THE ROLE OF ANTIOXIDANTS IN IMPROVING TREATMENT OUTCOME AND REDUCING DISEASE SEVERITY IN LATE STAGE HUMAN AFRICAN TRYPANOSOMIASIS

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DECLARATION AND RECOMMENDATION

DECLARATION

I declare that this thesis is my original work and has not been submitted wholly or in part in this form or any form for a degree in this or any other university

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RECOMMENDATION

We wish to confirm that this research thesis has been prepared under our supervision and have our approval to be presented for examination as per the Egerton University regulations.

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ABSTRACT

Human African trypanosomiasis (HAT) is a tropical disease caused by two subspecies of *Trypanosoma brucei*: *T. b. rhodesiense* and *T. b. gambiense*. Melarsoprol, an organic arsenical is the only drug used to treat late stage *T. b. rhodesiense* infection in spite of its toxic side effects. A mouse model was adapted to study the effects of melarsoprol which was apparently toxic at normal intravenous doses of 3.6mg/kg body weight. Results from this study show that melarsoprol markedly reduced blood PCV, aconitase-1 and GSH levels in the brain of uninfected mice. However, Kenyan purple tea anthocyanins or coenzyme-Q_{10}, orally administered prevented, to a significant degree, melarsoprol-induced decline in PCV and restored aconitase-1 and GSH levels. Notably, anthocyanin metabolites were detected in brain tissue of anthocyanin fed mice using HPLC. *T. b. rhodesiense* infection caused significant decreases in brain aconitase-1 and GSH levels while a general decrease in PCV levels was observed. Oral administration of Kenyan purple tea anthocyanins or coenzyme-Q_{10} in trypanosome-infected mice was found to impede these disease-induced anomalies. In PTRE studies, coenzyme-Q_{10} or Kenyan purple tea anthocyanins raised brain GSH and aconitase-1 levels when compared to untreated groups, coenzyme-Q_{10} treatment producing more beneficial effects compared to anthocyanin treatment. However, co-administration of both antioxidants caused a reduction of these beneficial effects implying a negative interaction. A histological study in the brain tissue of *T. b. rhodesiense* infected mice demonstrated neuroinflammatory pathology which was highly amplified in the PTRE groups. A prominent reduction in the severity of the neuroinflammatory response was detected when Kenyan purple tea anthocyanins or coenzyme-Q_{10} were administered separately. Moreover, degenerative changes in brain tissue of coenzyme-Q_{10} treated mice were less pronounced when compared to those given anthocyanins. Results of the present study provide evidence that oxidative stress in blood and brain is associated with the pathogenesis of HAT and PTRE. The present study also demonstrates a role of ROS-sensitive aconitase-1 and GSH in late stage HAT and PTRE. Evidence provided in this study implicates melarsoprol and *T. b. rhodesiense* parasites with interference of brain antioxidant systems and proposes that therapeutic intervention with Kenyan purple tea anthocyanins or coenzyme-Q_{10} may be useful in reducing disease severity and improve treatment outcome in late stage HAT and reduce PTRE occurrence.

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DEDICATION

How oblivious I was to the fact that reaching the finishing line needed strenuous effort, passion and hard work. However, owing to the vision of my beloved mother who always supported me through thick and thin, my thesis is finally complete. I would like to dedicate this thesis to my mother Dekah Yusuf whom without her contribution, this research and the entire MSc program in totality would not have been possible. I also dedicate this thesis to my sister Asha Anab and cousin Robert Okwaro.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACN’s	Anthocyanins
ACO-1	Aconitase 1
ACO-2	Aconitase 2
AP-1	Activator protein 1
ATP	Adenosine triphosphate
EAATs	Excitory amino acid transporters
EDTA	Ethylene diamine tetra acetic acid
ESG	EDTA saline glucose
GSH	Glutathione
HAT	Human African trypanosomiasis
$HO^{\bullet}$	Hydroxyl radical
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-10	Interleukin 10
IRE	Iron responsive element
IRP	Iron regulatory protein
MAP	Mitogen activated protein
MnSOD	Manganese superoxide dismutase
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of NADP+
NF-κB	Nuclear factor kappa beta
$NO^{\bullet}$	Nitric oxide
PAGE	Polyacrylamide gel electrophoresis
PDSS	Prenyl diphosphate synthase subunit
PI3K	Phosphoinoside-3-kinase
PTRE	Post treatment reactive encephalopathy
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
UCPs	Uncoupling proteins

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CHAPTER ONE
INTRODUCTION

1.1 Background Information

Human African trypanosomiasis (HAT) or sleeping sickness is a tropical disease transmitted through the bites of infected tsetse flies of *Glossina* species and is a typical example of a neglected disease (Remme *et al*., 2002). The disease is caused by infection with the protozoan parasites *Trypanosoma brucei gambiense* which is responsible for the chronic form in West and Central Africa or *Trypanosoma brucei rhodesiense* responsible for the acute form in Eastern and Southern Africa (Kennedy, 2004). Treatment of HAT is stage specific and relies on four parenteral drugs namely suramin for first-stage *rhodesiense*, pentamidine for first-stage *gambiense*, melarsoprol for the second stage of both forms of the disease and eflornithine and Nifurtimox-Eflornithine Combination Therapy (NECT), which are only effective in the second stage of the *gambiense* form (Legros *et al.,* 2002)

Melarsoprol is a melaminophenyl based organic arsenical introduced in 1949 and synthesized by complexing melarsen oxide with dimercaprol (Friedheim, 1949). It remains the most widely used drug against late stage HAT in spite of its extremely toxic side effects. Encephalopathy is the most life threatening complication of melarsoprol treatment of second stage HAT. Melarsoprol induces post treatment reactive encephalopathy (PTRE) in 10% of patients and results in death of up to 5% of patients (Pepin and Milord, 1994), and since melarsoprol results in neurological seizures in the absence of trypanosomes, it is clear that melarsoprol itself is responsible for the reactive encephalopathy (Soignet *et al*., 1999). This presents a big challenge to the management of late stage HAT because melarsoprol is the only late stage drug that can be used to treat the acute form of HAT caused by *T. b. rhodesiense*. The molecular processes that lead to PTRE are unclear however many hypotheses have been presented and include; immunological reactions to the parasite following therapy (Pepin and Milord, 1994), immune complex deposition (Lambert *et al*., 1981), toxicity of the arsenical moiety of melarsoprol (Hurst, 1959), autoimmune reactions or subcurative chemotherapy (Hunter *et al*., 1991; Hunter and Kennedy, 1992; Hunter *et al*., 1992). Because of the clinical importance of this complication, this study set out to understand some of the molecular events that contribute to PTRE.

The trivalent arsenical drug melarsoprol generates reactive oxygen species (ROS) and other reactive radicals which affects the body's innate defense mechanisms against these reactive moieties. Melarsen oxide, a metabolite of melarsoprol has been shown to interact with non protein thiols involved in antioxidant cellular defense such as glutathione and thioredoxin, forming a stable adduct (Fairlamb *et al*., 1989; Cunningham *et al*., 1994). The stable adduct formed is a competitive inhibitor of the respective flavoproteins namely glutathione reductase and thioredoxin reductase, responsible for maintaining intracellular glutathione and thioredoxin in the reduced form (Cunningham *et al*., 1994). These findings by Cunningham and Fairlamb formed the basis for determination of GSH levels in our experimental model. Moreover, it has also been shown that melarsen oxide can also bind to vital metabolic enzymes and thus results in oxidative stress (Fairlamb, 2003). This condition is further aggravated by the presence of trypanosome parasites which are known to produce enormous amounts of ROS during infection (Meshnick *et al*., 1977). Since considerable evidence points to oxidative stress as an important trigger in the complex chain of events leading to brain encephalopathies such as Alzheimer's disease (AD) and Parkinson's disease (PD), then it is possible that oxidative stress plays a key role in the development of PTRE following melarsoprol treatment in late stage HAT. From this observation, the present study evaluated the effects of the organic arsenical melarsoprol and *T. b. rhodesiense* strain KETRI 2537 parasites on cytosolic aconitase, an iron regulatory protein that is highly sensitive to oxidative stress (Pietrangelo, 2003; Hentze *et al*., 2004; Napier *et al*., 2005). It is important to note that inactivation of these iron regulatory proteins results in misregulation of iron homeostasis and consequently, an imbalance of brain iron homeostasis is considered an important contributing factor of neurotoxicity in several brain encephalopathies (Singh *et al.*, 2009). Since trypanosome parasites and arsenics are known to cause cell injury by oxidative stress, it is hypothesized that trypanosomiasis infection and melarsoprol treatment impairs these metabolic/antioxidant enzymes (aconitases) and therefore impacts negatively on iron homeostasis in the brain.

Furthermore, PTRE is associated with an intense inflammatory reaction especially at the terminal stages leading to the development of an acute meningoencephalitis. The neuropathological features involved include infiltration of various cells in the brain including macrophages, lymphocytes, plasma cells and occasionally, morular cells. Perivascular cuffings and severe astrocyte and microglia activation are also common histopathological features

associated with PTRE (Kennedy, 2004). Therefore, in view of the prominent inflammatory responses seen in PTRE and antioxidant system failure associated with melarsoprol treatment, employing compounds known to possess potent antioxidant and anti-inflammatory properties in ameliorating severity of this clinical complication seemed logical.

In this study Kenyan purple tea anthocyanins (ACN's) and Co-enzyme Q_{10} (Co-Q_{10}) were used as antioxidants. Anthocyanins from Kenyan purple tea cultivars were chosen as test antioxidants because tea has widely been studied owing to its wide consumption (Rietveld and Wiseman, 2003), health enhancing and medicinal properties including antioxidative (Shahidi and Alexander, 1998; Lin and Liang, 2000), anti-inflammatory (Karori *et al.,* 2008), antimicrobial (Taylor *et al.,* 2005) neuroprotective (Abd *et al.,* 2002) and cholesterol-lowering effects (Raederstorff *et al.,* 2003). While anthocyanins from fruits and flowers have been studied extensively, there is still paucity of data on the health benefits of tea anthocyanins despite their broad spectrum of health benefits including cardiovascular, neurological, urinary tract, and ocular protection as well as anti-carcinogenic (Lazzé *et al.,* 2004), anti-diabetic (Nizamutdinova *et al.,* 2009), anti-aging (Choi *et al.,* 2010), antioxidant and anti-inflammatory properties (Wang *et al.,* 1999). Furthermore, polyphenols in tea appear to be more potent than other antioxidants such as vitamins C and E (Rice-Evans *et al*., 1995) and other synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxytoluene (BHT) (Chen and Wan, 1994). Moreover, studies have associated ACN's from blueberries with several positive effects such as direct effects on signaling to enhance neuronal communication (Joseph *et al*., 2003), the ability to buffer against excess calcium (Joseph *et al*., 2004), enhancement of neuroprotective stress shock proteins (Galli *et al*., 2006) and reduction of stress signals such as nuclear factor kappa B (NF-κB) known to induce the expression of a wide array of inflammatory mediators (Goyarzu *et al*., 2004). In addition, ACN's contained in blueberries cross the blood brain barrier and their concentrations correlates with cognitive performance (Andres-Lacueva *et al*., 2005). The choice of Co-Q_{10} heavily relied upon increasing interest in the potential usefulness of this compound to treat neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Beal, 2002), hence the inclusion of this enzyme in this study.

However, studies on antioxidants as neuroprotective agents has not been impressive to say the least, primarily due to the impermeability of the blood brain barrier (BBB) which

controls entry of xenobiotics to a majority of the investigated compounds. While the bioavailability of Co-Q_{10}, a highly lipophilic compound known to transverse the BBB with concomitant neuroprotective effects is not in question, little is known about the bioavailability of Kenyan purple tea ACN's in the brain tissue. Therefore, this study endeavored to determine the bioavailability of ACN's from Kenyan purple tea and to evaluate their effects in the development of brain inflammation following infection with *T. b. rhodesiense* strain KETRI 2537 and post melarsoprol treatment in late stage HAT. However, to evaluate the bioavailability of ACN in the brain, it is necessary to consider the metabolites such as conjugated and methylated derivatives and ring-fission products produced by microorganisms. Based on previous literature, the concentration of the original forms of ACN's in the human body is much less than what is consumed (Manach *et al*., 1997). Therefore, metabolites may contribute, to some extent to the positive health effects.

1.2 Statement of the Problem

The only drug that is effective against the acute form of HAT once the CNS has become involved is the trivalent arsenical melarsoprol. Unfortunately, melarsoprol is highly toxic and treatment can result in the development of an extremely severe post treatment reactive encephalopathy in about 10% of patients with a 5% mortality rate, but the molecular mechanisms involved still remain elusive. However, melarsoprol has been implicated in the production of reactive oxygen species resulting in oxidative stress, a common gross pathological feature in other brain encephalopathies such as Alzheimer's and Parkinson's diseases. This study therefore intends to use antioxidants in ameliorating melarsoprol toxicity, reducing the prevalence of melarsoprol-induced post treatment reactive encephalopathy and improving treatment outcome in late stage HAT.

1.3 Objectives

1.3.1 General objective

To investigate the effect of melarsoprol and *T. b. rhodesiense* parasites on cellular metabolic processes including antioxidant systems in the mouse model.

1.3.2 Specific objectives

1. To determine the effect of melarsoprol and *T. b. rhodesiense* parasites on the metabolic/antioxidant protein, aconitase and the endogenous thiol, glutathione (GSH).

2. To determine whether native and/or metabolized ACN's from Kenyan purple tea cross the blood brain barrier and thus enhancing the brain's antioxidant capacity.
3. To determine the effect of coenzyme Q_{10} and ACN's from Kenyan purple tea on aconitase-1 and GSH in the brain following melarsoprol induced toxicity and/or *T. b. rhodesiense* infection.

1.4 Null Hypotheses (H_o)

1. Melarsoprol treatment and *T. b. rhodesiense* parasites have no effect on aconitase-1 and GSH and consequently do not impair the antioxidant system in the brain.
2. Native and/or metabolized ACN's from Kenyan purple tea does not cross the blood brain barrier neither do they enhance the brain's antioxidant capacity.
3. Coenzyme Q_{10} and ACN's from Kenyan purple tea have no effect on aconitase-1 and GSH in the brain following melarsoprol induced toxicity and/or *T. b. rhodesiense* infection.

1.5 Justification

Despite the fact that encephalopathy is the leading life threatening complication of melarsoprol treatment in second stage HAT, molecular processes that trigger PTRE still remain unclear. Given that melarsoprol is the only late stage drug used to treat the acute form of HAT prevalent in Eastern and Southern Africa due to *T. b. rhodesiense* infection, the need to fully understand molecular events that trigger PTRE cannot be overemphasized. However, melarsoprol has been documented to cause cell injury by inducing oxidative stress. This therefore means that understanding the mechanisms of antioxidant system impairment such as elevated oxidative stress, resultant protein alteration and mis-folding following melarsoprol treatment may putatively shed light on the processes that lead to PTRE. This study will present alternative approaches such as antioxidant therapy to improve treatment outcome and reduce PTRE prevalence in melarsoprol treatment of HAT.

CHAPTER TWO
LITERATURE REVIEW

2.1 Human African Trypanosomiasis

Human African trypanosomiasis is caused by the protozoan parasites *T. b. rhodesiense* or *T. b. gambiense*, and is a major cause of systemic and neurological disability throughout sub-Saharan Africa. Parasites are transmitted to humans by the bite of an infected tsetse fly of the *Glossina* species and can be distinguished from each other using molecular methods (Picozzi *et al*., 2008). *T. b. gambiense* causes a chronic form of the disease in West and Central Africa while *T. b. rhodesiense* is responsible for an acute form in East and Southern Africa (Kennedy, 2004). Both trypanosome species cause the same type of clinical disease, but infection progresses more rapidly into disease with *T. b. rhodesiense*, with an incubation period of 2-3 weeks and a course of several weeks. On the other hand, the *gambiense* form of the disease takes years to develop and represents more than 90% of reported cases of sleeping sickness (Simarro *et al*., 2008).

HAT develops in two stages, the early, or haemolymphatic stage when the parasites multiply and spread in the blood and lymph nodes, followed by the late or meningoencephalitic stage when the parasites migrate across the BBB and become established within the central nervous system (CNS) (Dumas and Bisser, 1999). A painless circumscribed trypanosomal chancre, characterized by local erythema, edema, tenderness and presence of dividing organisms occurs at the site of the bite within 2-5 days. Presence of trypanosome parasites in the blood stream is marked with waves of parasitaemia and intermittent bouts of fever that are separated by days or even months. This phenomenon is attributed to the antigenic variations of the variant surface glycoprotein (VSG) that renders antibodies in the host ineffective in eliminating parasites. This causes an expansion in the parasite population until the host develops novel antibodies against the new VSG coat of the parasite and the cycle continues (Vickerman, 1985). Other clinical features common in the haemolymphatic stage include among others malaise, fatigue, lymphadenopathy, splenomegally, cardiovascular alterations, anemia, muscle and joint pains, liver dysfunction and skin rashes. Current treatments for early stage HAT include pentamidine, a water-soluble aromatic diamidine and suramin, a sulfonated naphthylamine for *gambiense* and *rhodesiense* infections, respectively (Bacchi, 2009).

