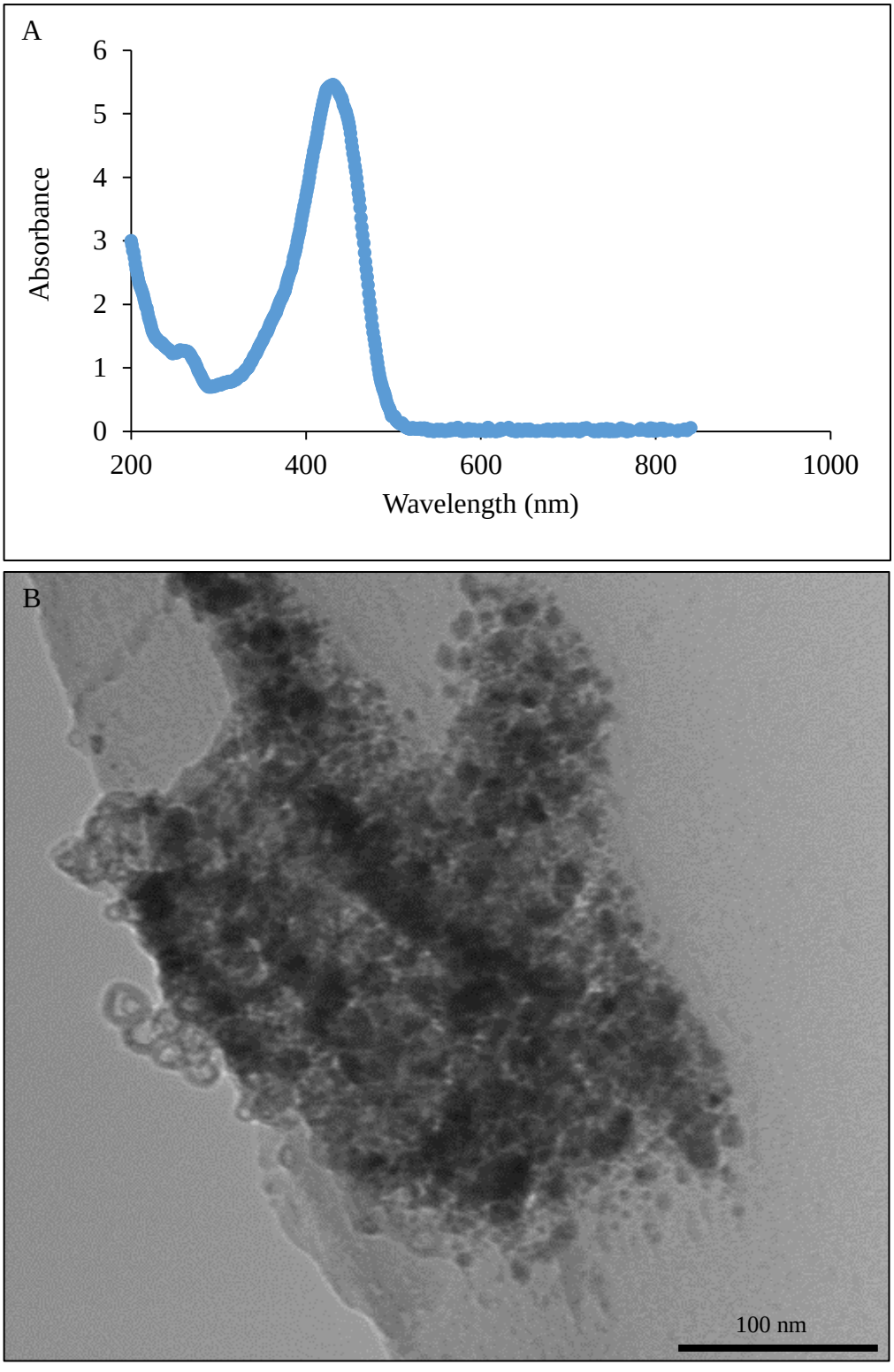


Figure 3.1: A) UV-Vis sprectra, and B) TEM imaging of aqueous dispersion of the synthesized CUR-nanoparticles.

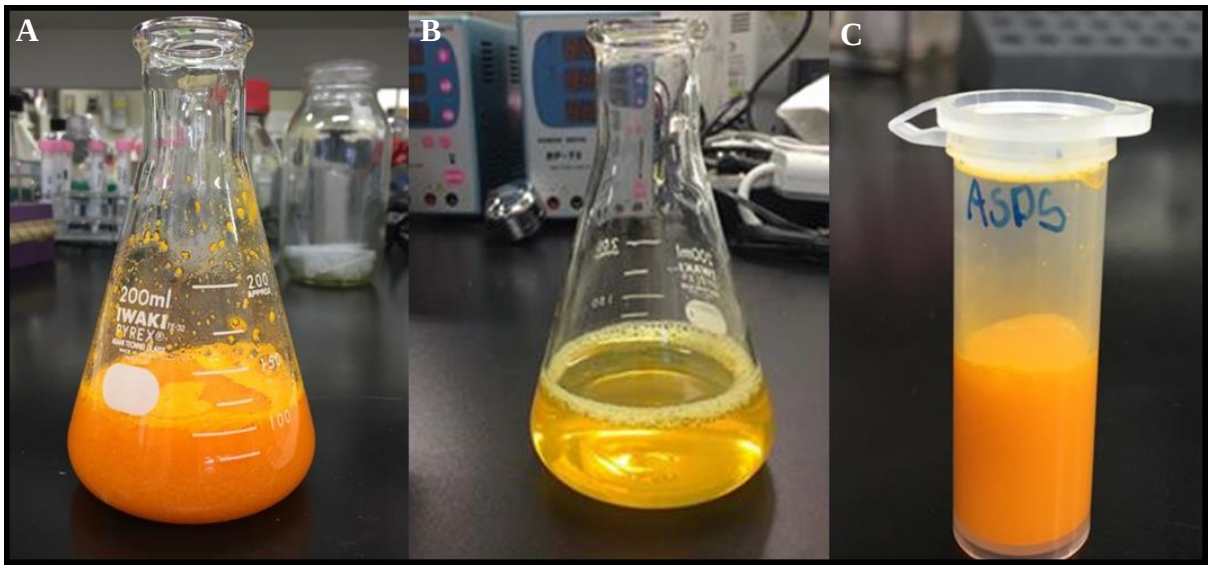


Figure 3.2: The production process of CUR-nanoparticle A) pure compound in water B) filtered end-product of CUR-nanoparticles C) non-filtered CUR-nanoparticles end-product.