Onset of the meningo-encephalitic form is marked by the presence and establishment of trypanosomes in the CNS. Up to date, it is not very clear how trypanosomes enter the CNS, but it

is likely that their entrance is through regions where the BBB is reduced and thus more fragile such as the area postrema, pineal gland and median eminence (Schultzberg *et al.*, 1988). Once in the CNS, the parasites cause a constellation of neurological disorders including tremors, motor weakness, walking difficulties, sensory disorders, headaches and disturbances in the sleep wake patterns hence the alternative name, "Sleeping sickness" (Kennedy, 2004; Fevre *et al*., 2008; Kennedy, 2010). In addition, late stage HAT is marked with neuropathological features which include diffuse, perivascular, cellular infiltrates in the white matter rich in lymphocytes, plasma cells and macrophages (Adams *et al*., 1986). Mott's also called morular cells, which harbor immunoglobulins of the IgM class in large intracellular vacuoles are also, occasionally found (Greenwood and Whittle, 1980). Severe astrocyte and microglia activation are common histopathological features associated with late stage HAT (Kennedy, 2004). Moreover, demyelinization occurs in the white matter but the cerebral cortex remains unchanged. Treatment of late stage HAT is hampered by the inability of a majority of compounds to cross the BBB and access the parasites in the brain and CNS in general. However, current treatments in use include eflornithine, an ornithine analogue and melarsoprol, an organic arsenical for *gambiense* and *rhodesiense* infections respectively. D, L-alpha-difluoromethylornithine (DFMO), which is far less toxic than melarsoprol, is also effective for late-stage *gambiense* disease but very costly. Moreover, SCYX-7158, an orally active benzoxaborole was also found to be efficacious and safe in the treatment of late stage HAT and was therefore recommended to enter pre-clinical studies with expected progression to phase 1 clinical trial (Jacobs *et al.*, 2011).

2.2.1 Human African trypanosomes

Trypanosoma brucei gambiense and *Trypanosoma brucei rhodesiense* are motile flagellated protozoan's unique to Africa. The genus *Trypanosoma* belongs to the order *Kinetoplastida*, characterized by a single large mitochondrion containing a kinetoplast at the base of the flagellum.

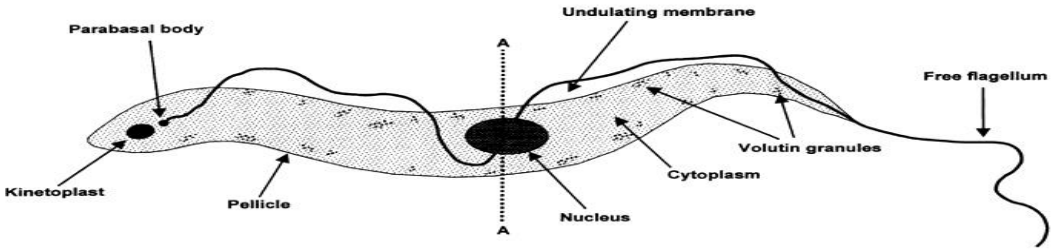


Figure 1: Diagram of a trypanosome

With the exception of a serum resistance associated protein (SRA) gene expressed in *T. b. rhodesiense*, the human infective trypanosomes are genetically identical (Xong *et al.,* 1998). Transmission of parasites to humans occurs through the bite of an infected tsetse fly of the *Glossina* species. Wild and domestic animals can act as reservoirs for human pathogenic trypanosomes without manifesting any disease symptoms (Brun and Brum, 2012). Furthermore, other related primates such as baboons, mandrills and sooty mangabeys are not susceptible to a *rhodesiense* infection (Thomson *et al.,* 2009). In the human bloodstream, the population of these human infective trypanosomes is pleomorphic, consisting of elongated, slender dividing forms, intermediate forms and short stumpy forms. Bloodstream trypanosomes actively divide after every 5-10 hours, and are able to transverse the walls of blood and lymph capillaries to connective tissue and ultimately, the CNS. Survival of bloodstream form trypanosomes largely depends on the sheath of glycosylphosphatidylinositol anchored glycoprotein known as the VSG. In fact, change in parasite cell surface is one of the most crucial changes that occur between the insect vector and the mammalian bloodstream forms (Gadelha, 2011).

Bloodstream forms of African trypanosomes are extremely dependant on glycolysis for energy production. The glycolytic enzymes are contained within a membrane bound microbody like organelle known as glycosome (Michels *et al.,* 2000). It is possible that this peroxisome related organelles enhance the parasite's high rate of glycolysis by the concentration of glycolytic substrates and enzymes in these compartments. *T. b. gambiense* parasites are also highly dependent on the polyamine biosynthesis, a situation that gave way to the production of a trypanocidal, eflornithine. Eflornithine is a potent inhibitor of the first enzyme involved in the biosynthesis of polyamine, ornithine decarboxylase. However, the drug is only effective against the *gambiense* infection but not the *rhodesiense* form (Barret *et al.,* 2003). Other metabolic pathways identified as important drug targets due to the presence of enzymes that are structurally different from their mammalian counterparts include pentose phosphate pathway, thiol metabolism and lipid and sterol metabolism (Barret *et al.,* 2003).

2.2.2 Epidemiology of human African trypanosomiasis

HAT occurs in geographic foci across sub-Saharan Africa in areas populated by tsetse flies and marked by weak or non-existent health systems. The disease occurs in 36 countries in sub-saharan Africa, and about 60 million people living in about 200 microfoci are at risk of developing it worldwide (WHO, 1986). *T. brucei* organisms are transmitted by tsetse flies of the

genus *Glossina* and order *diptera*, however *T. b. brucei* is not infective to man (Barret *et al.*, 2003). *T. b. gambiense* and *T. b rhodesiense* infections are geographically distinct, with Uganda being the only country facing the risk of an overlap of both parasite species (Brun and Brum, 2012). The *rhodesiense* HAT, endemic in eastern and southern Africa is a zoonotic disease and therefore, a major determinant of its epidemiology is the availability of cattle and game animals which harbor the parasites and ensure transmission to humans. On the other hand, *gambiense* HAT, endemic in western and central Africa relies on human-fly contact, rates of infection among tsetse flies and duration of infection in human hosts for its sustainability.

The first diagnosis of HAT was done more than 200 years ago (Atkins, 1734). After an increase in epidemics during the first decades of the 20th century, a massive decline was observed to an extent that sleeping sickness was considered to be under control at the beginning of the 1960's (Louis *et al*., 2002). Its incidence resurged alarmingly in the 1990s due to civil unrest and a lack of awareness of the disease, with major flare-ups in Angola, Democratic Republic of Congo (DRC), Uganda and Sudan (Stuart *et al*., 2008). The world health organization (WHO) reacted to the re-emergence of the disease by offering exclusive support to 24 out of the 36 countries listed as endemic either to assess the epidemiological status of HAT or to establish control and surveillance activities (Simarro *et al*., 2011). As a consequence of these activities, there were 9878 new HAT cases reported to the WHO in 2009, notably the first decline below the 10,000 mark for the first time in 50 years (WHO, 2010). Worth noting also is that only two countries reported more than 1,000 new cases in the same year of 2009, namely Central Africa Republic (CAR) and DRC representing, respectively, 11% and 73% of the total cases reported (figure 2) (Simarro *et al*., 2011). However, since most foci lie in the rural areas of Africa, then it is likely that most cases go undiagnosed and unreported (Michels *et al.*, 2000). Nevertheless, elimination of HAT as a public health problem is gaining new ground. In fact, *gambiense* HAT has in some foci already reached a prevalence rate that can enable it be termed as "an eliminated public health problem" (Simarro *et al*., 2011).

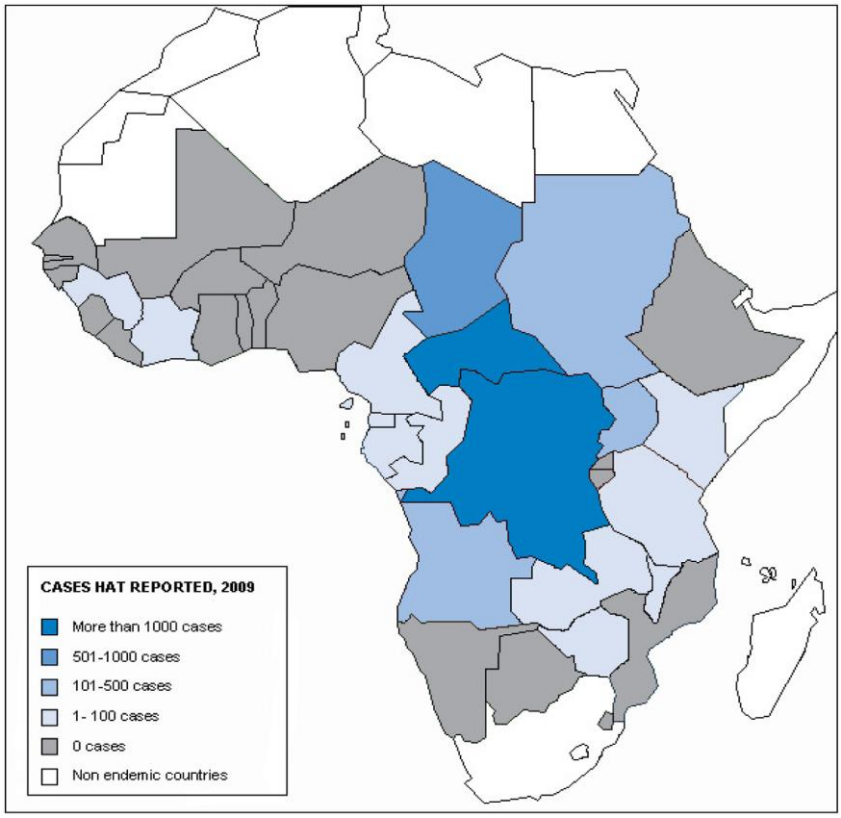


Figure 2: Classification of human African trypanosomiasis-endemic countries according to cases reported in 2009.

However, presenting an accurate picture of epidemiology of HAT has been hampered by variations occurring between classical disease forms and evolutionary forms, a phenomenon mostly attributed to the variability in host immunity (Truc *et al.,* 1997; Jamonneau *et al*., 2000; Jamonneau *et al.,* 2004). A particular attention has been paid in patients infected by the *gambiense* form, having complete absence of disease after 32 months despite presence of parasites in blood. This could be attributed to a strongly suited immune response highly mediated by inflammatory cytokines (Garcia *et al.,* 2000).

2.3 Role of Cytokines as Inflammatory Mediators in HAT

Increased production of inflammatory mediators is a common pathological feature of many neurodegenerative disorders including Alzheimer's and Parkinson's disease (Mog *et al.,* 1996; Griffin *et al.,* 1998). Similarly, one hallmark in the pathogenesis of HAT is the rapid and dramatic increase in inflammatory mediators and damaging free radicals including nitric oxide and ROS. CNS lesions in late stage HAT show a wide array of inflammatory features including perivascular cuffings and diffuse astrocytosis which appears to be important in initiating the

inflammatory process (Hunter *et al.,* 1992). There is also presence of macrophages, lymphocytes and plasma cells in cerebral white matter of infected patients. This inflammatory response can be greatly amplified in rodents by use of the trypanocidal diaminazine aceturate, which does not cross the BBB and therefore clears trypanosomes from the extravascular compartments but not the CNS (Jennings and Grey, 1983). Such phenomenon results in severe meningoencephalitis that closely mimics PTRE in human subjects.

Cytokines which are low molecular weight proteins act as intercellular mediators during inflammation and have widely been implicated in both early and late stage HAT. Increased cytokine levels have been directly correlated with anemia development following trypanosome infection and this has been attributed to their ability to inhibit haematopoiesis (Noyes *et al.,* 2001). Cytokines present during stage 2 HAT include interferon-gamma (IFN-γ) produced by lymphocytes and Interleukin 1 (IL-1), 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) produced by activated macrophages and which result in the activation of acute phase response (Paim *et al.,* 2011). It is important to note that pro-inflammatory cytokines IL-1, IL-6 and TNF-α are not only involved in inflammation during host immune response but also aid in parasite replication (Magez *et al.,* 2007). Presence of IL-10 has also been demonstrated in *T. b. gambiense* infections where it was shown to control the inflammatory response that results in neurodegeneration and therefore, plays a central role as a critical immunomodulator in both human and bovine trypanosomiasis (MacLean *et al.,* 2001). IL-6 also plays a vital role in the host immune response with IL-6 deficient mice being more susceptible to infections and registering higher parasitaemia loads and increased mortality rates than their counterparts expressing the same cytokine (Gao and Pereira, 2002). IL-1, present in high levels during acute phase of the disease has been shown to be an important mediator during trypanosomiasis inducing the production of acute phase proteins and stimulating lymphocyte mediated immune response (Reed *et al.,* 1989; Sileghem *et al.,* 1989; Eckersall *et al.,* 2001).

IFN-γ, the first inflammatory cytokine to be produced following infection plays a major role in the activation of macrophages and interestingly, has been implicated in parasite growth (Olsson *et al.,* 1991). In addition, IFN-γ has been correlated with disease severity in late stage HAT (Maclean *et al.,* 2007). TNF-α is involved in the activation of NF-κB which in turn is involved in the transcription of a wide array of inflammatory molecules, among them NO which is a potent trypanosome inhibitor (Vincendeau *et al.,* 1992). Hence, presence of TNF-α in the

choroid plexus is associated with a protective role during trypanosome infection evidenced by its ability to inhibit *T. b. brucei in vivo* (Magez *et al.,* 1993; MacLean *et al.,* 2001). Nevertheless, its overproduction during trypanosome infection which was first evidenced in *T. b. brucei* infected rabbits is implicated in the characteristic hypergammaglobulinemia observed during trypanosomiasis due to its role in the activation, proliferation and differentiation of B-cells (Rouzer and Cerami 1980; Roldan *et al.,* 1992).

Overall, it is evident that cytokines which are highly produced during trypanosomiasis are involved in both disease severity and regulation of immune responses and more studies are necessary to elucidate this delicate balance.

2.4 Melarsoprol Induced Reactive Encephalopathy

Treatment of human African trypanosomiasis currently relies on a limited number of highly toxic drugs, but untreated, is invariably fatal. Melarsoprol, a trivalent arsenical, is the only drug that can be used to cure both forms of the infection once the CNS has become involved, but unfortunately, this drug induces an extremely severe PTRE in up to 10% of treated patients, half of whom die from this complication (Pepin and Milord, 1994).

Melarsoprol results in neurological seizures in the absence of trypanosomes, and this suggests that melarsoprol itself is responsible for the reactive encephalopathy (Soignet *et al*., 1999). Notwithstanding this fact, the molecular mechanisms that lead to PTRE following melarsoprol treatment in late stage HAT are not well understood. However many hypotheses have been presented which include immunological reactions to the parasite following therapy (Pepin and Milord, 1994), immune complex deposition (Lambert *et al*., 1981), toxicity of the arsenical moiety of melarsoprol (Hurst, 1959), autoimmune reactions or subcurative chemotherapy (Hunter *et al*., 1991; Hunter and Kennedy, 1992; Hunter *et al*., 1992). It has also been shown that the trivalent arsenical drug melarsoprol generates ROS and free radicals like hydrogen peroxide (H_2O_2) (Barchowsky *et al*., 1996; Wang *et al*., 1996; Chen *et al*., 1998), hydroxyl radical species ($HO^{\bullet}$) (Wang *et al*., 1996), nitric oxide ($NO^{\bullet}$) (Gurr *et al*., 1998) and superoxide anion ($O_2^{\bullet-}$) (Barchowsky *et al*., 1996; Lynn *et al*., 2000). Moreover, melarsoprol and other arsenics have been shown to inhibit several antioxidant systems in the body (Vahter, 2007). Melarsen oxide, a metabolite of melarsoprol has been shown to interact with thiols such as glutathione and thioredoxin forming stable adducts which competitively inhibit the respective antioxidant flavoproteins namely glutathione reductase and thioredoxin reductase (Fairlamb *et*

al., 1989; Cunningham *et al*., 1994). Melarsen oxide also potently inhibits glutathione reductase and thioredoxin reductase directly by interacting with catalytically active sulphydryl groups present in both enzymes (Cunningham *et al*., 1994). The disruption of these selenoenzymes which are major oxidative stress protection systems in the brain is expected to result in a wide range of neurological complications which share oxidative and nitrosative stress as common pathological features. Furthermore, it has also been reported that arsenics have the ability to complex with sulphydryl groups depleting cellular reduced glutathione (GSH) levels as illustrated in figure 3 (Del-razo *et al.,* 2001). Other antioxidant systems in the body inhibited by arsenics include catalase and manganese superoxide dismutase (MnSOD) (Lin *et al*., 2001; Mazumder, 2005; Dwivedi *et al.,* 2011), but how this is achieved still remains elusive. Moreover, the role of arsenic-associated antioxidant failure in HAT patients on melarsoprol has not been investigated.

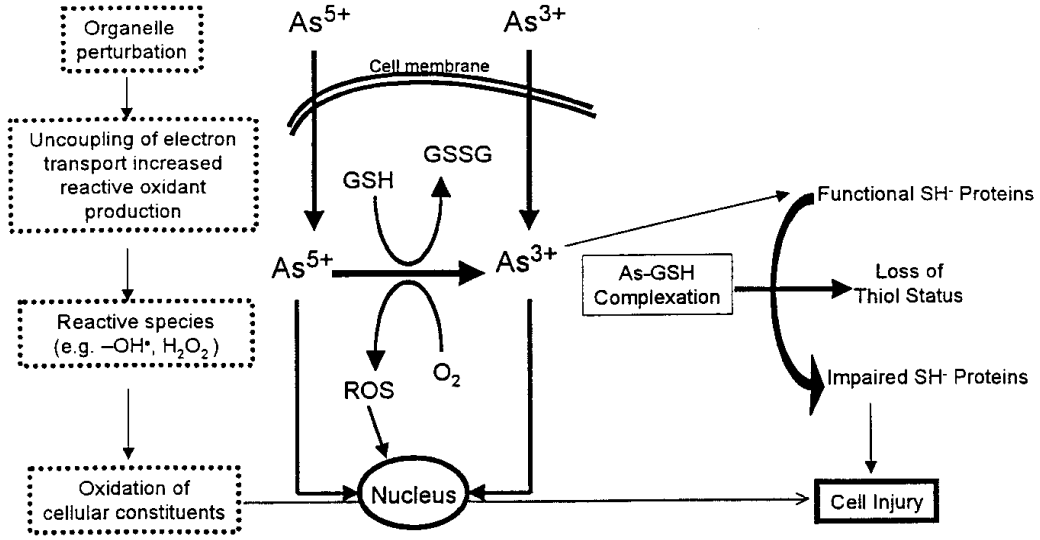


Figure 3: Mechanism of cell injury by arsenic.

Since considerable evidence points to oxidative stress as an important trigger in the complex chain of events leading to brain encephalopathies such as Alzheimer's disease and Parkinson's disease, then it is possible that oxidative stress plays a key role in the development of PTRE following melarsoprol treatment in late stage HAT.

2.5 Brain Energy Metabolism and Antioxidant Defense

Energy metabolism in mammalian cells occurs in the mitochondria and this process is intimately linked to antioxidant capacity protection in the brain. The primary role of

mitochondria is generation of adenosine triphosphate (ATP) through oxidative phosphorylation and oxygen consumption, and since the brain exhibits high oxidative metabolism, it is most sensitive to mitochondrial dysfunction (Orina *et al*., 2007). Brain cells have very high energy requirements and therefore exhibit a high rate of production of ROS from the mitochondrial electron transport activities coupled to oxidative phosphorylation (Chan, 1996). Antioxidant systems are therefore vital for normal cell function and survival of these cells.

Mitochondrial dysfunction has been attributed to oxidative stress and this is the case in neurodegenerative diseases such as Parkinson's disease (Ikebe *et al*., 1990; Copeland 2002). Mitochondria are highly susceptible to oxidative damage with ROS being able to damage mitochondrial enzymes directly (Lenaz, 1998) and/or cause mutation in mitochondrial DNAs. Since organic arsenics such as melarsoprol trigger generation of ROS, it is possible that PTRE following melarsoprol treatment during late stage HAT occurs as a result of mitochondrial dysfunction and impaired mitochondrial enzymes.

During aerobic respiration to generate ATP in mitochondria, leakage of electrons frequently produces mitochondrial superoxide anions that are rapidly reduced to H_2O_2 by MnSOD (Seung-Hee *et al*., 2001). Reduced glutathione (GSH), which is an efficient antioxidant and free radical scavenger, metabolizes H_2O_2 and is the best defense against potential toxicity of H_2O_2 in mitochondria (Seung-Hee *et al*., 2001). Indirectly, GSH acts as a substrate for glutathione peroxidase (GPx) and glutathione S transferase which are enzymes involved in other ROS detoxification reactions (Michael *et al*., 2002). Its activity is however mediated by the enzyme glutathione reductase which is responsible for the nicotinamide adenine dinucleotide phosphate reduced (NADPH)-dependent reduction of the oxidized form glutathione disulphide (GSSG) to the reduced form (GSH). Cytosolic aconitase and cytosolic $NADP^+$-dependent isocitrate dehydrogenase metabolize citrate to α-ketoglutarate with concomitant release of NADPH which is an essential cofactor for the reduction of oxidized glutathione disulphide (GSSG) (Tong and Rouault, 2006). Thus cellular inactivation of aconitases which is largely attributed to the enzyme's sensitivity to oxidative stress may have at least two major consequences in the brain. First, aconitase deficiency causes glutamate auxotrophy because of the lack of α-ketoglutarate generation. α-ketoglutarate, which is formed from glucose, constitutes the carbon backbone of glutamate (figure 4) (Hassel and Dingledine, 2006). Additionally, the fact that neurons cannot synthesize citrate due to absence of pyruvate carboxylase and therefore

depend on neighboring cells such as astrocytes for glutamate further underscores the effects of aconitase deficiency in the brain (Hertz and Zielke, 2004).

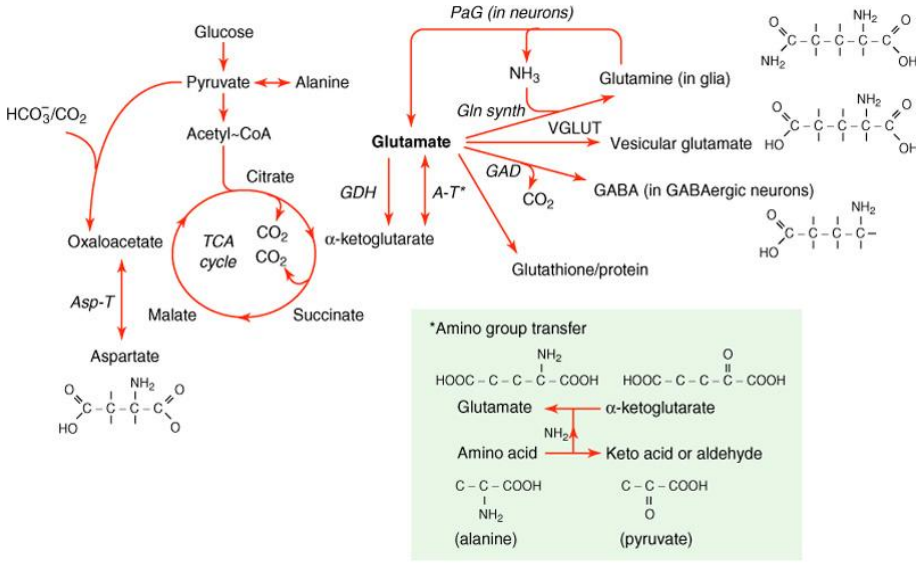


Figure 4: Formation and fate of glutamate in the brain.

Secondly, inhibition of aconitases could decrease ATP production via the tricarboxylic acid (TCA) cycle and also decrease production of cytosolic NADPH (figure 5), a major source of reducing equivalents and an important defense against oxidative stress (Tong and Rouault, 2006).

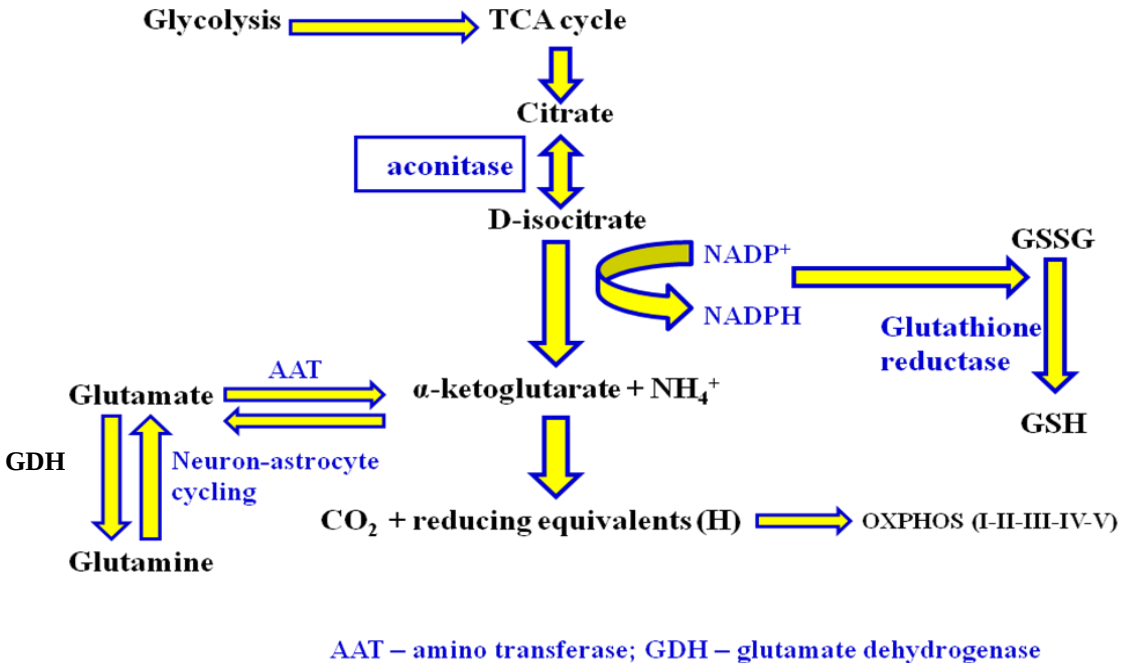


Figure 5: Role of aconitase in ATP production and as an antioxidant

It is therefore clear that oxidative imbalance is related to impairment in the capacity of ATP synthesis in the brain with concomitant decrease in astrocytic function. This is validated by the fact that injection of the aconitase inhibitor fluorocitrate into glial cells results in the selective damage of astrocytes (Paulsen *et al.*, 1987). Given the important role astrocytes play in neuroprotection and as a source of reactive nitrogen species which are cell signaling molecules that can induce neuronal damage (Arnaudo *et al*., 1991; Nekhaeva *et al*., 2002), it is evident that a defect in normal astrocytic function will result in increased vulnerability of neurons to oxidative stress.

2.5.1 Aconitases

Aconitase belongs to a group of iron-sulphur proteins which are the most ubiquitous and functionally versatile classes of metalloproteins (Beinert *et al*., 1997; Johnson *et al*., 2005). Aconitase contains a [4Fe–4S] cluster and the enzymatic reaction catalyzed by aconitase involves substrate coordination to a specific iron atom in this cluster (Beinert *et al*., 1996). Eukaryotes possess two forms of the protein aconitase: A cytoplasmic form or aconitase 1 (ACO 1) and a mitochondrial form or aconitase 2 (ACO 2). The primary function of mitochondrial aconitase is enzymatic, converting citrate to isocitrate via a cis-aconitate intermediate in the TCA cycle (Breusch, 1937). The cytoplasmic aconitase protein, however, is bifunctional, serving as both an enzyme and an iron regulatory protein (IRP) (Gruer *et al*., 1997).

When cellular iron levels are high, the cytoplasmic protein retains its [4Fe-4S] cluster, and is enzymatically active (Philpott *et al*., 1994), with the [3Fe-4S] form also present at equilibrium (Beinert *et al*., 1996). When iron levels are low, the Fe-S cluster is completely disassembled and cytoplasmic aconitase loses its enzymatic function (Philpott *et al*., 1994). The apoprotein functions to bind specific stem-loop sequences of mRNA called iron responsive elements (IREs), and is thus called the iron-responsive element binding protein (IRE-BP) which regulates the uptake, transport, and storage of cellular iron. IREs are found at the 3’ ends of transferrin receptor mRNAs encoding for proteins involved in iron transport into the cell (Philpott *et al*., 1994). Binding of IRE-BP at this site prevents the degradation of this mRNA, allowing the cell to import more iron in times of need. IREs are also found at the 5’ ends of ferritin mRNAs, where IRE-BP binding prevents translation into protein (figure 6) (Beinert *et al*., 1996). Therefore, proteins involved in iron sequestration, such as ferritin, have IRE sequences at the 5’ end of mRNA (Tong and Rouault, 2006).

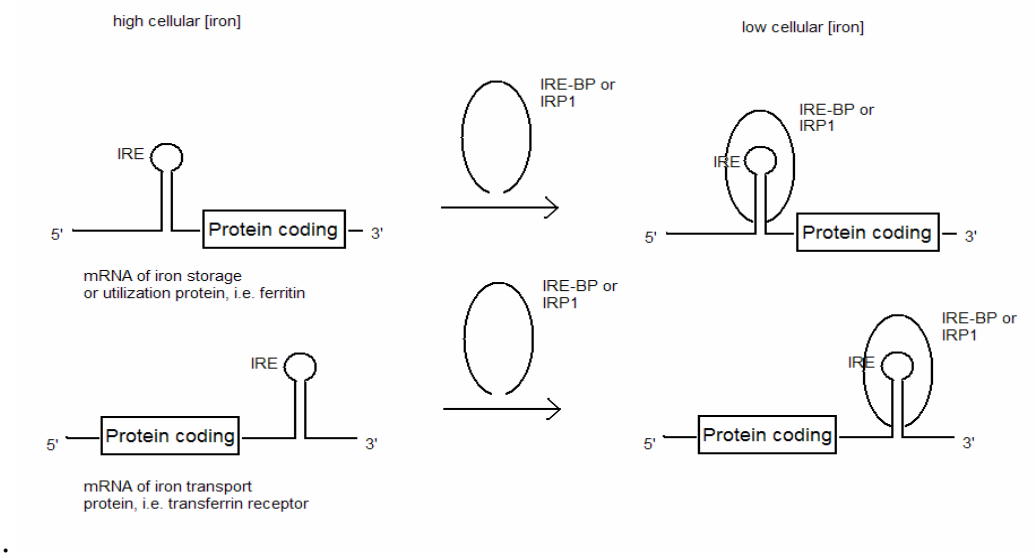


Figure 6: Iron responsive protein binding of 5' and 3' IRE sequences.

Cellular inactivation of aconitases is largely attributed to oxidative stress. The presence of an unligated acid labile iron atom (Fe_a) in the active site of the enzyme renders these dehydratases especially sensitive to oxidative inactivation by nucleophilic ROS, involving the univalent oxidation of the [4Fe-4S] cluster (Flint *et al*., 1993). Consequently an unstable intermediate is formed which fails to keep the unligated Fe_a bound to the cluster. This instability promotes the loss of Fe_a from [4Fe-4S] forming [3Fe-4S] cluster with concomitant loss of enzyme activity. Formation of an inactive [3Fe-4S] cluster results in the simultaneous release of Fe^{2+} and H_2O_2. Superoxide (O_2^-) mediated inactivation of Fe-S-containing enzymes may pose a significant oxidative burden because it provides equimolar amounts of H_2O_2 per mole of O_2^- (Liochev and Fridovich, 1999). The release of Fe^{2+} and H_2O_2, ingredients of the Fenton reaction, can result in generation of the potent OH^- radical, which can oxidize mitochondrial proteins, DNA, and lipids thereby amplifying O_2^- initiated oxidative damage.

2.5.2 Energy dependent functions of astrocytes

Energy dependent functions of astrocytes circumvent around synthesis of glutamine and extracellular glutamate and K^+. Astrocytic cells synthesize glutamine from glutamate which is then taken up by the neurons through specific uptake carriers. In the neuron, glutamine is converted to glutamate through glutaminase, a phosphate-dependent enzyme preferentially localized to synaptosomal mitochondria (Yudkoff *et al*., 1989; Westergaard *et al*., 1995). Glutamate uptake by the astrocytes is an energy dependent process, which is mediated by

different plasma membrane carriers, five of which have been cloned and named excitory amino acid transporters (EAATs) 1–5 (Gegelashvili and Schousboe, 1998; Danbolt, 2001) and a vast majority of which are localized on astrocytes (Lehre *et al*., 1995; Lehre and Danbolt, 1998; Levy, 2002).

Glutamate uptake is driven by the electrochemical gradient of Na^+, which is maintained by action of Na^+, K^+-adenosine triphosphatase (ATPase). However, although the binding of glutamate to the carrier is not in itself energy dependent, the capacity for binding is exhausted in case the transport cycle is interrupted by energy failure and collapse of the transmembrane Na^+ gradient (Wadiche *et al*., 1995). Impairment of glutamate uptake by the astrocyte in the setting of ATP depletion, as occurs with hypoxia, ischemia, or hypoglycemia, is considered a primary mechanism of excessive accumulation of glutamate in the synaptic space, leading to neuronal injury and finally brain neurodegeneration (Eduardo, 2005).

Astrocytes play a similar active role in the clearance of extracellular K^+ (Walz, 2000). The K^+ uptake into astrocytes occurs by two different mechanisms namely activation of the extracellular K^+ sensitive site of the Na^+, K^+-ATPase and stimulation of the Na^+, K^+, Cl^- co-transporter. Uptake of K^+ by the astrocytic Na^+, K^+-ATPase occurs by direct stimulation at its extracellular K^+-sensitive site by any increase in extracellular K^+ concentration and requires an immediate supply of ATP. In contrast, uptake by the co-transporter is driven by the Na^+ gradient (Leif *et al*., 2007).

2.6 Tea and Health

Tea, an evergreen plant native to China and belonging to the family *Theaceae* , is one of the most commonly consumed beverages in the world and is manufactured from the young tender leaves of the plant *Camellia sinensis* (Cabrera *et al*., 2003). Two types of tea products most widely consumed include green and black tea. Other teas available include oolong, pu'erh, white, red and purple tea. Green tea favored in Asia, is prepared from unfermented leaves while black tea which accounts for about 76-78% of the world's production is fully oxidized and fermented, loosing present antioxidants and gaining new ones in the process (Sajilata *et al.,* 2008). Oolong is a partially fermented tea with a characteristic flavor of both green and black teas. Red or roobios tea, which is naturally caffeine free, comes from a shrub in South Africa (Sharangi, 2009). Pu'erh tea is a unique type of tea in that once the leaves are picked at any time of the year, they are piled and aged for as long as a half or a full century and eventually

processed in a similar fashion to black tea (Sharangi, 2009). The chemical composition of tea is complex and includes polyphenols, amino acids, carbohydrates, proteins, chlorophyll, volatile compounds, minerals, trace elements and alkaloids such as caffeine, theophylline and theobromine. Among these, polyphenols constitutes the main bioactive molecules in tea (Cabrera *et al*., 2003). The major polyphenols found in tea include catechins namely epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG).