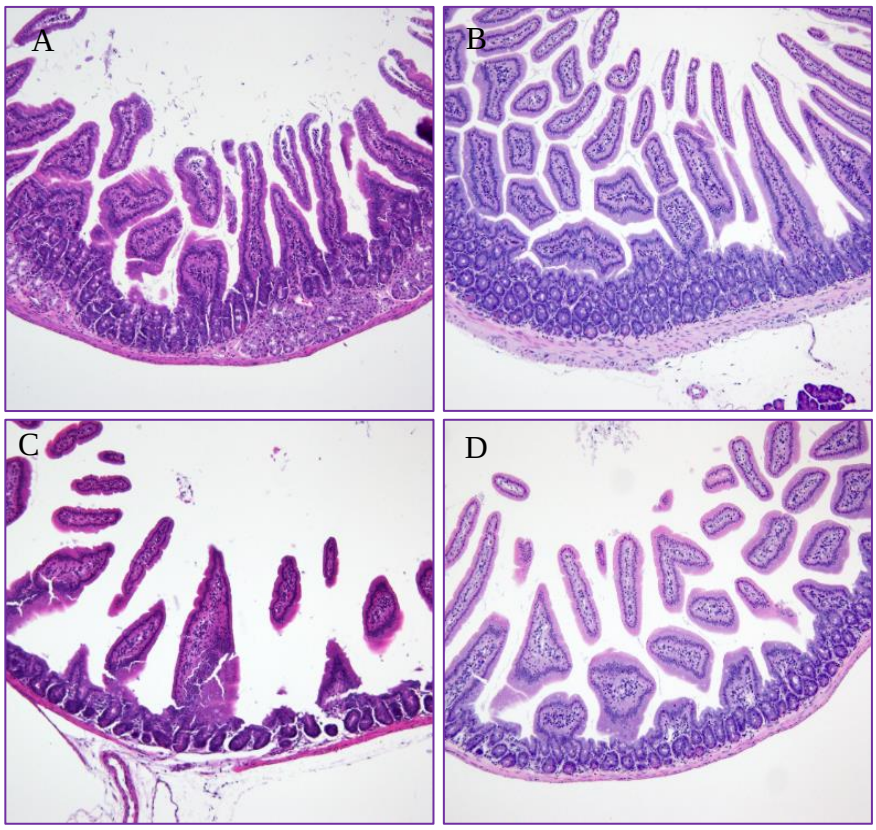


Figure 3.3: Histology of the duodenum of the control mice and those exposed to various doses of CUR for 28 days: A) control; B) 100 mg/kg; C) 500 mg/kg and D) 1,000 mg/kg.

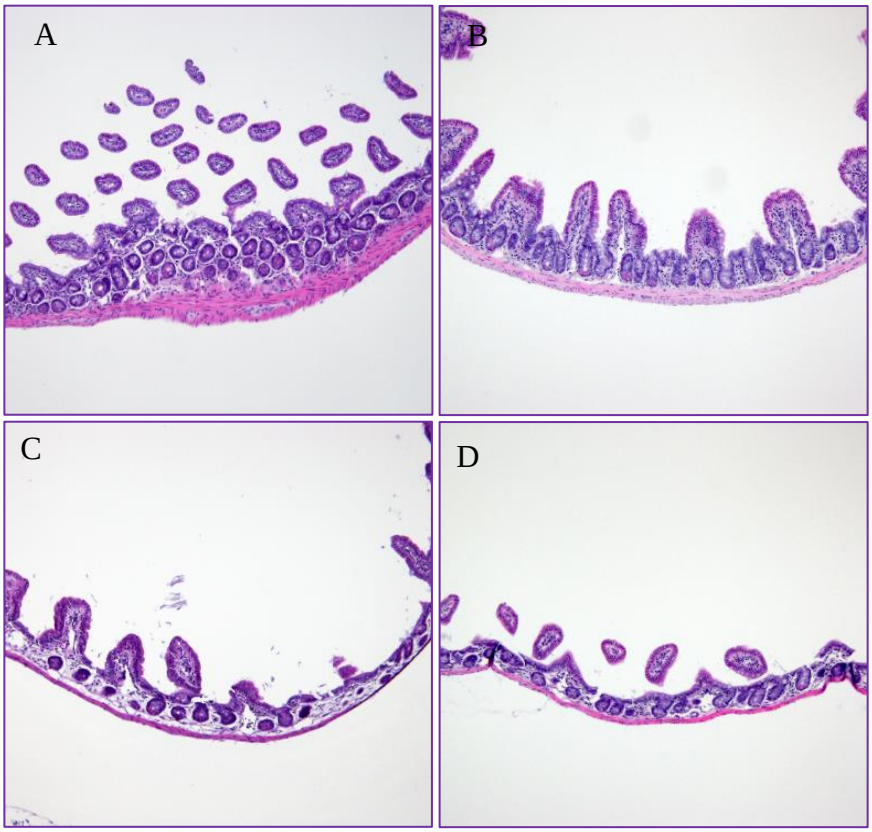


Figure 3.4: Histology of the ileum of the control mice and those exposed to various doses of CUR for 28 days: A) control; B) 100 mg/kg; C) 500 mg/kg and D) 1,000 mg/kg.

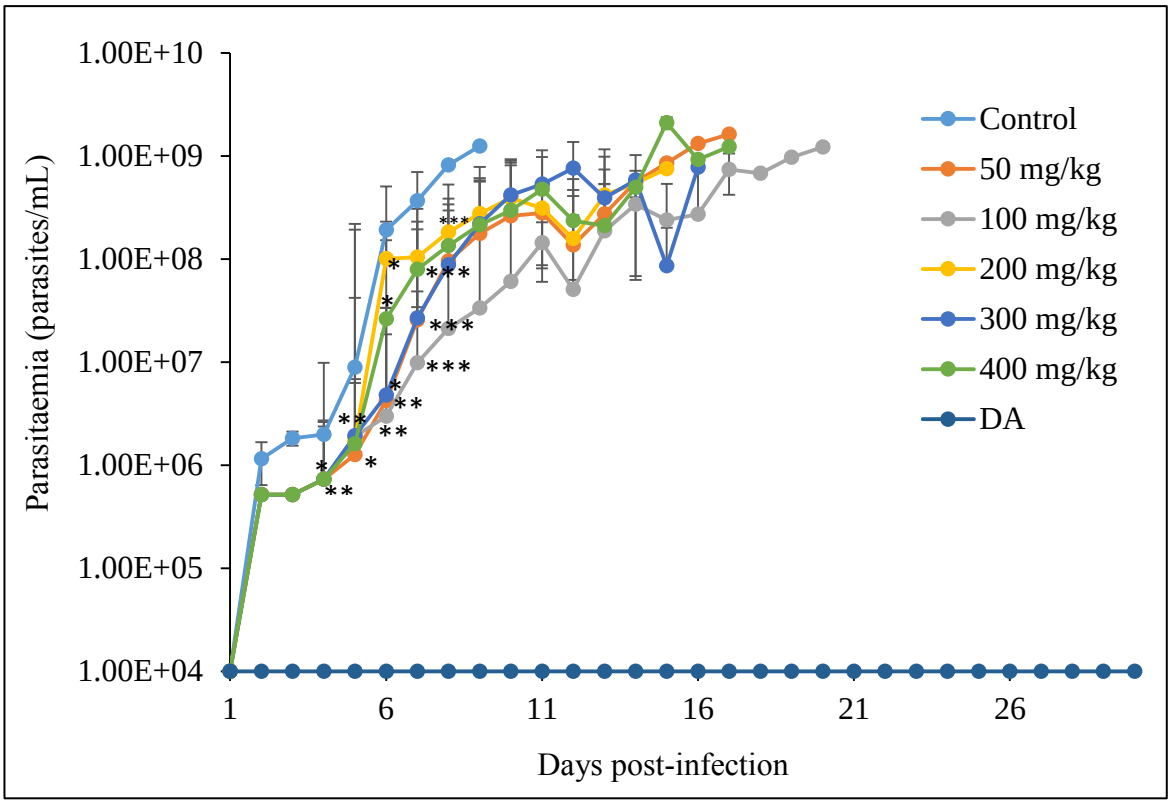


Figure 3.5: The evaluation of parasitaemia in mice infected with *T. congolense* and orally treated with different concentrations of CUR for 7 days. *$p < 0.05$; ** $p < 0.001$ and *** $p < 0.0001$. The data are expressed as the mean ± S.D. The 1×10^4 represents parasitaemia below the detection levels.

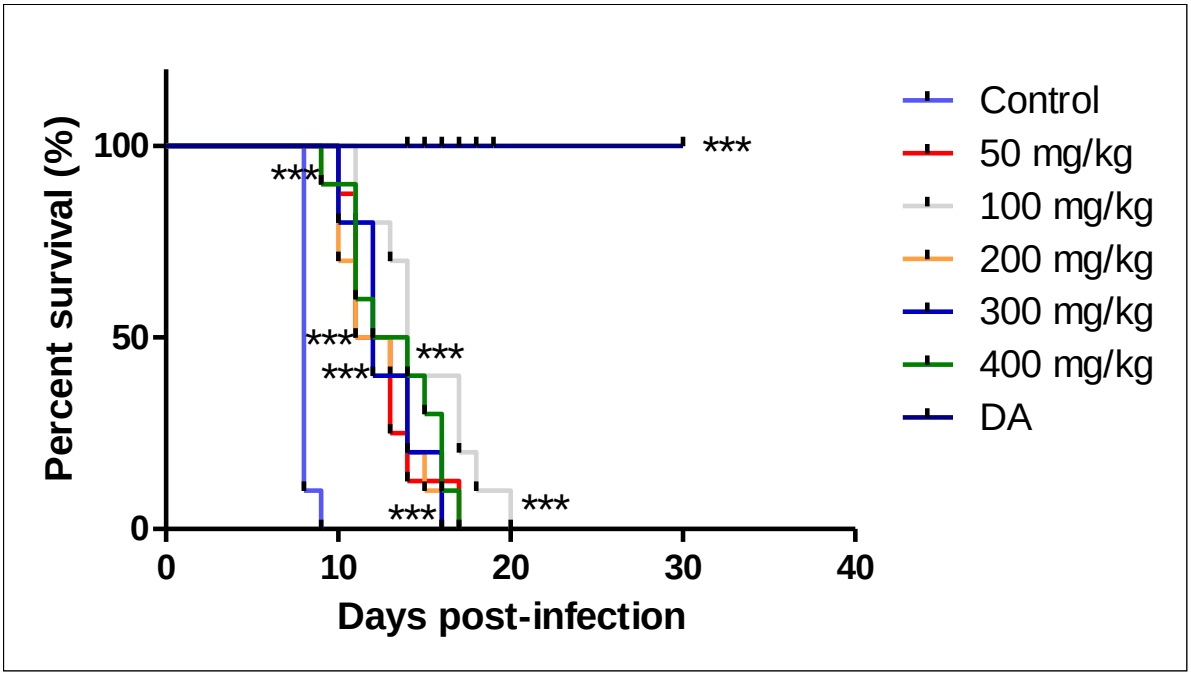


Figure 3.6: Survival curves of mice infected with *T. congolense* and orally treated with different concentrations of CUR. The survival rate was significantly different from that of the control group (n = 10), *** $p < 0.0001$ (Log-rank test).