Centuries after centuries, the Chinese community has been using the tea beverage to treat a myriad of diseases (Sato and Miyata 2000). This myth has seen the last decade witness increased interests in the use of tea polyphenols as health enhancing or medicinal agents. The pharmacological value of tea has heavily relied upon its antioxidative properties known to surpass that of major antioxidants. Rice-Evans *et al.* (1995) were able to show that green tea polyphenols exerted greater antioxidant activity on a molar basis than vitamin C. Shahidi and Alexander (1998) corroborated these results by establishing that tea polyphenols had radical quenching abilities greater than α-tocopherol. The ability of tea to scavenge for free radicals is associated with the possession of a phenolic hydroxyl group attached to the flavan-3-ol structure of these compounds (Amie *et al*., 2003). However, the importance of polyphenols in enhancing resistance to oxidative stress goes beyond simple scavenging activity and is also due to amplified activity of most detoxifying enzymes such as glutathione peroxidase and glutathione reductase (Sharangi, 2009). Indeed, as a result of their free radical squelching strengths, tea polyphenols have widely been credited with therapeutic action against free radical mediated diseases (Amie *et al*., 2003).

Numerous *in-vitro* and *in-vivo* studies have provided evidence for the anti-carcinogenic abilities of tea polyphenols, cancer being one of medical conditions widely associated with oxidative stress. Tea consumption may inhibit development of cancers of the skin, lung, liver, pancreas, bladder, prostate and mammary gland (Yang and Wang 1993; Zhong *et al.,* 2001; Jia *et al.,* 2002; Qanungo *et al.,* 2005; Bettuzzi *et al.,* 2006; Sun *et al.,* 2006). Numerous mechanisms for the action of tea polyphenols against cancer have been put forth including inhibition of mitogen activated protein (MAP) kinases and the phosphoinoside-3-kinase/protein kinase B (PI3K/PKB) pathway, inhibition of nuclear factor kappa beta (NF-κB) and activator protein (AP)-1-mediated transcription, inhibition of growth factor-mediated signaling with concomitant inhibition of tumor cell growth, angiogenesis and induction of apoptosis (Yang *et al.,* 2009).

However, the effects of polyphenols on health cannot be solely explained on the basis of their antioxidant properties. Based on current evidence, tea polyphenols possess potent anti-inflammatory properties (Karori *et al.*, 2008). Effects of tea polyphenols against inflammation are directed towards activities related to the arachidonic acid dependent pathways, NOS and NF-κB being classical targets **(**Miles *et al.*, 2005; de Mejia *et al.*, 2009). Tea polyphenols, in particular EGCG suppresses the activation of NF-κB inhibiting cyclooxygenase 2 (COX-2) and inducible nitric-oxide synthase (iNOS) expression (Kundu and Surh, 2008). The polyphenols thus ultimately, inhibit the production of arachidonic acid metabolites with pro-inflammatory properties resulting in a decreased inflammatory response with concomitant risk reduction in the development of many inflammatory diseases such as diabetes, arthritis, cancer and asthma.

There has also been intense interest in the potential of polyphenols to prevent neurodegeneration. This is because previous studies have shown that ACN's such as cyanidin-3-rutinoside and pelargonidin-3-glucoside, tea catechins such as EGCG, which is the major and most active component of green tea catechins and EC metabolites including epicatechin glucuronide and 3'-O-methylated epicatechin glucuronide, formed after oral ingestion of EC by rats, can cross the blood brain barrier (Nakagawa and Miyazawa, 1997; Youdim and Joseph, 2001; Abd *et al*., 2002) causing neuroprotection. In addition, previous studies have shown that tea polyphenols are able to protect nerve cells from ROS-induced cell death (Matsuoka *et al*., 1995). Other neuroprotective properties associated with tea polyphenols include preventing loss of dopaminergic neurons and preservation of striatal levels of dopamine, decreased expression of neuronal nitric oxide synthase (nNOS), inhibition of pro-apoptotic genes and protection against beta-amyloid induced neurotoxicity strongly suggesting that tea polyphenols have potential application in the treatment of neurodegenerative disorders such as Alzheimer's and Parkinson's disease (Choi *et al.*, 2001; Choi *et al.*, 2002; Zaveri, 2006). However, despite the growing body of data on the pharmacological properties of tea extracts, most studies have heavily relied upon the use of catechins, the major polyphenols found in tea and little work has been carried out to establish the health benefits of tea-derived ACN's.

2.7 Anthocyanins

Anthocyanins are a diverse group of naturally occuring polyphenol compounds conferring hues of blue, purple or deep red to several plants especially in leaves, flowers and fruits (Crozier *et al*., 2006). Anthocyanins which belong to a group of compounds known as

flavonoids are synthesized from phenylalanine and are characterized by a benzo-γ-pyrone structure (Yao *et al*., 2004). They consist of an aglycone anthocyanidin part and a glycone sugar part. The most common anthocyanidins include delphinidin, cyanidin, peonidin, petunidin, malvidin and pelargonidin, with individual differences in the number of hydroxyl groups and nature and number of attached sugar moieties (Kong *et al.,* 2003). Anthocyanins, though soluble in water, are highly soluble in polar solvents and are normally extracted from plants using acidified methanol (Kong *et al.,* 2003).

Biological activities of anthocyanins are closely linked to their absorption and metabolism. Anthocyanins share common metabolic properties with other flavonoids, undergoing extensive glucuronidation and methylation in humans (Mazza *et al*., 2002; Kay *et al*., 2005). In fact glucuronidated and methylated anthocyanin metabolites appear in humans at levels more than twice that of the parent (intact) compounds (Kay *et al*., 2005). However, glucuronide conjugation is regarded as the major conjugation reaction involved in anthocyanin metabolism (Oliveira *et al*., 2002). There are two main reasons for the widespread utilization of the glucuronidation pathway. First, glucuronic acid is derived directly from glucose, and its stored form, glycogen, and is therefore readily available. Secondly, glucuronic acid has the capacity to be conjugated with a wide range of compounds (Kay, 2006). As a result of these extensive bio-transformations, the forms reaching target organs are different from those present in food sources and have lower biological activity and health benefit effects than their parent compounds (Day and Williamson, 2001; D'Archivio *et al.*, 2007).

Human consumption of anthocyanins is increasing because of their potential health benefits including being powerful antioxidants (Kahkonen and Heinonen, 2003) and having antimicrobial, anti-carcinogenic and anti-inflammatory properties (Youdim and Joseph, 2001; Viskelis *et al.,* 2009). Moreover, currently available synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxytoluene (BHT) and gallic acid esters have been suspected to cause or prompt negative health effects, hence the need to substitute them with naturally occurring antioxidants (Amie *et al*., 2003; Aqil *et al*., 2006; Pourmorad *et al*., 2006). Therefore anthocyanin supplements are widely marketed for their health enhancing properties. In line with this development, the Tea Research Foundation of Kenya (TRFK) has pre-released a purple tea variety, clone TRFK 306 characterized as having exceptionally high levels of anthocyanins (figure 7) (Kerio *et al.,* 2012).

Figure 7: Anthocyanin rich Kenyan purple tea plants clone TRFK 306/1.

Nevertheless, it has now been widely established that isolated individual antioxidants do not explain the observed health benefits of diets rich in phytochemicals, implying that interactions between antioxidants may yield positive synergistic effects (Chu *et al.,* 2002). Indeed, Lachman *et al.* (2000) were able to demonstrate that antioxidative activities of natural anthocyanin extracts of colored potatoes are much higher than that of pure individual anthocyanin compounds, indicating synergistic effects of the mixture of the anthocyanins and other antioxidants contained in potato tubers. Hosseini-Beheshti *et al.*, (2012) confirmed these results and reported antioxidant synergy between black-current and wine grape berries. Therefore, the health benefits of a diet rich in phytochemicals is attributed to the complex mixture of phytochemicals present in it, an observation which clearly suggest that to improve their nutrition and health, consumers should be getting antioxidants from diverse sources. However, only a very limited number of studies have investigated combinations of purified anthocyanin extracts with other chemical components of food.

2.8 Coenzyme-Q_{10}

Coenzyme-Q_{10} (Co-Q_{10}), or ubiquinone, is an endogenously synthesized lipid, which shuttles electrons from complexes I and II to complex III (ubiquinol cytochrome c oxidase) of the electron transport chain. It is present in all organs, but found in the highest concentrations in brain, heart, kidney and liver tissue (Ogawa *et al*., 2002; Bonakdar and Guarneri, 2005). The high concentrations of Co-Q_{10} in heart and brain, two organs with exceptional demand for energy demonstrates the importance of Co-Q_{10} in energy metabolism and the resultant oxidative demand in the cells. Intracellular synthesis that occurs in the inner mitochondrial membrane via the

mevalonate pathway is the major source of Co-Q_{10}, although small amounts can be obtained from the diet (Mancusso *et al.,* 2010). Co-Q_{10} is commercially available in many forms including powder, tablets and soft gel capsules (Spindler *et al.,* 2009). In addition, a short chain synthetic analogue of Co-Q_{10} similarly involved in the transfer of electrons from complex-I and II to complex–III of the electron transport chain, has been produced (Geromel *et al.,* 2002). Co-Q_{10} is an essential cofactor involved in mitochondrial oxidative phosphorylation and when reduced, it is a powerful antioxidant that prevents oxidative damage by free radicals including oxidation of lipids within the mitochondrial membrane (Matthews *et al*., 1998; Geromel *et al*., 2002). Co-Q_{10} also serves as an antioxidant by activating and increasing expression of mitochondrial uncoupling proteins (UCPs), an effect which is anti-apoptotic (Shults and Haas, 2005) and thus leads to a reduction in free radical generation.

Previous studies have also implicated Co-Q_{10} as having potent anti-inflammatory properties by inhibiting the expression of IL-6, TNF-α, and NF-κB (Sharma *et al.,* 2006). In addition, Co-Q_{10} exerts its anti-inflammatory effects by gene expression modification reducing the activity of inflammatory markers (Schmelzer *et al.,* 2008). This has seen the supplementation of clinical populations with Co-Q_{10} to modulate inflammatory conditions such as cancer and diabetes. More importantly, Co-Q_{10} crosses the blood brain barrier exerting a multitude of neuroprotective effects in the brain and protecting against pathophysiology associated with neurodegenerative disorders (Matthews *et al.,* 1998). Co-Q_{10} has been shown to protect against dopamine depletion, development of α-synuclein aggregates, and loss of tyrosine hydroxylase immunostained neurons induced by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) in a mouse model of Parkinson's disease (Beal *et al.,* 1998). In a mouse model of Huntington disease, Co-Q_{10} reduced brain atrophy, hungtingtin inclusions and improved motor performance as well as extending survival period (Ferrante *et al.,* 2002). In transgenic mice models of Alzheimer's disease, Co-Q_{10} conferred protection against plaques and memory loss and attenuated brain atrophy (Yang *et al.,* 2008). It is against these findings that the present study sought to determine if Co-Q_{10} would be neuroprotective in HAT infection and melarsoprol neurotoxicity.

Primary Co-Q_{10} deficiency is a rare phenomenon which manifests itself in several variants including pure myopathy, myopathy characterized with encephalopathy, cerebellar ataxia and severe infantile multisystem disease including encephalopathy and nephropathy (Quinzii *et al*., 2008). Distinctive features associated all phenotypic variants include generalized

weakness, exercise intolerance and recurrent myoglobinuria. Out of the nine genes believed to be involved in the biosynthesis of Co-Q_{10}, only three have been shown to result in primary Co-Q_{10} deficiency namely prenyl diphosphate synthase subunit (PDSS)1, PDSS2, and COQ2 (Dimauro *et al.,* 2007). Pathophysiology symptoms of Co-Q_{10} deficiency, which are partially reversed on Co-Q_{10} supplementation, include reduced activities of complexes I to IV in the electron transport chain, reduced expression of mitochondrial proteins involved in oxidative phosphorylation, decreased mitochondrial membrane potential and increased levels of ROS (Rodriguez-Hernandez *et al.,* 2009). It is no surprise therefore that Co-Q_{10} deficiency is associated with a myriad of neurological disorders.

Therefore the goal of the present study was to investigate the potential usefulness of Co-Q_{10} as an antioxidant and an anti-inflammatory agent in a mouse model (Swiss white) for HAT and PTRE. This model was chosen to understand the impact of Co-Q_{10} following invasion of the brain by African trypanosomes and/or melarsoprol-induced PTRE, clinical conditions highly associated with the rapid and dramatic increase in inflammatory mediators and damaging free radicals including nitric oxide and ROS (Kennedy, 2004). It was hypothesized that Co-Q_{10} would offer neuroprotection attenuating the adverse effects associated with late stage trypanosomiasis and post melarsoprol treatment.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Purple Tea Anthocyanins

Purple tea anthocyanins were extracted from a mixture of the purple tea clones namely TRFK 306/1, 306/2, 306/3 and 306/4 obtained from the Tea Research Foundation of Kenya, Timbilil Estate in Kericho (latitude 0 ^{o}22'S, longitude 35^{o}21'E, altitude 2180m a.m.s.l).. Anthocyanins were extracted from a mixture of the purple tea clones namely TRFK 306/1, 306/2, 306/3 and 306/4. Young tender shoots comprising of two leaves plus a bud were harvested, dried using a microwave and pulverized with a grinder into fine powder.

3.1.1 Extraction of anthocyanins

The modified method of Donner *et al*. (1997) was employed to extract anthocyanins. Five grams of powdered leaves of purple *Camellia sinensis* were weighed into 250ml conical flasks covered with aluminum foil to prevent photo degradation after which was added 50ml methanol/formic acid at a ratio of 99/1 volume/volume (v/v). The sample was magnetically stirred for four hours at room temperature at a speed of 900rpm. The resultant solution was filtered and methanol and formic acid were removed using a rotary evaporator (Buchi Rotavapour R-300, Switzerland) at 35^{o}C under vacuum, and the residue was reconstituted in 10mls distilled water. The extract was then passed through a membrane filter 0.45µM and stored at 4^{o}C until required for analysis.

3.1.2 Purification of anthocyanin fraction

The tea extracts were passed through a C-18 reverse phase octadecylsilane **(**ODS; Phenomenex Inc. Torrance CA, USA) cartridge previously activated with acidified methanol (10% HCl/methanol v/v). Anthocyanins were adsorbed onto the column while sugars, acids and other water soluble compounds were washed out using 0.01% HCl in distilled water. The anthocyanins were recovered using acidified methanol (10% formic acid/methanol v/v). The catridges were washed with ethyl acetate (Fischer Scientific, UK) to remove phenolic compounds other than anthocyanins. The purified extracts were then stored at -10ºC until required for analysis.

3.1.3 HPLC analysis of anthocyanins

Qualitative analyses of the extract and anthocyanin profiles of purple tea variety TRFK/306 were carried out by high performance liquid chromatography (HPLC) using a

modified method of Guisti and Wrolstad (1996). One ml of the anthocyanin sample was pipetted into separate tubes and diluted to 2ml with mobile phase A solution (87:3:10 water/acetonitrile/formic acid v/v/v) filtered and loaded into 2ml vials. A Shimadzu LC 20 AT HPLC fitted with a SIL 20A autosample and a SPD-20 Ultraviolet-visible detector with a class LC10 chromatography workstation with UV detection at 520nm was used for analysis of the prepared samples. A Luna TM 5 μM, C18, 25cmx4.6mm internal diameter (Phenomenex, Torrance, CA, USA) column fitted with a Rheodyne precolumn filter of 7335 model was used. Mobile phase solutions were filtered through a 0.45μm nitrocellulose filter on a membrane filter disk and degassed before injection into the HPLC system.

Gradient elution was employed for analysis using the following solvent: The eluents were mobile phase A (water/acetonitrile/formic acid at a ratio of 87/3/10 v/v/v) and mobile phase B (100% HPLC grade acetonitrile). The flow rate of the mobile phase was set at 1ml/min, column temperature at 35±0.5ºC and injection volume at 20μl. Chromatographic conditions were set as follows: 3% B in A at the time of injection, at 45minutes 25% B in A, at 46 minutes 30% B in A and at 47 minutes 3% B in A. The conditions were set at 3% B for 10 minutes before the next injection to allow for equilibration.

Identification of individual anthocyanidins was carried out by comparing the retention times from sample chromatographs and absorbances of unknown peaks with the peaks obtained from the individual and mixed standards under similar conditions. The standards used were cyanidin-3-O-galactoside, cyanidin-3-O-glucoside, cyanidin chloride, delphinidin chloride, petunidin chloride, pelargonidin chloride and malvidin chloride purchased from Sigma Aldrich, (UK). Quantification of anthocyanins was performed at 520nm using external anthocyanin standards each with its determined calibration curve. The identified individual anthocyanin content calculated on a dry matter basis was determined by the formula outlined in appendix I.

3.1.4 Lyophilization of anthocyanin extract

Prior to the lyophilization process, methanol and formic acid were removed using a rotary evaporator at 35ºC under vacuum and the residue was reconstituted to distilled water. Pre-freezing of the extract was done before being placed on the drying accessory. A volume of 200ml of the tea sample were placed in dehydration flasks and rapidly frozen by spinning the round bottom flasks in a dry ice-acetone bath. Temperature and pressure of the lyophilizer were allowed to reach appropriate levels of -40ºC and $100x10^{-3}$ M Bar, respectively before the freeze

drying process was initiated. Lyophilization was done using a Modulyo freeze dryer (Edwards, England) producing a free flowing powder that was weighed and stored in airtight containers at room temperature until use.

3.2 Experimental Animals

All experimental protocols and procedures involving use of mice as experimental animals adhered to rules and regulations approved by Institutional Animal Care and Use Committee (IACUC) of the Trypanosomiasis Research Centre of Kenya Agricultural Research Institute (KARI-TRC) Muguga, Kenya and Egerton University as well as the National Regulations of the Kenya Veterinary Association. A total of 73, eight weeks old female adult healthy Swiss white mice weighing between 21-30g were obtained from the TRC colony and used in all experiments. The animals were housed in standard mice cages at a temperature of 21-28^{o}C and were provided *ad libitum* access to water and standard mice cubes obtained from Unga Feeds Ltd Kenya. Wood-chippings were provided as bedding material. All mice were treated with 0.02ml of Ivermectin (Ivermectin®, Anupco, Suffolk, England) injected subcutaneously to each mouse to eradicate endoparasites and ectoparasites infestation. All animals were marked using picric acid for identification.