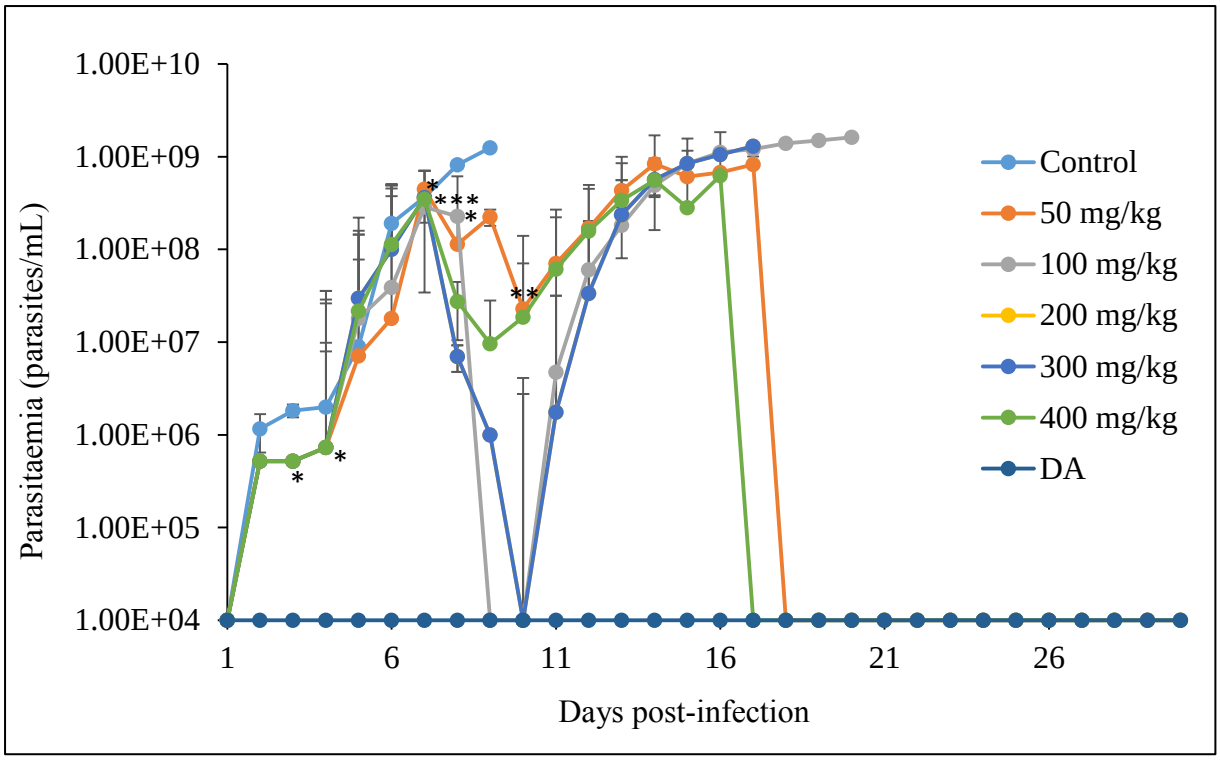


Figure 3.7: The evaluation of parasitaemia in mice infected with *T. congolense* and orally treated with different concentrations of CUR-nanoparticle for 7 days. *$p < 0.05$; ** $p < 0.001$ and *** $p < 0.0001$. The data are expressed as the mean ± S.D. The 1×10^4 represents parasitaemia below the detection levels.

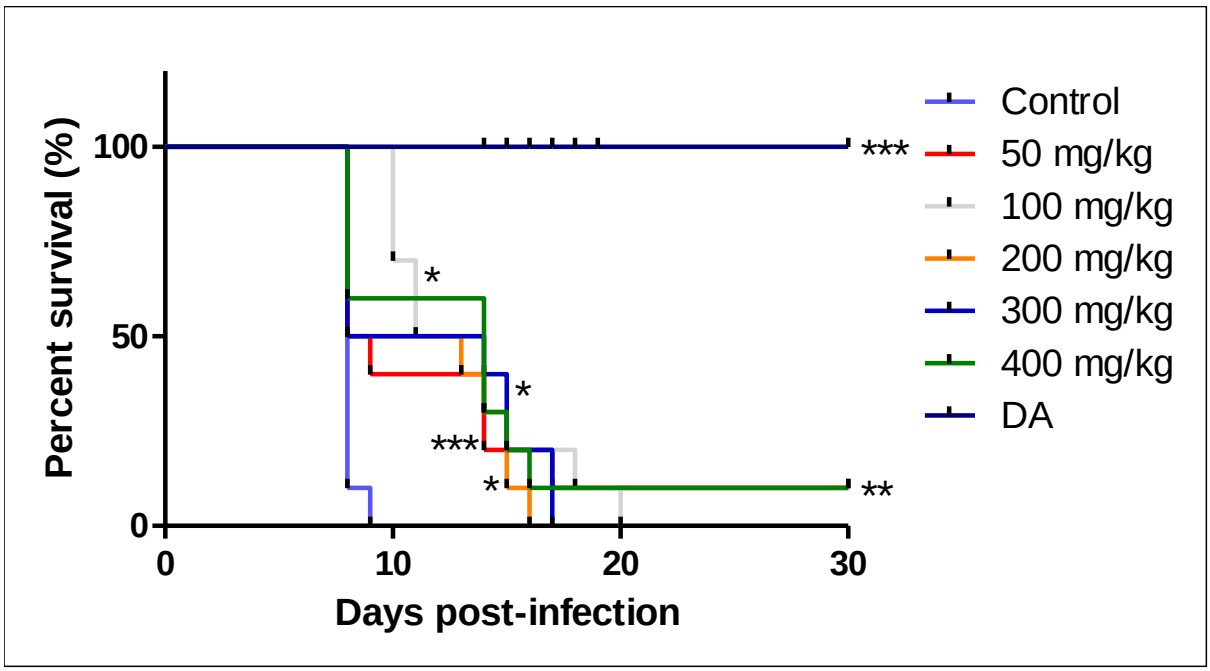
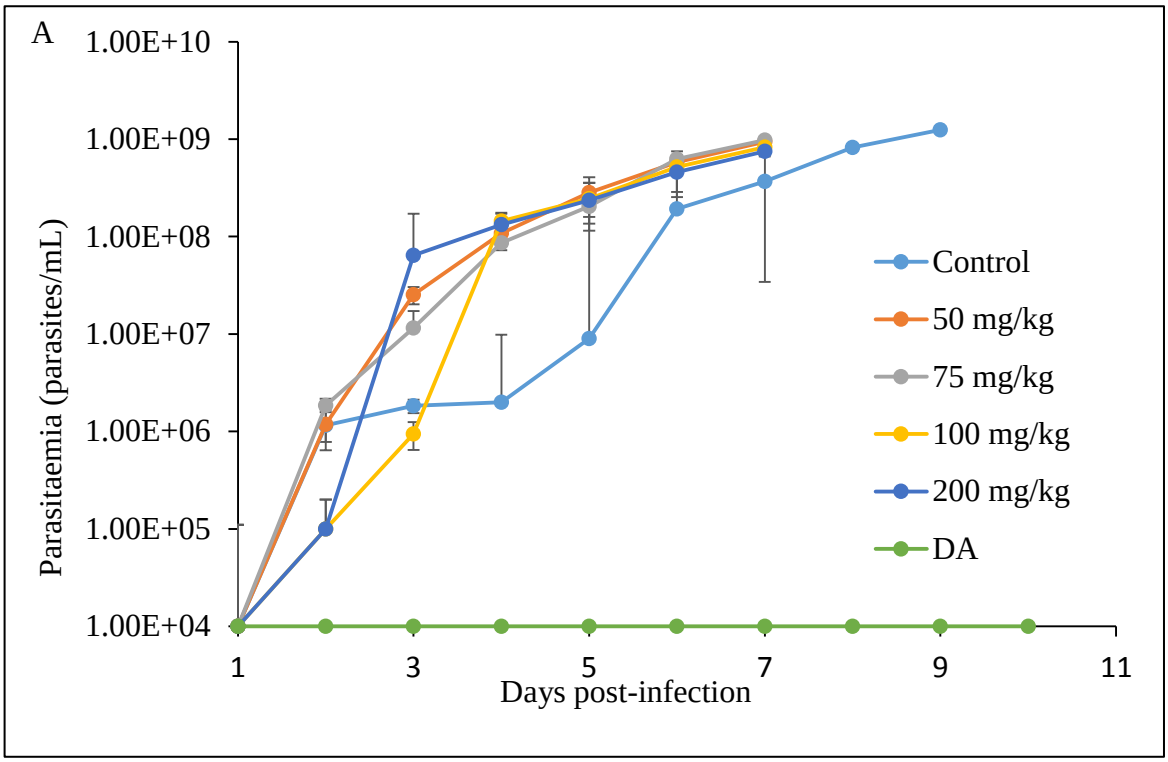


Figure 3.8: Survival curves of mice infected with *T. congolense* and orally treated with different concentrations of CUR-nanoparticle. The survival rate was significantly different from that of the control group (n = 10), ** $p < 0.001$ (Log-rank test).



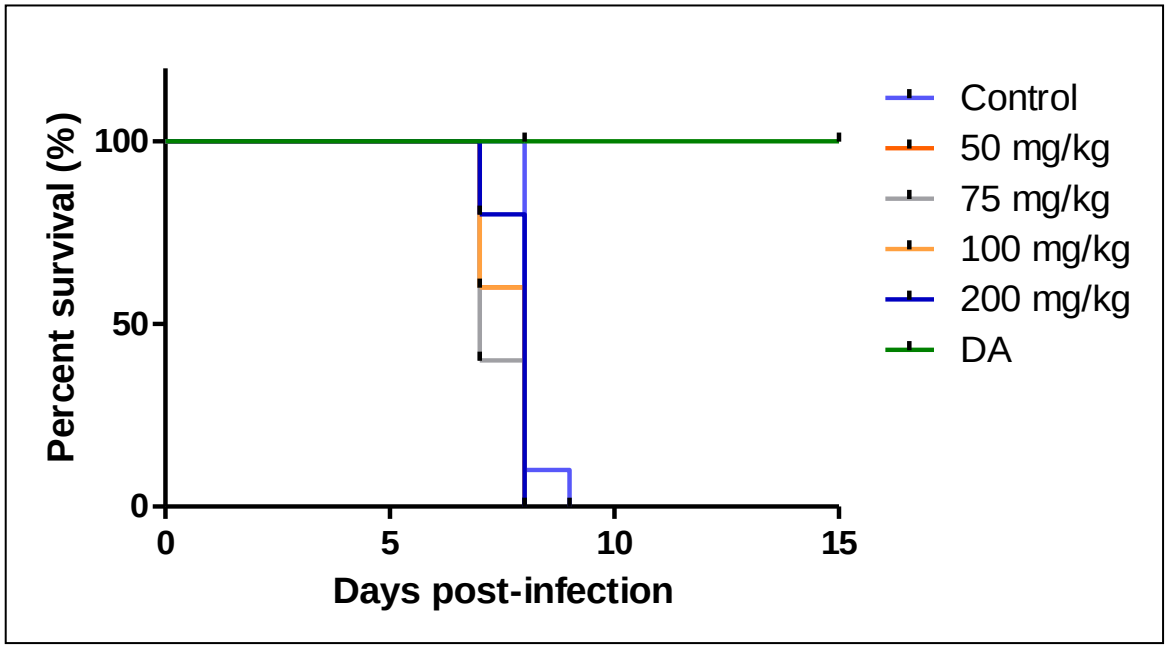


Figure 3.9: The evaluation of A) parasitaemia; B) survival rate of the mice infected with *T. congolense* and intraperitoneally treated with different concentrations of CUR for 7 days. The 1×10^4 represents parasitaemia below the detection levels.

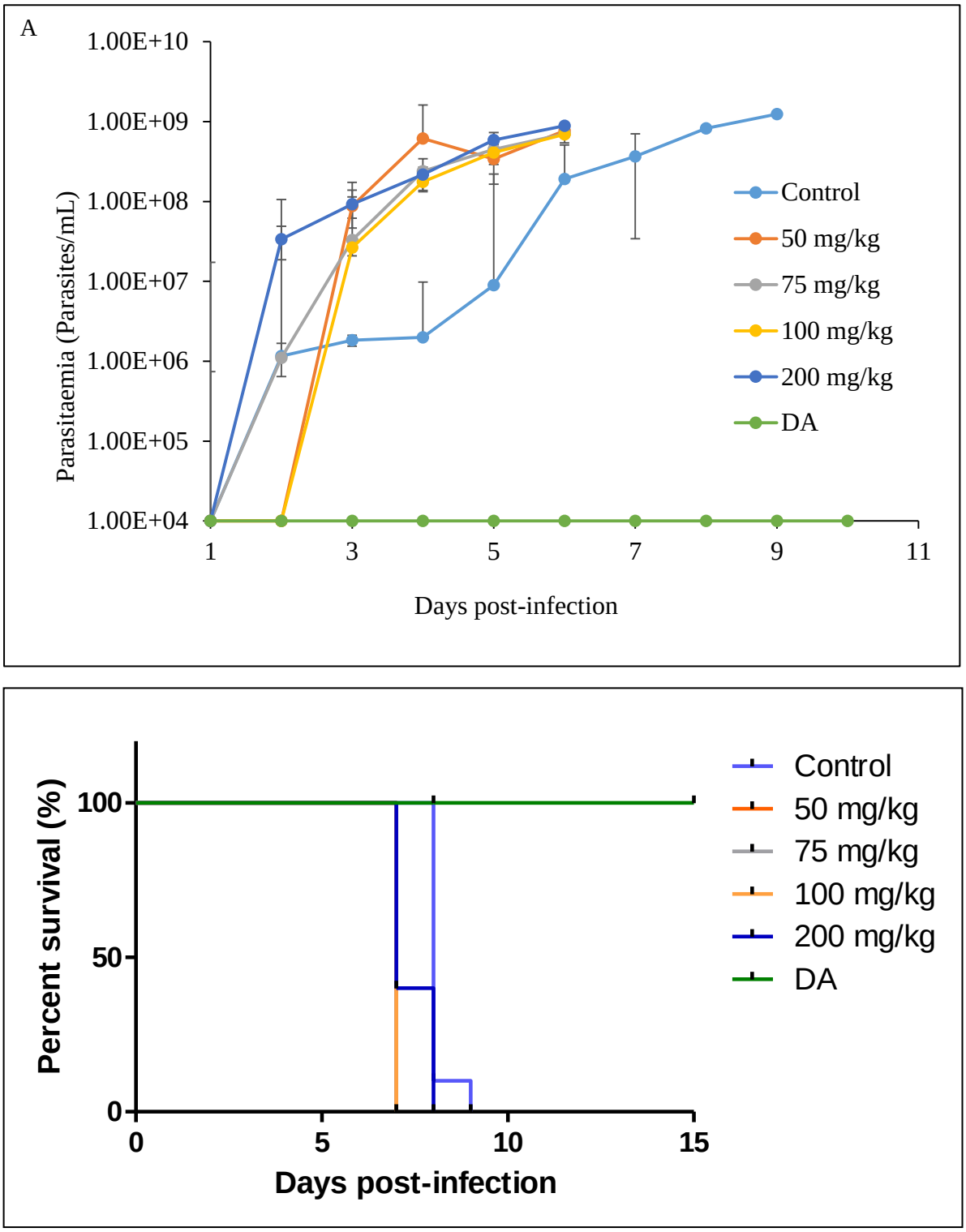


Figure 3.10: The evaluation of A) parasitaemia; B) survival rate of the mice infected with *T. congolense* and intraperitoneally treated with different concentrations of CUR-nanoparticle. The 1×10^4 represents parasitaemia below the detection levels.