3.3 Experimental Design

After two weeks of acclimatization, mice were randomly selected and divided broadly into two experimental groups; Group (I) employing uninfected animals and Group (II) employing trypanosome infected animals (figure 8). Uninfected animals were further subdivided into two groups: (i) Uninfected animals challenged with the organic arsenical melarsoprol with or without the intervention of antioxidants. These animals were important in investigating the potential involvement of antioxidant capacity failure in the brain during melarsoprol treatment; (ii) Uninfected animals supplemented with tea ACN's or a combination of tea ACN's and Co-Q_{10}. These animals were important in investigating the effects of antioxidants on the cellular antioxidant capacity of the brain and possible synergistic or antagonistic effects between the two test antioxidants.

Infected animals were also subdivided into two groups: (i) Infected animals where the disease was allowed to progress in the absence of trypanocidals with or without antioxidant intervention. These animals were used to analyze the effect of trypanosome infection on the

integrity of the brain antioxidant system; (ii) Infected animals employed for PTRE studies. These animals were employed to study severe late CNS stage infection.

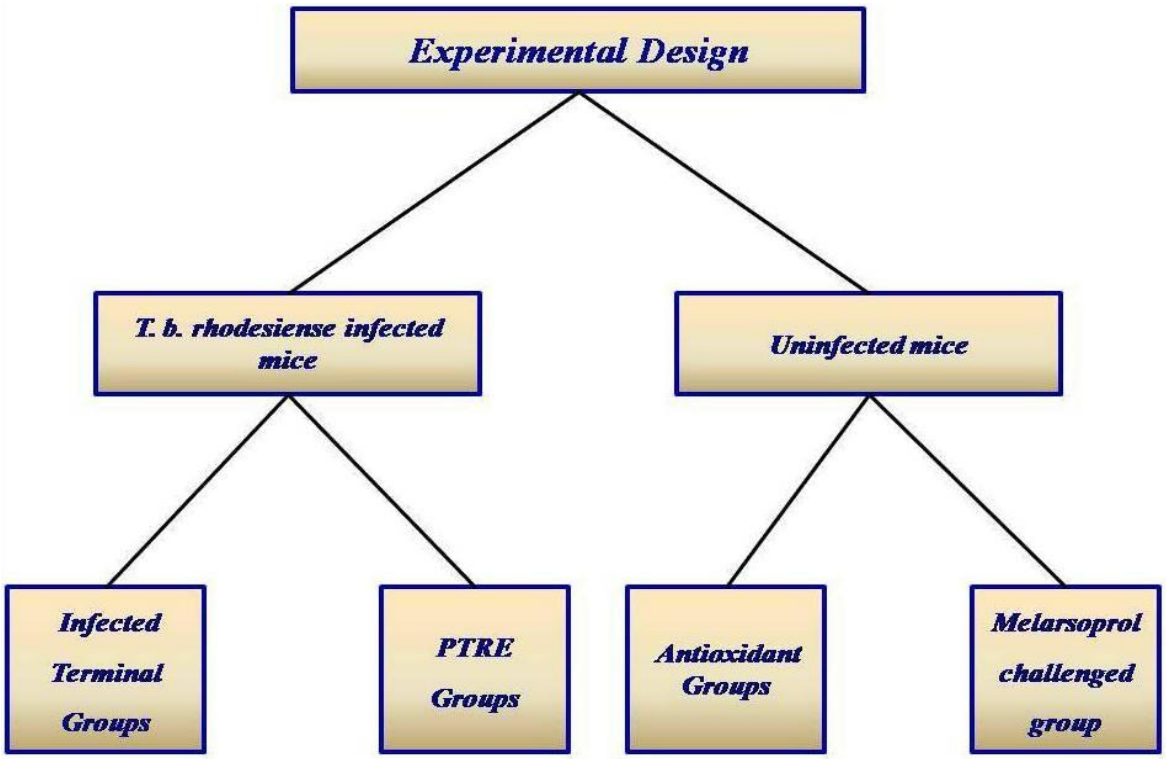


Figure 8: Schematic representation of the study design

3.4 Uninfected Melarsoprol Groups

Experimental animals in this study were randomly divided into five groups (figure 9). Administration of antioxidants commenced ten days prior to Melarsoprol (Mel B) treatment to allow concentration of the antioxidants in the brain and was continued until the last day of the experiment. The test antioxidants, Co-Q_{10} and tea ACN's were administered orally at a dosage of 200mg/kg body weight (bwt) after every second day using a gavage needle. After lapse of the ten day period, animals were administered with melarsoprol intraperitoneally at a dosage of 3.6mg/kg bwt for four days and sacrificed 24 hours post the last dosage to obtain brain samples. Chloroform was used to euthanize the mice after which the brain was excised and cut into two halves. Brain samples used to determine histopathological changes were fixed in 10% formal saline while brain samples for biochemical analyses were snap frozen in dry ice and stored in liquid nitrogen until required.

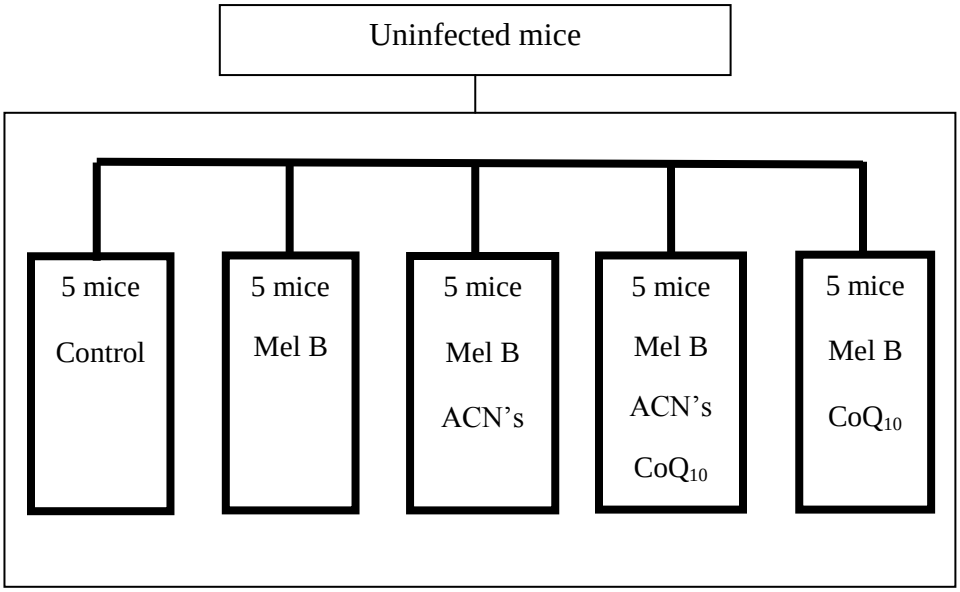


Figure 9: Schematic representation of the uninfected melarsoprol groups

3.4.1 Packed cell volume and body weight

At one week interval, blood was taken from each mouse by tail snip into 100µl microhaematocrit capillary tubes for PCV determination as per the method of Woo (1970). After blood collection, the capillary tubes were sealed with plasticin at one end and centrifuged in a haematocrit centrifuge (Hawksley H England) at 10,000 revolutions per minute (RPM) for 5 minutes. PCV was then read using a micro-haematocrit reader and expressed as a percentage (%) of the total blood volume. Body weight of each mouse was determined every two days using the analytical electronic balance (Mettler PM34, DoltaRange®).

3.4.2 Brain sample preparation

Snap-frozen whole brains were homogenized on ice water (4ºC) in 0.5mls of 0.25M sucrose, 5mM Hepes-Tris, pH 7.4, with protease inhibitor cocktail to a final concentration of 10% (w/v). The homogenates were aliquoted into 1.5ml microfuge tubes to avoid repeated freeze-thaw process and stored in liquid nitrogen until analysis.

3.4.3 Glutathione assay

Glutathione assay was performed as described by Rahman *et al.*, (2007) with slight modifications. A volume of 50µl of brain homogenates were mixed with 50µl solution containing sulphosalicylic acid (5% w/v) and 0.25mM ethylene diamine tetra acetic acid (EDTA) and the mixture centrifuged at 8000 x*g* for 10 minutes at 4^{o}C. A volume of 200µmol/l of GSH standard solution was prepared in 0.5% sulphosalicylic acid (SSA) and serial dilutions made

using the same solution (0.5% SSA) to final concentrations of 100, 50, 25, 12.5, 6.25, 3.13 and 1.56µmol/l. 5,5'-Dithiobis (2-nitrobenzoic acid (DTNB), also known as Ellman's reagent, was prepared by dissolving in 0.1M potassium phosphate buffer with 5mM EDTA disodium salt, pH 7.5) (KPE buffer) to a final concentration of 0.6mg/ml. A volume of 25µl of each standard were loaded on a 96-well microtitre plate to wells B–H in column 1, 2 and 3 followed by 25µl of the sample to the remaining wells in triplicate. To each well, 100µl of freshly prepared DTNB was then added and the absorbance measured at 405nm at intervals of 30seconds up to 2 minutes using a multi-detection microtitre plate reader.

3.4.4 Immunodot blots

The method of Pourmarat *et al.,* (1991) was employed to detect protein for the immunodot blot technique with slight modifications. A volume of 10µl of the homogenized brain samples were spotted onto 0.45µm nitrocellulose membrane (NC) and thereafter air dried at room temperature (RT) to fix IRP-1 protein onto the membrane. The strips were incubated in buffer containing 5% skimmed milk at RT for 1 hour to block the unoccupied sites on the NC membrane. The blots were then washed in wash buffer (0.05% Tween 20 in phosphate buffered saline (PBS) pH 7.4) and incubated for 2 hours in IRP-1 primary antibody solution (IgG-mouse monoclonal antibody, Santa Cruz Biotechnology) diluted in PBS buffer containing 1% skimmed milk at a ratio of 1:250. The blots were again washed three times in the washing buffer to remove the excess unreacted primary antibody, each wash lasting five minutes on an orbital shaker. Secondary antibody (anti-mouse IgG-conjugated horseradish peroxidase (HRPO), Santa Cruz Biotechnology), diluted on a ratio of 1:1,000 using PBS buffer containing 1% skimmed milk as diluent was added and left at room temperature (RT) for another 2 hours while shaking on an orbital shaker. Washing was then done five times before the visualization system was added. The development system consisted of 0.01% 3, 3'-diaminobenzidine (DAB) in PBS pH 7.4 containing 150µl of 30% hydrogen peroxide. Brown colour formed at the point of the immobilized IRP-1 protein onto which primary and secondary antibodies were bound. The reaction was then stopped by washing the blots using distilled water spiked with a few drops of 3M HCl just before a brown background formed.

3.4.5 Sodium dodecyl sulphate –poly acrylamide gel electrophoresis and western blotting

One volume of each homogenate was mixed with two volumes of 3x sample buffer (500mM Tris/HCl 6.8, 4.6% w/v SDS, 20% v/v glycerol, 10% v/v 2-mercaptoethanol and

0.004% bromophenol blue), boiled for 5 minutes and centrifuged for 5 seconds. The homogenates were then loaded onto a 1.5mm thick, 5% stacking gel and 10% sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) separating gel (appendix II). Electrophoresis was performed at a constant current of 20mA (Laemmli, 1970). The running buffer (pH 8.3) used contained 25mM Tris, 192mM glycine and 0.1% SDS. After electrophoresis, the gel was removed and left to equilibrate in the transfer buffer as the blotting membrane was precisely cut to fit the size of the gel. The nitrocellulose membrane was then prewetted in transfer buffer as the transfer apparatus was set. The gel and the blotting membrane were assembled into a sandwich along with two sheets of Whatman 3MM placed on either side. The gel and nitrocellulose sandwich was also sandwiched between two scotch-brite pads before being placed in a transfer blot cell apparatus (Hoeffer Scientific instruments, San Francisco, Model SE 600) filled with the transfer buffer. The separated proteins were electro transferred from the gel onto 0.45μm nitrocellulose membrane for twelve hours at a constant voltage of 25V at RT as described by Towbin *et al*. (1979).

Prior to immunodetection of the transferred proteins on the nitrocellulose paper, blocking was done by incubating the blotting membrane in 5% skimmed milk PBS (pH 7.4) for 1hour at RT on a rocking platform. This action was meant to prevent non specific binding of antibody to the blotting membrane. After blocking, the blots were washed in washing buffer (0.05% Tween 20 in PBS pH 7.4) and then incubated for 2 hours at RT in dilute solution of antibody in antibody diluent (1% skimmed milk in PBS, pH 7.4). The primary antibody (IRP-1 IgG-mouse monoclonal, Santa Cruz Biotechnology) was then removed, the blots washed in washing buffer three times for five minutes intervals and then incubated for 1 hour in secondary antibody (anti-mouse IgG-conjugated HRPO, Santa Cruz Biotechnology) diluted to a final concentration of 1:1000. The blots were again washed repeatedly at five minutes intervals for twenty five minutes and the bound antibodies visualized by addition of 0.01% 3, 3-DAB in PBS (pH 7.4) containing 150μl of 30% hydrogen peroxide. The reaction was stopped before a brown background developed by washing in distilled water spiked with few drops of 3M HCl. The blots were then scanned using Hewlett Packard (HP) scan jet 5590 and the relative levels of the proteins determined using pixel density analysis software (NIH, UN-SCAN-IT).

3.4.6 HPLC analysis of brain homogenates for detection of anthocyanins

A volume of 500µl methanol/ formic acid (99/1) was mixed with 500µl brain homogenate in a 1.5ml microfuge tube and the mixture centrifuged at a speed of 5000g for 10 minutes. One ml of the supernatant was then pipetted into separate tubes and HPLC analysis of the samples carried out as described in section 3.1.3.

3.5 Uninfected Antioxidant Groups

Experimental animals were divided into two groups and appropriate controls were used for this experiment (figure 10). The animals received antioxidants for fourteen days after every second day at a dosage of 200mg/kg using a gavage needle. Twenty four hours post the last dosage of antioxidants, experimental animals were sacrificed and brain samples preserved as described in section 3.7.

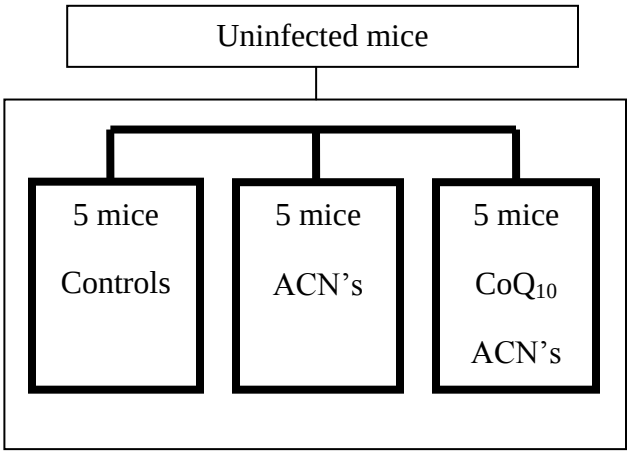


Figure 10: Schematic representation of the uninfected antioxidant groups

3.5.1 Packed cell volume and body weight

PCV levels and body weight parameters were carried out as described earlier in section 3.4.1.

3.5.2 Biochemical analysis

Glutathione assay and western blot analysis were carried out as described earlier in sections 3.4.3 and 3.4.5, respectively.

3.5.3 HPLC analysis of brain homogenates for detection of anthocyanins

HPLC analysis of the brain homogenates was carried out as described in section 3.4.6.

3.6 Infected Untreated Groups

Experimental animals in this group were subdivided into three sub-groups as presented in figure 11. Antioxidant administration commenced ten days prior to infection with trypanosomes and were administered as described in section 3.7. Animals in this group were projected to survive for 60 days post infection (dpi) after which they were sacrificed at termination of experiment and samples harvested and preserved as described in section 3.7.

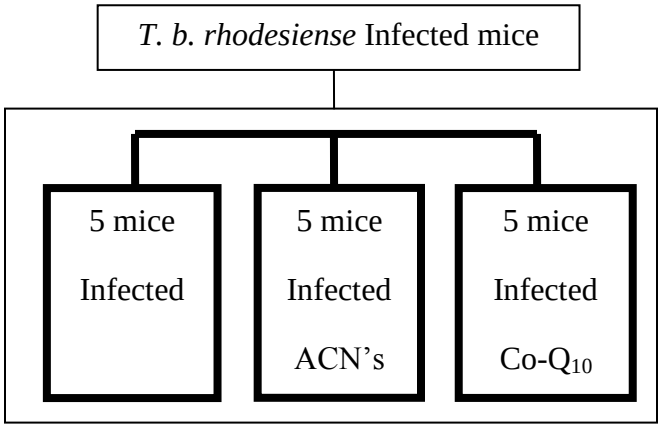


Figure 11: Schematic representation of infected untreated groups

3.6.1 Immunosuppression of donor mice and trypanosome expansion

Three female Swiss white mice were used to expand the trypanosome isolate *Trypanosoma brucei rhodesiense* KETRI 2537, a derivative of EATRO 1989, isolated from a patient in Uganda by direct inoculation of blood and lymph node aspirates into a monkey and later cryopreserved. Donor mice were immunosuppressed using cyclophosphamide at a dose of 8.3mg/kg of body weight injected intraperitoneally daily for three days before infection. Cryopreserved *Trypanosoma brucei rhodesiense* isolate KETRI 2537 was obtained in small capillary tubes of 20µl each from Trypanosomiasis Research Centre (TRC) trypanosome bank and left to thaw allowing time for the parasites to adapt to the room temperatures before infection. Phosphate buffered saline (PBS), pH 8.0 containing 1.5% (w/v) glucose (PSG) was used to dilute the parasites in the capillary tube and viability of the parasites was checked by putting a drop of its contents on a slide, covered with a cover slip and viewing at ×400 magnification by phase-contrast microscopy to check for trypanosome motility before injecting 0.2ml of the dilution to each immunosuppressed donor mouse.

3.6.2 Infection of experimental animals

Follow up of the parasitaemia on the donor mice was done by taking a drop of blood from the tail and a wet smear prepared for examination at ×400 magnification by phase-contrast microscopy. On the first peak of parasitaemia a few drops of blood were taken from the tail of the donor mice and mixed with 2ml of EDTA saline-glucose (ESG) buffer in a bijou bottle (Kagira *et al*., 2006). The number of trypanosomes was estimated using an improved Neubauer chamber viewed under the microscope at x400 magnification. This blood solution was then diluted 10 times with ESG buffer pH 8.0 using a leukopipette to a final concentration of $5.0x10^4$ trypanosomes/ml. To each experimental mouse, 0.2ml of this dilution was inoculated intraperitoneally so that each mouse received 10^4 trypanosomes (Gichuki and Brun, 1999).

3.6.3 Parasitaemia levels

Parasitaemia levels for each infected mouse were determined daily for the first seven dpi to determine the prepatent period and thereafter at two days interval. Parasitaemia was estimated by microscopic examination of a blood smear collected by tail snip using the "Rapid Matching" method of Herbert and Lumsden (1976). Briefly, the method involves matching the microscopic appearance of a wet film of infected blood with reference charts which represent different concentrations of trypanosomes. Each reference chart and table is assigned a logarithm value which when converted to antilog provides the absolute number of trypanosomes per ml of blood. When parasites could not be seen by direct microscopy of wet film, the buffy coat technique was employed (Murray *et al*., 1977). This technique involves preparation of a buffy coat in a microhaematocrit capillary tube which is centrifuged in a similar fashion as for measurement of PCV. The buffy coat/plasma interface is expressed onto a microscope slide and examined microscopically for presence of parasites using a dark-background or phase contrast illumination.

3.6.4 Packed cell volume and body weight

PCV levels and body weight parameters were determined as described in section 3.4.1

3.6.5 Biochemical analysis

Glutathione assays and western blot analysis were carried out as described in section 3.4.3 and 3.4.5, respectively.

3.6.6 Evaluation of pathological changes

Following sacrifice of the mice**,** brains were removed from the cranial cavity, excised into two halves and the forebrain fixed in 10% formal saline before being embedded in paraffin

wax. The paraffin blocks were then sectioned at a thickness of 3 to 4µm. The sections were then stained using haematoxylin and eosin stain (H&E) and examined histologically under a light microscope to evaluate the effects of Co-Q_{10} or Kenyan purple tea ACN's on the development of brain inflammation following infection with *T. b. rhodesiense* strain KETRI 2537 and/or post melarsoprol treatment.

3.7 Infected PTRE Groups

Experimental animals were divided into four sub-groups as presented in figure 12. Expansion of the trypanosome stock and immunosuppression of donor mice was done as described in section 3.6.1. Administration of the antioxidants commenced ten days prior to infection to allow concentration of the antioxidants in the mice tissues including the brain and was continued until the last day of the experiment. At the end of the ten days, animals were inoculated intraperitoneally with approximately 10^4 trypanosomes diluted with ESG buffer pH 8.0 and parasitaemia estimation carried out as described in section 3.6.2. The infection was allowed to progress with or without antioxidant intervention until 21 dpi when animals were treated sub-curatively with 5mg/kg diminazene aceturate (DA) daily for three days. Treatment with DA clears the parasites from the extravascular compartment but leaves residual trypanosomes in the CNS (Jennings and Gray, 1983) and thus is used to induce severe late CNS infection that closely mirrors PTRE in human subjects. Thereafter mice were monitored for relapse of parasitaemia after which they were treated with melarsoprol at a dosage of 3.6mg/kg for four days and sacrificed 24 hours post the last dosage to obtain brain samples. Sacrifice of animals and preservation of samples was established as described in section 3.4.

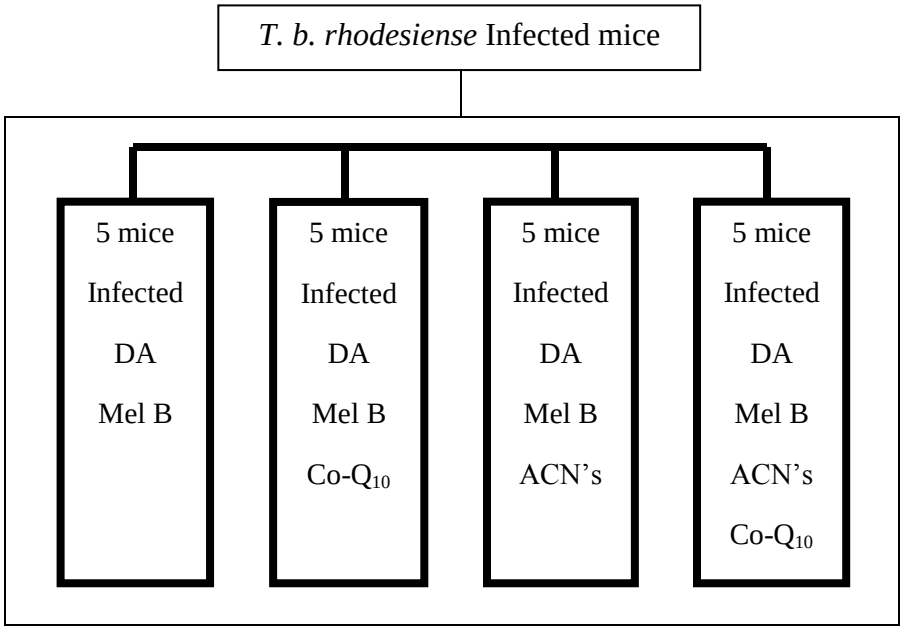


Figure 12: Schematic representation of infected post treatment reactive encephalopathy groups.

3.7.1 Parasitaemia levels

Parasitaemia levels were determined as described earlier in section 3.6.3.

3.7.2 Packed cell volume and body weight

PCV levels and body weight parameters were determined as described in section 3.4.1.

3.7.3 Biochemical analysis

Glutathione assay and Western blot analysis were carried out as described in section 3.4.3 and 3.4.5, respectively.

3.7.4 Evaluation of pathological changes

Following mice sacrifice, brains were excised into two halves and the forebrain processed as described in section 3.6.6. The sections were examined histologically under a light microscope to evaluate the effects of Co-Q_{10} and/or Kenyan purple tea ACN's on the development of brain inflammation following infection with *T. b. rhodesiense* strain KETRI 2537 and treatment with diminazene aceturate at 21 dpi to exacerbate the inflammatory reaction.

3.8 Data Analysis

Data was analyzed using Prism Graph pad version 5.0 and a *P* value of <0.05 considered to be statistically significant. Significance of difference between means for PCV, prepatent and relapse periods, parasitaemia, glutathione and aconitase (1RP) 1 was determined by one way ANOVA and Tukey post hoc test was performed to evaluate differences among group means.

Graphs were plotted to show the trend of the various response variables. The data are expressed as the mean ± standard error of the mean.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Purple Tea Anthocyanin Profile

Following lyophilization of the ACN's extracts, a free flowing powder bright red in colour, with a characteristic smell of berries was produced (figure 13). ACN's profiling of Kenyan purple tea variety TRFK 306 using HPLC revealed presence of anthocyanidins; cyanidin, peonidin, pelargonidin, delphinidin and malvidin (figure 13). From the anthocyanidins profile, cyanidin was highest in concentration (1755.60µg/ml) and delphinidin was least (122.85µg/ml) (Table 1).

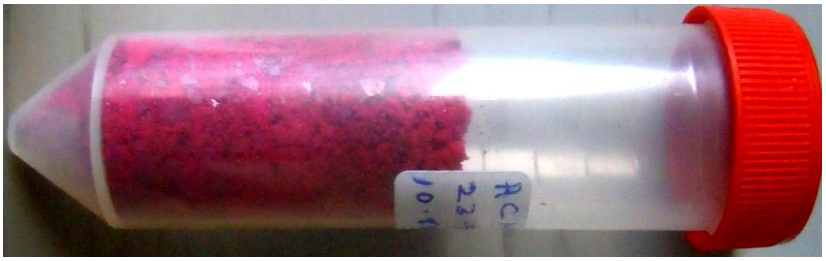


Figure 13: Anthocyanins extracted from Kenyan purple tea variety TRFK 306 and lyophilized to produce a free flowing powder

Table 1: Concentration of anthocyanins (µg/ml) estimated using HPLC in non-aerated (green) tea derived from Kenyan purple leaf colored variety TRFK 306

Individual anthocyanins/anthocyanidins	**Concentrations (µg/ml)**
Cyanidin-3-O-Galactoside	139.25
Cyanidin-3-O-Glucoside	50.26
Delphinidin	122.85
Cyanidin	1755.60
Pelargonidin	840.08
Peonidin	371.36
Malvidin	304.83
Total anthocyanin content	3584.23

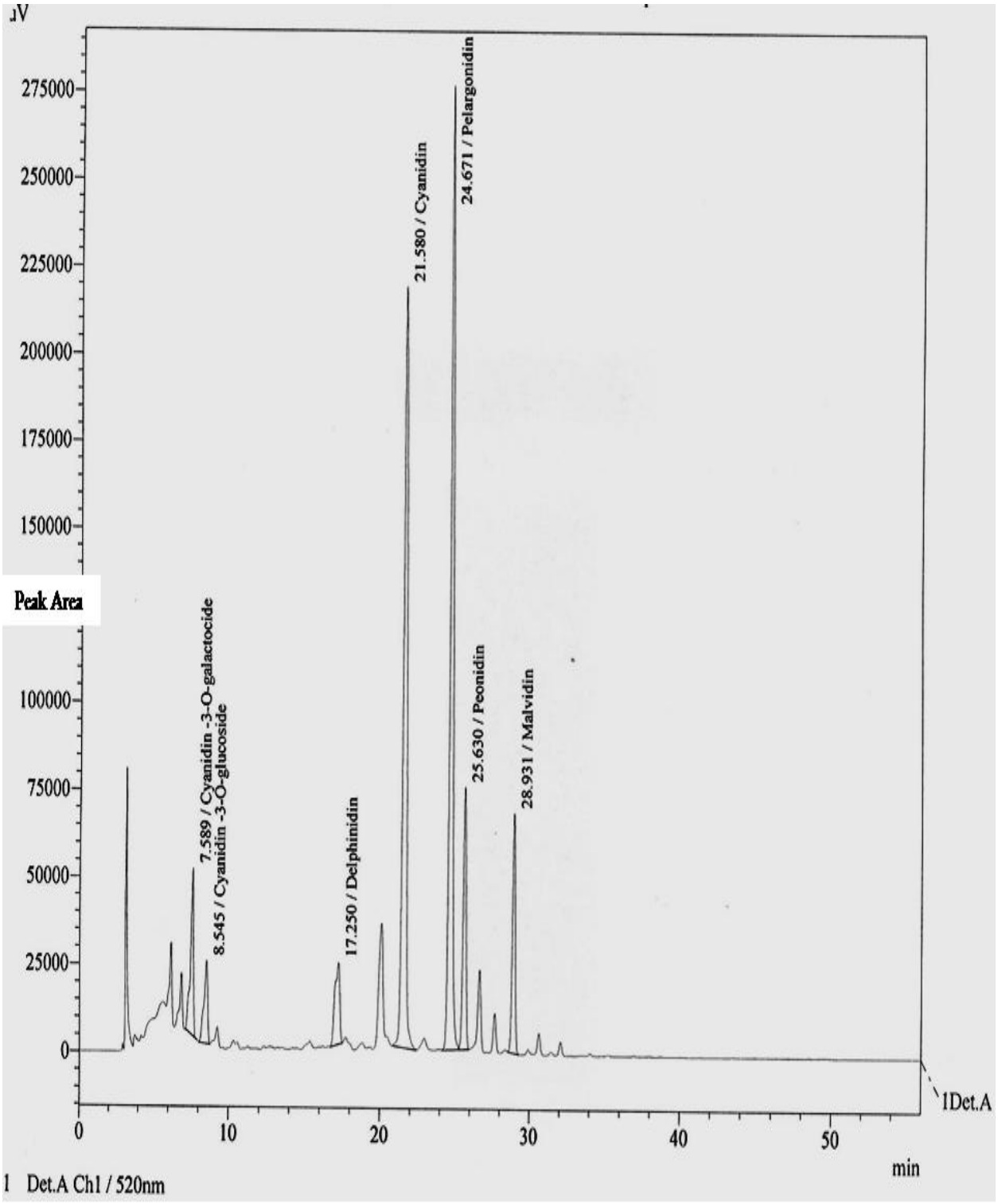


Figure 14: A representative HPLC chromatogram of anthocyanin extracts from Kenyan purple tea variety TRFK 306.

4.2 Anthocyanin in Brain Tissue

HPLC analysis of ACN's in brain tissue was done as described out to identify intact and/or metabolized ACN's. ACN's metabolites, vividly absent from animals not supplemented with ACN's and having very close retention times to individual intact ACN's (Table 2) were detected in brain tissue of animals from the various ACN's groups (figure 15).

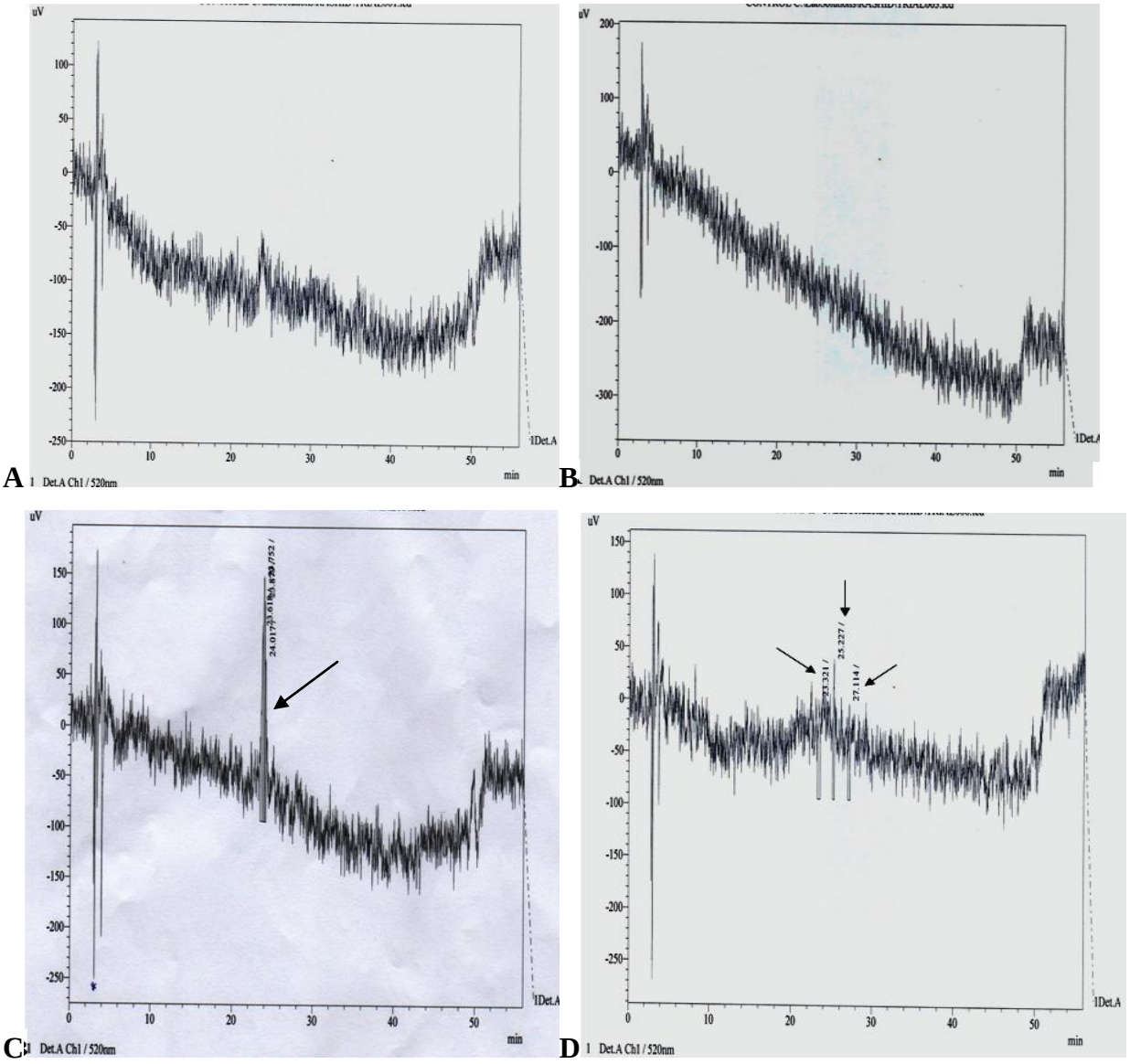


Figure 15: Representative HPLC chromatograms of brain homogenates from; untreated mice (A and B), animal challenged with melarsoprol and treated with ACN's (C) and animal supplemented with ACN's and Co-Q_{10} in the absence of melarsoprol (D). Chromatogram C and D show presence of possible ACN's metabolites in the brain tissue indicated by arrows.