Table 3.1: *In vitro* summary of the CUR and CUR-nanoparticle efficacy against *T. congolense, T. b. brucei* and *T. evansi* and the cytotoxicity on the MDBK and NIH 3T3/ HFF cells.

Test samples	Trypanocidal tests			Cytotoxicity tests		Selectivity index		
	T. congolense	***T. b. brucei***	***T. evansi***	**MDBK**	**HFF**	**T. c**	**T. b. b**	**T. e**
CUR	1.36 ± 0.31	2.82 ± 1.22	2.37 ± 3.07	18.71 ± 1.97	4.06 ± 3.09 (NIH 3T3)	8.37	4.0	12.8
ASPS	0.58 ± 0.50	10.43 ± 9.43	4.86 ± 0.13	20.1 ± 0.3	26.99 ± 1.7	40.60	2.26	4.85
WM	0.54 ± 0.49	17.13 ± 2.45	4.88 ± 0.81	21.19 ± 1.082	26.92 ± 0.68	44.55	2.81	4.93
EPN	0.63 ± 0.54	16.48 ± 8.1	14.80 ± 14.43	21.08 ± 0.89	21.1 ± 2.17	33.48	1.28	1.00

Data expressed as µg/mL ± S.D. ASPS – Antisolvent recipitation with a syringe pump, WM – Wet milling method, EPN – Evaporative precipitation of nanosuspension. T.c: *T. congolense;* T. b. b: *T. b. brucei;* T. e: *T. evansi.*

General discussion

The compounds used in this study were selected based on the previously documented effects on other parasites, whereby they were now assessed for their trypanocidal efficacy. From 8 compounds (artesunate; melatonin; 2-hexadecenoic acid; triclosan; curcumin; azithromycin; pepstatin A and nerolidol) tested at the beginning of the study only AZM, TCS and CUR were effective on the trypanosomes and possessed less cytotoxicity resulting in an acceptable selectivity index values. The objective of these selection of the compounds is due to the shared metabolic pathways between numerous protozoans and other parasites. This hypothesis was justified by shared chemotherapy effects between most protozoans for instance, pentamidine, a trypanocidal compound used for the first stage of *T. b. gambiense* infection which is also effective on *Leishmania* (Steverding, 2010; Silva-Jardim *et al.*, 2014). In addition, diminazene aceturate (DA), a trypanocidal compound, which binds irreversibly to a double-stranded DNA in the grove between complementary strains has shown a tremendous effect on *Babesia* spp. (Nehrbass-Stuedli *et al.*, 2011; Mosqueda *et al.*, 2012).

AZM and CUR *in vitro* shown more efficacy against *T. congolense* than *T. b. brucei* and *T. evansi* whilst TCS was uniformly effective on all the species alike. As mentioned in the chapters 1 and 3, *T. congolense* belongs to the subgenus *Nannomonas* while *T. b. brucei* and *T. evansi* belong to the subgenus *Trypanozoon*.

AZM showed more efficacy against *T. congolense* than *T. b. brucei in vivo*. Tissue tropism of the trypanosomes subgeneraplays a major role in the distinguished efficacy between the 2 subgenera. The *T. congolense* is strictly an intravascular parasites, binding on to the red blood cells and the endothelial cells through the flagellum and thereby causing damage at the binding sites. It is therefore always in contact with the drugs absorbed into the circulating system as well as the immune defence of the host. On the contrary, *T. brucei* sub-species (*T. b.*

brucei, *T. b. gambiense* and *T. b. rhodensiense*) are highly tropic and have the ability of penetrating in to the extravascular system of the hosts, sites inaccessible to the drug effects such as the CNS, adipose tissues and the reproductive organs (Giordani *et al.*, 2016). Recently though, this pathogen was recorded in the adipose tissues as well as the reproductive organs of the hosts (Biteau *et al.*, 2016; Trindade *et al.*, 2016). One more factor contributing to the different efficacy between the two trypanosome subgenera is the mode of action of AZM. Even though the mode of action of AZM on the trypanosomes has not yet been established, the current study through TEM analysis recorded the possibility of apoptosis and/or autophagy. In the instance of *T. congolense*, the development of a vacuole like structure was observed whilst for *T. b. brucei* an increase in glycosomes and the presence of acidocalcisomes were detected in the *T. b. brucei* analysis (Luder *et al.*, 2010; Brennand *et al.*, 2012). These differences should be used for further analysis in order to determine the exact pathway differences in these species of trypanosomes. Nonetheless, this study has demonstrated that *T. congolense* might possess a pathway susceptible to AZM action.

The pharmacokinetics of AZM include great bioavailability from the GI tract and absorption into the cells with an advantage of a long half-life (Lode *et al.*, 1996; Taylor *et al.*, 1999; Sagara *et al.*, 2014; Rosenthal *et al.*, 2016). AZM therefore has made a good prophylaxis for the obligate intracellular parasites such as malaria. Currently, in the market for malarial prophylaxis is Austell-Azithromycin®. Regardless, AZM does not make a good prophylactic agent for trypanosomes (data not shown), due to the fact that AZM accumulates better in the cells. The extracellular factor of the trypanosomes contribute significantly into the development of vaccine and/or prophylaxis. Isometamidium chloride (ISM) is currently in use for the prophylactic and the chemotherapeutic effects in cattle. Unlike AZM that is rapidly absorbed into the cells within 8 hours of intramuscular administration (Escudero *et al.*, 2006),

the mean absorption time of ISM takes up to 282 hours after intravenous and intraperitoneal injection (Peregrine *et al.,* 1988; Peregrine *et al.,* 1997).