Table 2: Retention times in minutes of the detected metabolites against intact individual anthocyanins

Chromatogram C- Retention times in minutes of the detected metabolites	**Possible metabolites**
23.618 23.752 23.879	Cyanidin-21.580 Pelargonidin- 24.671 Peonidin-25.630
24.017	Pelargonidin- 24.671 Peonidin-25.630
Chromatogram D- Retention times in minutes of the detected metabolites	**Possible metabolites**
23.321	Cyanidin-21.580 Pelargonidin-24.671
25.227	Pelargonidin-24.671 Peonidin-25.630
27.114	Malvidin-28.931

4.3 Effects of Antioxidants on Uninfected Mice Treated with Melarsoprol

4.3.1 Clinical symptoms and survival

Animals supplemented with Co-Q_{10} were marked with hyperactivity from the onset of Co-Q_{10} supplementation to the last day of the experiment. One animal supplemented with both test antioxidants died 6 days after start of antioxidant administration. This animals lost weight consistently from start of the experiment to the last day.

4.3.2 Packed cell volume and body weight

Figure 16 shows the effects of melarsoprol on the PCV levels of uninfected mice with or without antioxidants supplementation. All groups recorded a drop in the PCV levels following melarsoprol treatment. However, the level of drop in PCV was dependent on the antioxidant(s) co-administered with melarsoprol. The ACN's and melarsoprol treated group had the highest percentage drop in PCV of 11.5%, dropping from 59.2±1.24% to 52.4±0.6%. Animals challenged with melarsoprol and supplemented with Co-Q_{10} only had the lowest percentage drop

(2.9%) in PCV as compared to other animals challenged with the trypanocidal, dropping from 56±1.52% to 54.4±2.46%. The beneficial effects of Co-Q_{10} in protecting against drop in PCV level following melarsoprol treatment was evident even in the presence of ACN's, where the PCV level dropped from 57.8±0.86% to 53±0.91%**.** This translated to a percentage drop of 8.3% as compared to 11.5% in ACN's only group. Untreated animals recorded a marginal increase in the PCV level rising from 57.4±0.51% to 58.4±2.50% by the end of the experiment.

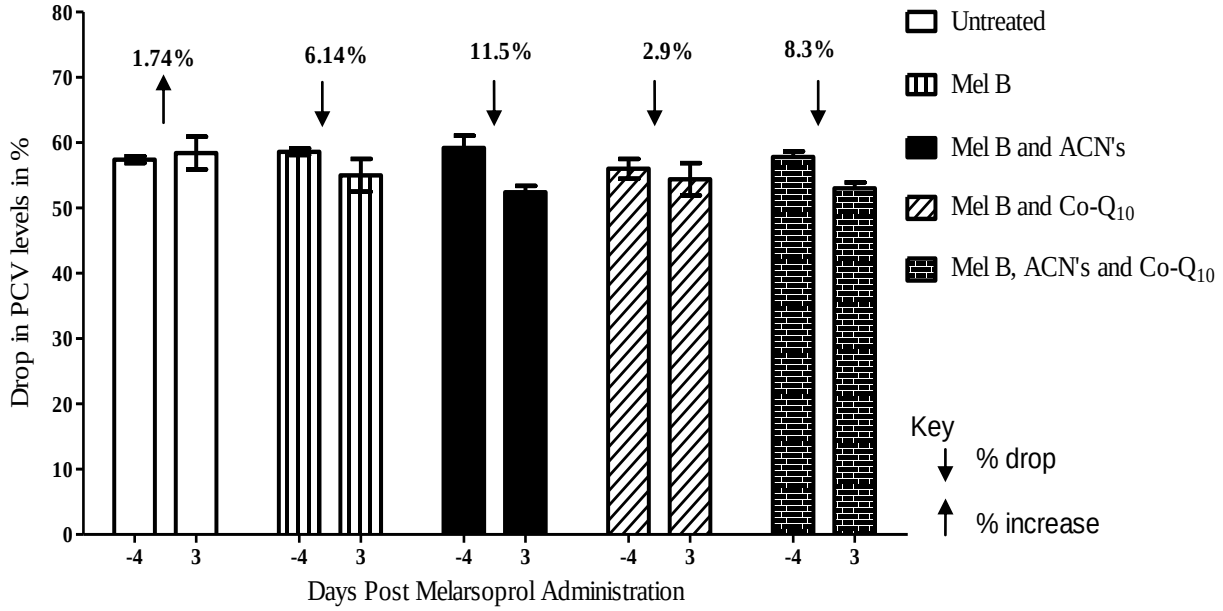


Figure 16: Change in PCV levels of uninfected mice challenged with melarsoprol and supplemented with ACN's, ACN's and Co-Q_{10} or water only (negative controls). Data are means± standard error of the means (SEM).

Animals treated with ACN's only and a combination of ACN's and Co-Q_{10} registered a sharp decrease in body weight following start of antioxidant administration and continued consistently for seven days declining from 23.48±0.65g and 26.76±0.77g to 19.60± 0.44g and 23.3±1.67g respectively, before stabilizing by 8 days post antioxidant administration (figure 17). A marginal increase was then observed in the ACN's group reaching 20.2±0.53g by 14 day post antioxidant administration. No major changes in body weight were observed after animals were challenged with the trypanocidal melarsoprol. However, animals supplemented with both test antioxidants had significantly lower body weights ($p<0.05$) than other groups by the end of the experiment that lasted for fifteen days.

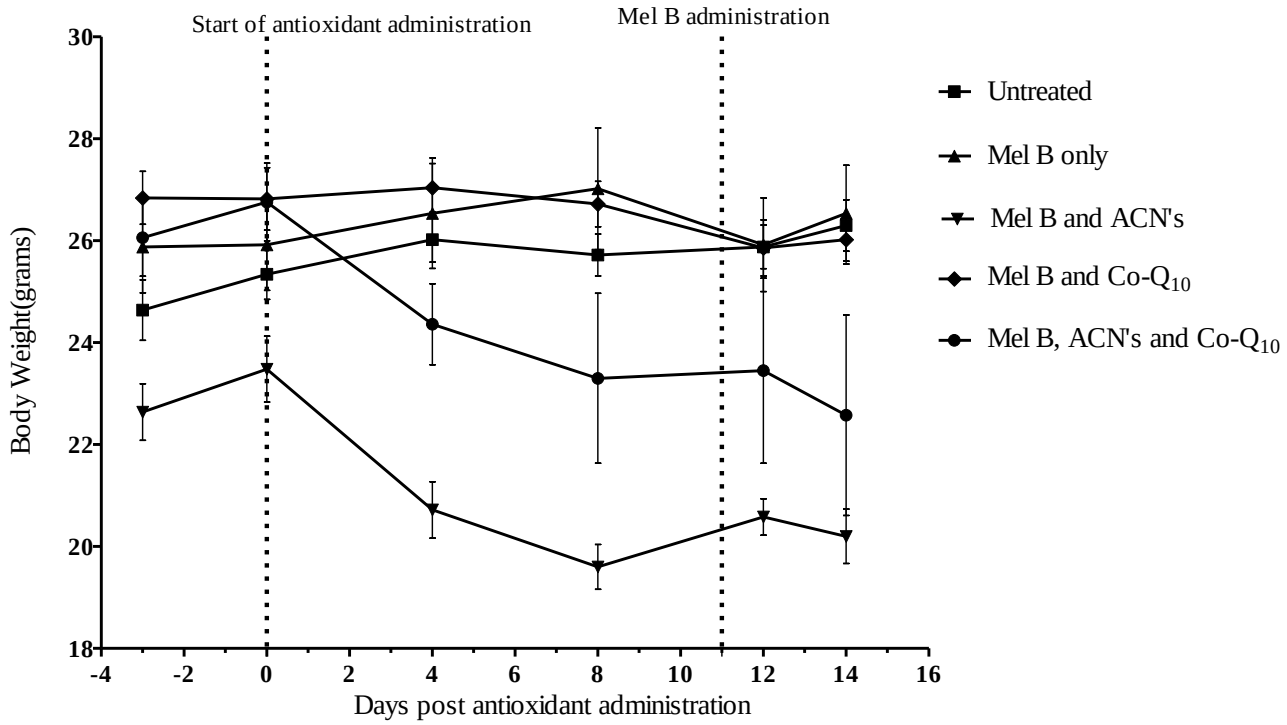


Figure 17: Changes in body weight of uninfected mice challenged with melarsoprol and supplemented with ACN's, ACN's and Co-Q_{10} or water only.

4.3.3 Glutathione assay

Levels of endogenous total GSH levels were determined in brain tissue of mice challenged with the organic arsenical melarsoprol in uninfected mice (figure 18). The experimental animals were supplemented with test antioxidants or water in the placebo group. Untreated animals had a GSH concentration of 1.26±0.12μM while animals challenged with melarsoprol had much lower GSH concentrations reaching 1.04±0.08μM. Test antioxidants employed in this experiment were able to reverse the adverse effect of melarsoprol on GSH, significantly raising the levels of this endogenous thiol to 2.72±0.20μM and 3.02±0.25μM in ACN's and Co-Q_{10} groups, respectively ($p<0.05$) (figure 16). However, the antagonistic effects of the two antioxidants were observed as in PCV levels, GSH levels in this group being significantly lower (1.905±0.098 μM) than in the ACN's only or Co-Q_{10} only groups.

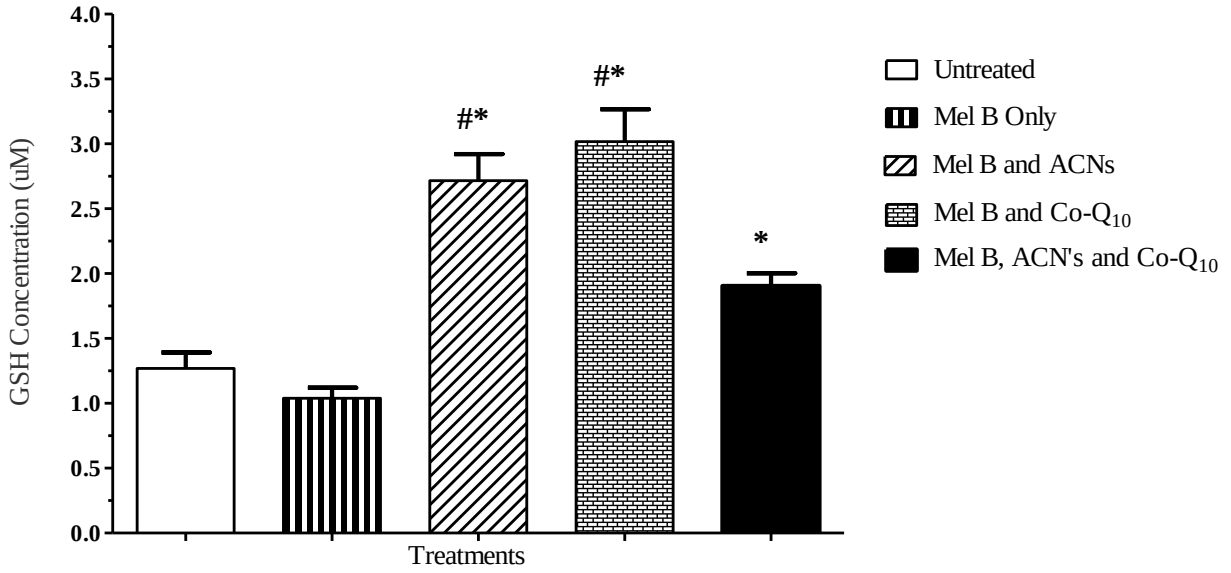


Figure 18: Total GSH levels in uninfected mice challenged with melarsoprol and supplemented with ACN's, Co-Q_{10}, ACN's and Co-Q_{10} or water only. $^{\#}p<0.05$, statistically significant versus untreated group. *$p<0.05$, statistically significant versus melarsoprol only group.

4.3.4 Aconitase (IRP) 1 levels

Prior to western blotting, immunodot blots were conducted for initial screening of the test antibodies and samples. Development of the blots confirmed the specificity of the antibodies and presence of the test protein in the samples (figure 19).

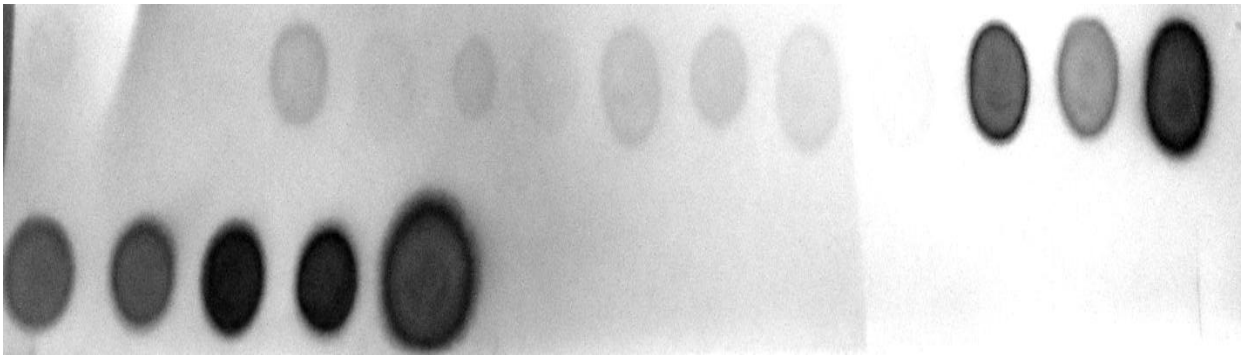
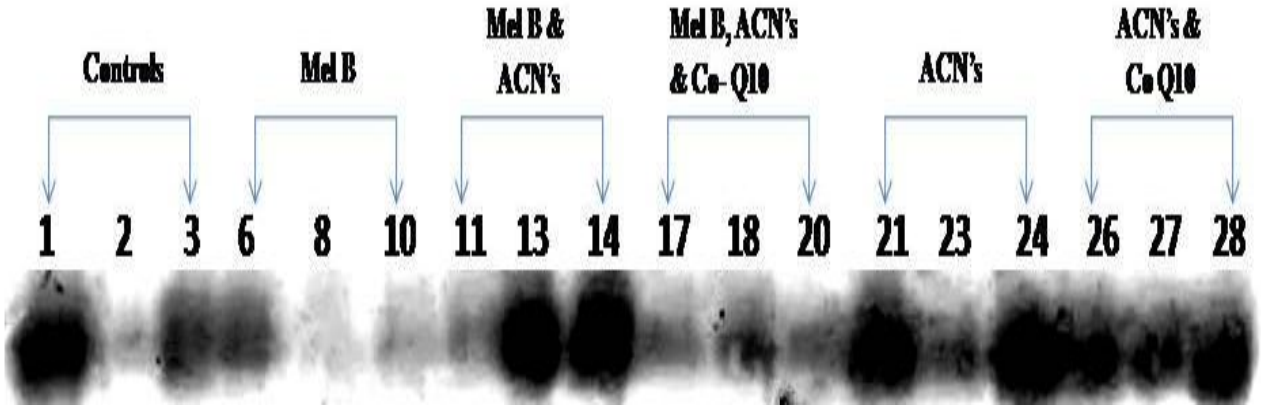


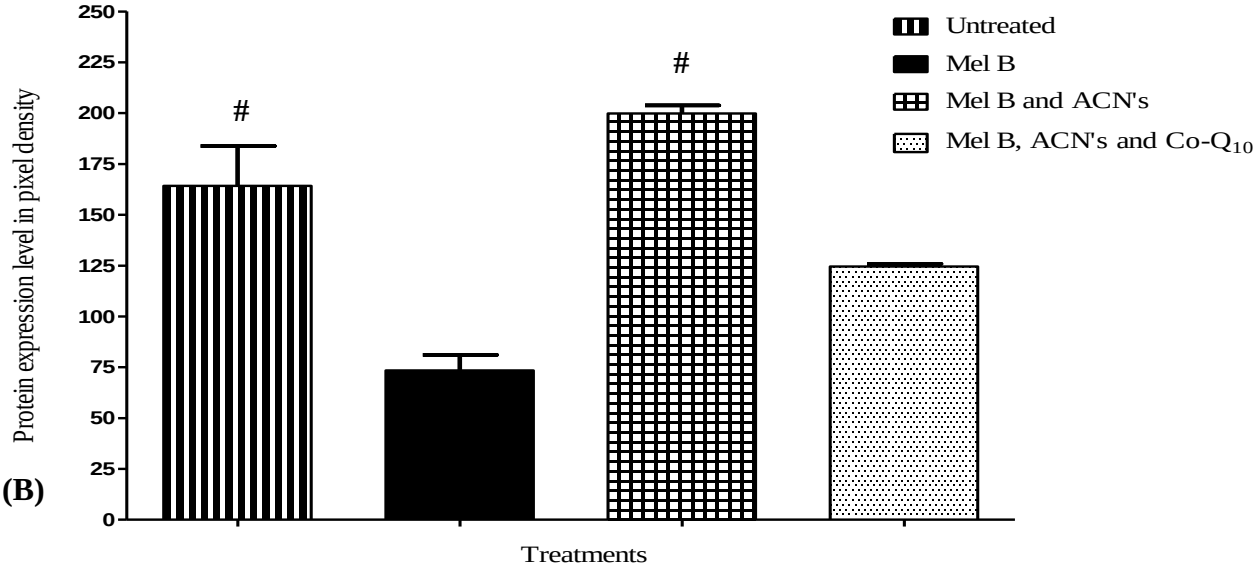
Figure 19: Immunodot blots processed using IRP-1 primary antibody and probed using anti-mouse secondary antibody.