As per the final ruling of the FDA regarding the removal of TCS (Halden *et al.,* 2017) from over the counter components, this study determined the *in vivo* dose, safe for BALB/c mice. Observed was the hepatotoxicity of TCS in a long-term treatment in mice manifested with the presence of hepatomegaly, increase liver enzymes, fluctuating levels of haematological parameters, and histological changes that included the dose dependent hydropic degeneration, extramedullary haematopoiesis and megakaryocytes (Thoolen *et al.,* 2010). Judging from these results, a long-term treatment of TCS in mice was not feasible and/or safe even though several studies have shown its efficacy. Nonetheless, mild trypanocidal effects on *T. conoglense*- infected mice were recorded in this study with a prolongation of treated mice as compared to the non-treated control group. The current study results were more or less similar to results that were obtained by (Paul *et al.,* 2004) who determined the effectivity of TCS on *T. brucei.* The TCS was effective *in vitro* on *T. brucei* but not *in vivo*. A conclusion was reached that TCS mode of action on trypanosomes differs from that of bacteria and other documented microorganisms such as *Toxoplasma, Babesia* and *E. coli* (Heath *et al.,* 1999; Bork *et al.,* 2003; El-Zawawy *et al.,* 2015a).

Lastly, with CUR and CUR-nanoparticle, this study has shown that the efficacy of free CUR was improved *in vitro* by the usage of CUR-nanoparticle. Nanoparticles are absorbed faster and better by the parasites due to their size, however, it is very important to select the appropriate sizes for the intended pathogen given the fact that bigger particles remain hydrophobic (Singh *et al.,* 2008). Regardless of the high excretion and/or removal of CUR, a certain degree of efficacy was observed in the *T. congolense*-infected mice, when administered orally. For intraperitoneally administration, there was no efficacy whatsoever in both the CUR and CUR-nanoparticles. The efficacy of CUR and CUR-nanoparticles was influenced by

external factors namely; the solvent used to dissolve the compound when preparing the regimens, whether the mice received food or not and lastly the beginning of the treatment period. Nanoparticle results observed in this study was not before the treatment administration, which means that extensive studies are still needed in order to determine the correct range of particle size, shape and solvent necessary to improve the effectivity of this compound (Nguyen, 2011; Desai, 2012; Kakran *et al.*, 2012).

The current study has demonstrated better efficacy of orally administered AZM, TCS and CUR on *T. congolense* as compared to the intraperitoneal administration. The oral efficacy of these compounds is attributed and justified by the pharmacokinetics. According to Lukas *et al.* (1971), intraperitoneal administration does not guarantee a complete absorption of the compound even though it is the most used route, as the intraperitoneally administered compounds are primarily absorbed through the portal circulation and are therefore not exempted from liver metabolism before reaching the target organs. Another possible factor influencing the inefficiency intraperitoneally administered compounds could be the better absorption into the cells that induced toxicity and/or immune defence defect. Toxicity effect of the compound is influenced by the route of administration (Amlacher and Hoffmann, 1984; AbuKhader, 2012).

The oral efficacy of these compounds, however, raises possible hurdles regarding the applicability of these compounds especially in the veterinary field. Oral administration of compounds would be challenging but possible, particularly for big animals. The initial step towards the applicability of oral trypanocides in the veterinary system would involve the treatment of dogs which are more susceptible to *T. congolense* nagana than the rest of the animals. Dogs are believed to be the link for parasite exchange between livestock and human and responsible for the re-emerging of the infections. Moreover, the cases of trypanosomosis in dogs are under-reported (Lisulo *et al.*, 2014). A study reported the potential ability of dogs

to harbour human African trypanosomosis, *T. b. rhodensiense* and *T. b. gambiense* (Abenga *et al.,* 2005). In addition pigs also play a reservoir host role for the most pathogenic trypanosomes of livestock, *T. congolense* and *T. vivax* (Simukoko *et al.,* 2007; Hamill *et al.,* 2013). Furthermore, in order to address the oral efficacy of AZM, this study determined the efficacy of AZM on *T. b. brucei* with human pathogens in mind for which oral administration will not be a problem.

Lastly, monotherapy is no longer advisable for most of the infectious disease treatment based on factors such as the in-efficacy in the second stage, relapse cases, cross resistance, high toxicity levels, long treatment and high concentrations required (Barrett *et al.,* 2007; Barbokhov *et al.,* 2013). The tested compounds demonstrated a certain degree of trypanocidal efficacy, the combination of these compounds with the currently available trypanocides would be beneficial in reducing the time necessary for the treatment, concentrations and therefore limiting toxicity effects and enhance the efficacy of both the compounds. Additionally, these compounds possess no active sides similar to those of the currently available trypanocides, pentamidine, suramin, diminazene aceturate or merlasoprol, due to structural differences, therefore no cross resistance is likely to develop.

Conclusion

This study has identified 3 possible compounds for the treatment of trypanosomosis. AZM, TCS and CUR possessed a trypanocidal effect on *T. congolense, T. b. brucei* and *T. evansi in vitro,* with less or no cytotoxicity effects on the host cells.

The above mentioned results raised enough interest to propel the experiments further into the animal experiments. AZM possessed a trypanocidal effect on *T. congolense* and *T. b. brucei in vivo.* The efficacy was increased with a prolonged treatment period. Long term treatment with AZM executed trypanocidal effects at 300 and 400 mg/kg as compared to the short term treatment.

TCS possessed a slight effect on *T. congolense in vivo,* however, due to the observed toxicity effects in mice, the long term treatment of TCS was not conducted. The effect was much better after the oral administration of TCS in comparison to the intraperitoneal administration. The toxicity effects of TCS was dose dependent with the presence of hepatomegaly, fluctuations haematological and biochemical parameters, presence of megakaryotes, extramedullary haematopoiesis and the hydropic degeneration of the hepatocytes.

CUR and CUR-nanoparticles also possessed trypanocidal effects when administered orally in *T. congolense* infected mice. The obtained particle size influenced the solubility and the efficacy of the compounds in mice. Furthermore, various factors have shown the ability to affect the efficacy of the CUR and CUR-nanoparticles, which are the solvent, the route of administration, treatment period and the presence or absence of food during treatment.

AZM, TCS and CUR have shown their potential trypanocidal effects in this study, however, follow up studies are needed to determine and confirm the mode of action of these compounds in trypanosomes. This study demonstrated the development of a vesicle in AZM

treated *T. congolense*. This morphological change shall be confirmed by follow up studies in order to obtain factors responsible for the development of the vesicle and those responsible for the eventual death of the parasites.

These compound could be beneficial in the combination therapy of the treatment of trypanosomosis, even though it is still premature to ascertain the applicability in animals, especially ruminant.

学位論文要旨

ヒト及び動物トリパノソーマ病はトリパノソーマの感染により引き起こされる致死的疾病である。トリパノソーマ病は「顧みられない熱帯病」の一つであり、流行国の多くが発展途上国であることから製薬会社による新規創薬のインセンティブに乏しく、新規トリパノソーマ病治療薬の開発は進んでいない。また、現在までに使用されているトリパノソーマ病治療薬の多くが開発から 40 年以上経過し、薬剤耐性トリパノソーマの出現も多数報告されている。さらに既存のトリパノソーマ病治療薬は経口投与での治療が行えないため、医療インフラが整備されていない地方部ではトリパノソーマ病の治療が十分に行えない。そのため経口投与が可能な新規トリパノソーマ病治療薬の開発が、トリパノソーマ病対策の喫緊の課題である。ドラッグ・リポジショニングとは、ある疾病で有効な既存薬を、別疾病の治療に適応することである。ドラッグ・リポジショニングによる新規創薬の場合、すでにヒトあるいは動物で有用性が確立されている化合物のため、新規化合物による創薬に比べ短期間で対象疾病の治療に適応可能な利点がある。そこで本研究では 3 種類の化合物、アジスロマイシン（第 1 章）、トリクロサン（第 2 章）及び植物抽出物であるクルクミン（第 3 章）のトリパノソーマ病経口治療薬としての有用性を *in vitro* 及び *in vivo* で解析した。

第 1 章では、細菌性疾病の治療に広く用いられている抗生物質であるアジスロマイシンのトリパノソーマ病経口治療薬として有効性を検証した。まず *in vitro* における 3 種類のトリパノソーマ（*Trypanosoma congolense, T. evansi* 及び *T. brucei brucei*）に対する抗トリパノソーマ活性及び 2 種類の哺乳類細胞（MDBK 細胞及び NIH 3T3 細胞）に対する細胞毒性を評価した。その結果、アジスロマイシンは 3 種類すべてのトリパノソーマに対して抗トリパノソーマ活性を有していることが明らかとなった。中でも *T. congolense* に対しては 50%阻害濃度（Inhibitory Concentration 50: IC_{50}）が 0.19 μg/mL と最も強い抗トリパノソーマ活性を示した。続いてマウス感染モデル（*T. congolense* 及び *T. brucei*）を用いて、感染後 7 日間（短期間治療群）もしくは 28 日間（長期間治療群）のアジスロマイシン経口投与によるトリパノソーマ病治療効果を評価した。*T. congolense* 感染マウスモデルを用いた治療実験の結果、短期及び長期治療群どちらにおいても、アジスロマイシン投与濃度に依存してパラシテミアの有意な低下に伴い生存率の有意に上昇が認められた。一方、*T. brucei* 感染マウスモデルを用いた治療実験の結果、短期治療群ではアジスロマイシンによる有意なトリパノソーマ病治療効果は認められなかったが、長期投与群において生存率の有意な上昇が認められた。これらの治療効果の差は 2 種類のトリパノソーマの分類学的な差異（*T. congolense*: *Nannomonas* 亜属、*T. b. brucei*: *Trypanozoon* 亜属）に基づ

く薬剤感受性の違い及び寄生部位の違い（*T. congolense*: 偏性血管内寄生、*T. b. brucei*: 血管内、組織及び脳脊髄液内寄生）に起因するものと考えられた。さらにアジスロマイシンのトリパノソーマに対する薬理作用を解析するため、アジスロマイシン処理したトリパノソーマを透過型電子顕微鏡により解析した。その結果、アジスロマイシン処理後の *T. congolense* においては空胞状の構造物が、*T. b. brucei* においてはアシドカルシソーム様の構造物がそれぞれ認められた。これら微細構造の変化はオートファジーと関連して形成されることが報告されているため、アジスロマイシンの坑トリパノソーマ作用機序としてオートファジーの過剰誘導が示唆された。以上の結果より、アジスロマイシン経口投与によるトリパノソーマ病治療薬としての可能性が明らかとなった。

第 2 章では一般的な抗菌成分として広く用いられているトリクロサンのトリパノソーマ病治療薬として有効性を検証した。まずトリクロサン経口投与がマウスに与える影響を病理組織学的に解析した結果、投与量依存的に肝臓の空胞性変性が認められた。*in vitro* における坑トリパノソーマ活性評価試験の結果、トリクロサンは 3 種類のトリパノソーマに対して 1.0 - 2.0 μg/mL 程度の IC_{50} を示した。*T. congolense* を用いた *in vivo* 試験の結果、短期治療群及び長期治療群ともに非治療群と比較してパラシテミアの有意な低下と生存率の有意な上昇が認められた。以上の結

果から、高濃度のトリクロサン経口投与は特に肝臓に悪影響を与えうるが、適切な投与量であればトリクロサンはトリパノソーマ病治療薬となりうることが示唆された。

第 3 章では植物抽出成分であり香辛料として広く用いられるクルクミンのトリパノソーマ病治療薬として有効性を検証した。まずクルクミン経口投与がマウスに与える影響を病理組織学的に解析した結果、クルクミン濃度依存的に小腸粘膜及び微絨毛の菲薄化が認められた。続いてクルクミンの親水性を向上させ消化管からの吸収性を向上させることを目的として、ナノパーティクル化クルクミンを作製した。クルクミン及びナノパーティクル化クルクミンの *in vitro* における坑トリパノソーマ活性試験の結果、*T. congolense* における坑トリパノソーマ活性がクルクミン(IC_{50} = 1.36 μg/mL)と比較し、ナノパーティクル化クルクミン（IC_{50} = 0.54-0.63 μg/mL）では 2-3 倍程度向上することが明らかとなった。一方 *T. b. brucei* 及び *T. evansi* ではそのような IC_{50} 値の低下は認められなかった。*T. congolense* を用いた *in vivo* 試験の結果、クルクミン及びナノパーティクル化クルクミンを用いた短期治療群及び長期治療群ともに非治療群と比較してパラシテミアの有意な低下と生存率の有意な上昇が認められた。一方腹腔内投与では、経口投与群で認められた治療効果は認められなかった。以上の結果から、高濃度のクルクミン経口投与は、特に小腸に悪影響を

与えうるが、適切な投与量であればクルクミン及びナノパーティクル化クルクミンはトリパノソーマ病治療薬となりうることが示唆された。

以上より、３種類の化合物の経口投与によるトリパノソーマ病治療効果が明らかとなった。さらに、他の２種類の化合物に比べアジスロマイシンは、短期間及び長期間の経口投与により強いトリパノソーマ病治療効果を示した。今後、本研究成果をもとにしたドラッグ・リポジショニングによる経口投与が可能な新規トリパノソーマ病治療薬の開発が期待される。

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Dedication

To the pillar of my strength, my all-time best friend, a shoulder to cry on, my everything, my mother, Dieketseng Josephine Molefe. For the struggles we have been through together, you have always put us first, giving yourself up so that we could be something in life. I have seen you in pain because of us and lacking things so that we may have the good things in life. I wouldn't have asked for a better mother than you. I hereby dedicate this efforts to you.

"We have made it!" – Nthatisi

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