Melarsoprol significantly reduced IRP-1 concentration ($p<0.05$) in the brain and this could be an important contributory factor to the molecular mechanisms that lead to the high levels of toxicity due to this trypanocidal drug. However, purple tea anthocyanins were able to ameliorate this effect significantly enhancing the concentration of this endogenous antioxidant

protein even beyond normal levels (figures 19A and 19B). A combination of the two antioxidants also partially raised the IRP-1 concentration though not significantly. Moreover, the difference between melarsoprol and ACN's group and animals challenged with melarsoprol and supplemented with both test antioxidants was statistically significantly different at $p<0.05$.



(A)



(B)

Figure 20: Immunoblotting for aconitase-1 expression**.** (A) The photomicrograph shows a representative of IRP-1 protein expression in three independent homogenates from brains of uninfected mice; (i) challenged with melarsoprol and treated with antioxidants or water for the placebo group (ii) supplemented with antioxidants and/or water in the absence of melarsoprol. (B) Pixel intensities of brain IRP-1 protein in uninfected mice challenged with melarsoprol and treated with ACN's, ACN's and Co-Q_{10} or water only. $^{\#}p<0.05$, statistically significant versus melarsoprol only group.

4.4 Effects of Kenyan Purple Tea Anthocyanins and/or Co-enzyme Q_{10} on Mice

4.4.1 Clinical symptoms and survival

One animal supplemented with tea ACN's and Co-Q_{10} died 9 days post start of antioxidant administration. No clinical signs were detectable in the ACN's only groups signifying that the tea polyphenols were well tolerated in the experimental animals.

4.4.2 Packed cell volume and body weight

PCV levels and body weight parameters were measured prior to the start of the experiment to establish baseline data (Table 3). There was a gradual increase in PCV levels of the untreated animals throughout the experimental period of 15 days rising from 55.2±0.49% to 58.4±2.50%. Experimental animals supplemented with ACN's only and ACN's and Co-Q_{10} showed a steady increase in PCV rising from 53.4±1.03% and 53.4±1.36% three days prior to antioxidant administration to 58.6±1.08% and 59.25±2.03% on the sixth day post start of antioxidant administration (DPSAA), respectively. This was followed by a steady decrease in PCV levels to the last day of the experiment, reaching 53.2±1.93% and 55±0.71% in ACN's only and ACN's and Co-Q_{10} groups respectively. However, these differences were not statistically significant ($p>0.05$).

Table 3: Changes in PCV levels of mice supplemented with ACN's, ACN's and Co-Q_{10} or water only.

Days post start of antioxidant administration	PCV levels in %		
	Controls	ACN's	ACN's and Co-Q_{10}
-3	55.2 ±0.49	53.4±1.03	53.40±1.36
6	57.4±0.51	58.6±1.08	59.25±2.03
12	58.4±2.50	53.2±1.93	55.00±0.71

Mice receiving both antioxidants showed a significant decrease in mean body weight during the experimental period ($p>0.05$) (figure 21). The decrease commenced immediately after start of antioxidant administration, falling from 26.76±0.77g to 23.30±1.67g by the seventh DPSAA after which a marginal increase was observed reaching 23.45±1.82g. Animals supplemented with ACN's lost weight consistently from the first DPSAA to the last day of the

experiment, dropping from 27.88±1.60g to 25.76±1.69g. Untreated animals registered an unsteady but gradual increase in mean body weight rising from 24.64±0.59g to 26.3±0.50g by the end of the experimental period. However, no significant differences in mean body weight were recorded between untreated animals and animals supplemented with ACN' ($p>0.05$).

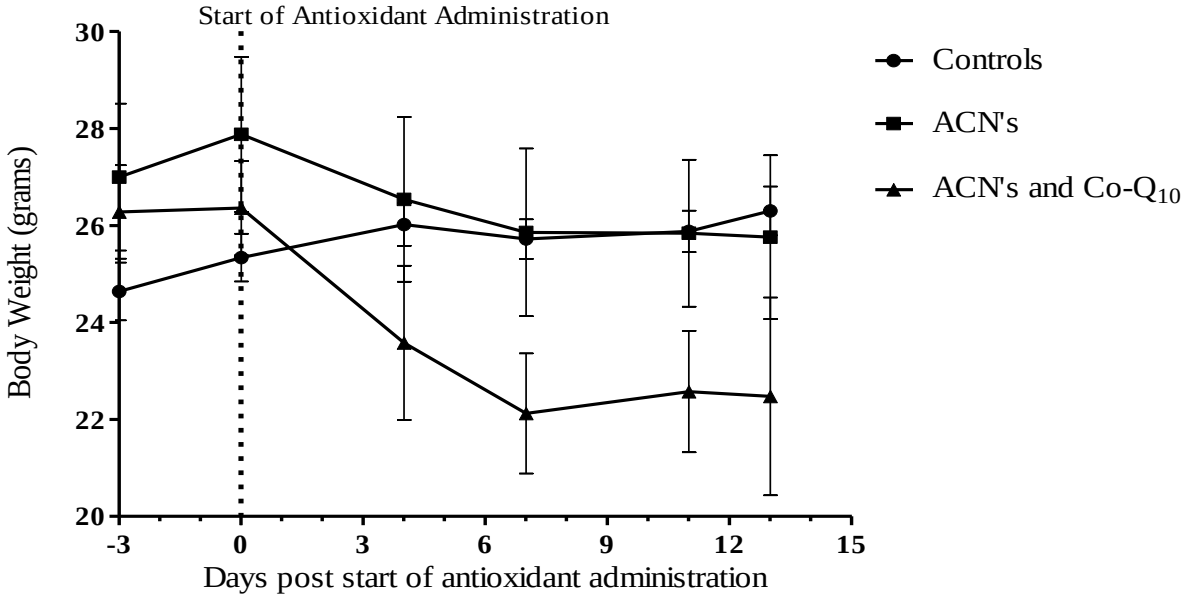


Figure 21: Changes in body weight of uninfected mice supplemented with ACN's, ACN's and Co-Q_{10} or water only.

4.4.3 Glutathione assay

Levels of GSH, the largest non-protein thiol in the cell responsible for quenching reactive oxygen species and other free radicals, were determined in brain tissue of mice supplemented with either one or both antioxidant supplements (figure 22). Results indicate that supplementing experimental animals with Kenyan purple tea anthocyanins significantly boosted the levels of endogenous total GSH levels in the brain tissue (2.22±0.18μM) when compared to the controls (1.27±0.12μM) ($p=0.0006$). However, when both test antioxidants were administered to the experimental animals, there was a decline in total GSH decreasing the amounts below even the levels recorded in healthy animals not supplemented with antioxidants (0.97±0.06μM).

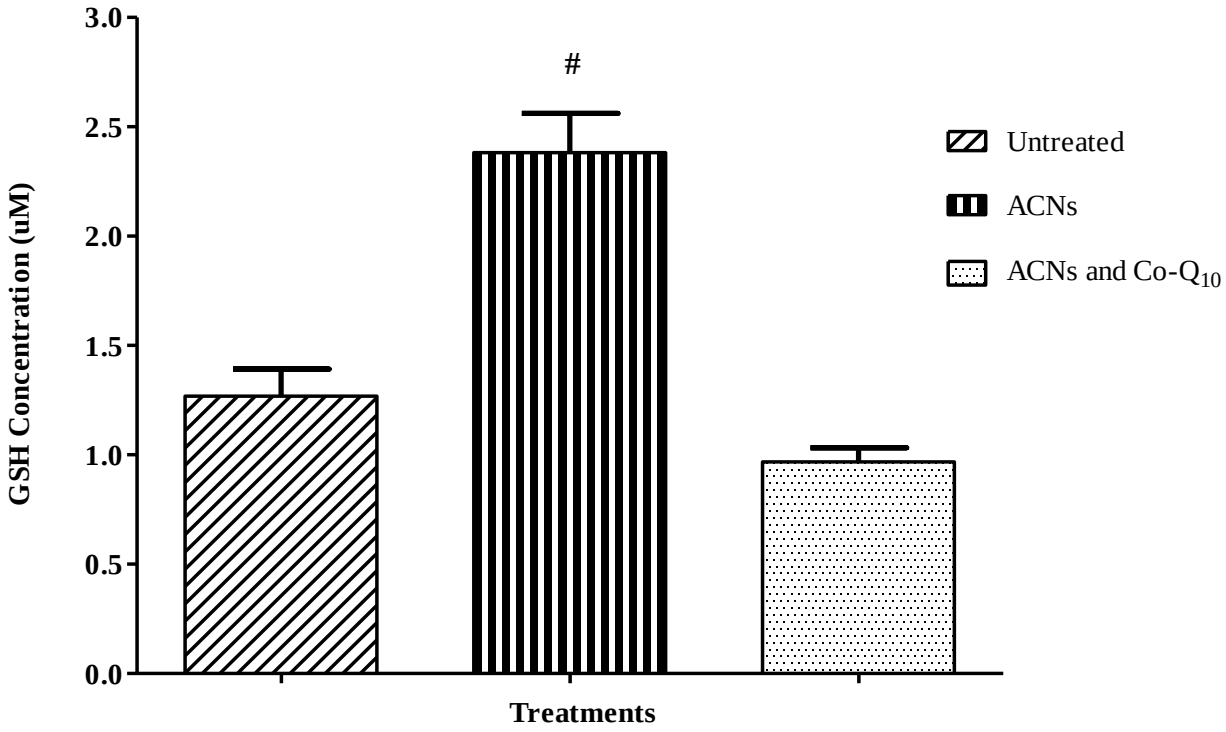


Figure 22: Total brain GSH levels in uninfected mice supplemented with ACN's, ACN's and Co-Q_{10} or water only. $^{\#}p<0.05$, statistically significant versus untreated group.

4.4.4 Aconitase (IRP) 1 levels

Purple tea anthocyanins marginally up-regulated IRP-1 protein, though not significantly ($p>0.05$) (figures 19A and 23). However, combination of the two antioxidants eliminated the positive effects observed when tea anthocyanins were administered in isolation.

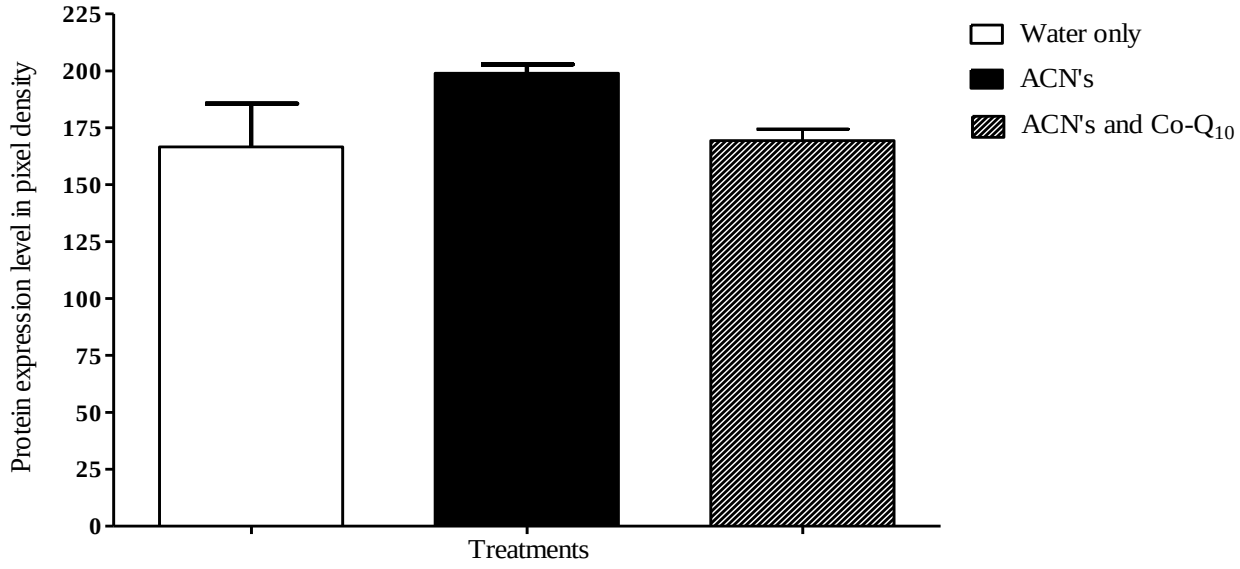


Figure 23: Differences in expression of brain IRP-1 protein in uninfected mice supplemented with ACN's, ACN's and Co-Q_{10} or water only.

4.5 Effects of Antioxidants on Infected Terminal Groups

4.5.1 Clinical symptoms and survival

Clinical symptoms of all *T. b. rhodesiense* KETRI strain 2537 infected mice included weight loss, enlarged abdomen, raised hair coats, reduced activity and hind limb paralysis during the meningo-encephalitic phase. On termination, hepatosplenomegally was evident. Infected terminal groups were expected to survive for 60 dpi but this was not the case. These animals developed severe illness by 40 dpi and this was followed by loss of 4 animals on 45, 47 and 49 dpi having parasite densities of antilog 8.7, 8.4, and 9.0 trypanosomes/ml. Animals that died were distributed in the groups as follows; 1 animal from infected controls, 2 from infected animals supplemented with ACN's and Co-Q_{10} and 1 from infected animals supplemented with ACN's only. The remaining infected animals were sacrificed at 51 dpi before becoming moribund.

4.5.2 Parasitaemia levels

Parasite appearance in peripheral blood commenced on 4 dpi in all groups and no significant differences ($p>0.05$) in the pre-patent period was observed (Table 3). However, the infected group supplemented with ACN's had the highest pre-patent period (PPP), with one animal in this group being positive for parasites on the 7 dpi.

Table 4: Values (Mean ± SEM) of pre-patent period in days in mice infected with *Trypanosoma brucei rhodesiense.*

Mice Group	Mean Pre-patent period (days) ± SEM	Range
Infected controls	4.75±0.25[a]	4-5 days
Infected and anthocyanins	5.00±0.55[a]	4-7 days
Infected and Co-Q_{10}	4.50±0.22[a]	4-5 days

Treatment marked with the same letters are not significantly different at $p<0.05$

First peak of parasitaemia occurred on the 6th dpi in infected untreated groups (antilog 8.1 trypanosomes/ml) and on the 7th dpi in both infected antioxidant groups, having mean parasite densities of antilog 6.75 and 7.34 trypanosomes/ml in infected Co-Q_{10} and infected ACN's groups respectively. This was followed by a gradual drop in trypanosome levels and then a consistent increase until the end of the experiment in all animal groups (figure 24). Infected untreated group reached a maximum parasitaemia of antilog 8.7 trypanosomes/ml while both antioxidant groups reached maximum parasitaemia of antilog 9.0 trypanosomes/ml. However, no significant differences in parasitaemia were observed between the three animal groups ($p>0.05$). Parasitaemic waves were however evident throughout the experiment.

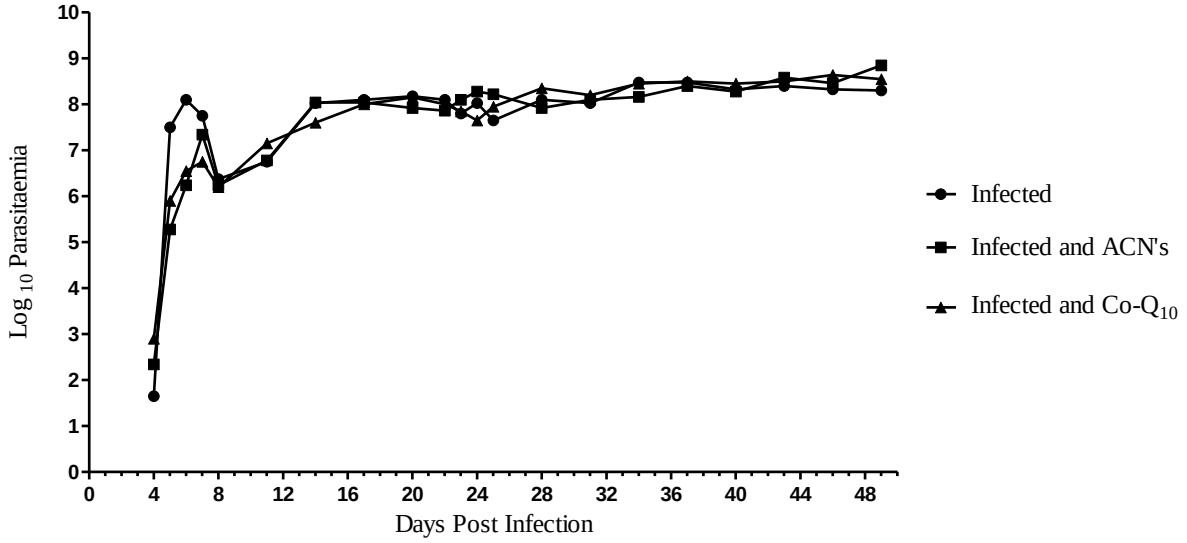


Figure 24: Parasitaemia of *T. b. rhodesiense* infected mice supplemented with ACN's, Co-Q_{10} or water only.

4.5.3 Packed cell volume and body weight

Anemia, observed by the drop in the PCV levels was evident immediately after infection and progressed consistently until the second week post infection, coinciding with the first wave of parasitaemia (figure 25). Following infection, PCV levels dropped from 55.4±0.98%, 55.2±0.8% and 51.17±1.38% to 50.2±2.22%, 46.6±1.99% and 49.0±1.13% in infected untreated, infected ACN's and infected Co-Q_{10} groups, respectively. The drop in PCV was significantly higher ($p=0.0059$) in infected controls and infected animals supplemented with ACN's when compared to infected animals supplemented with Co-Q_{10} (figure 26). A general increase with periodic fluctuations was then observed in infected ACN's group up to the fifth week post infection. This was followed by a general decrease observed in all groups reaching 45.8±2.82%, 47.6±2.05% and 43.67±1.28% in infected untreated, infected ACN's and infected Co-Q_{10} groups respectively by the last day of the experiment.

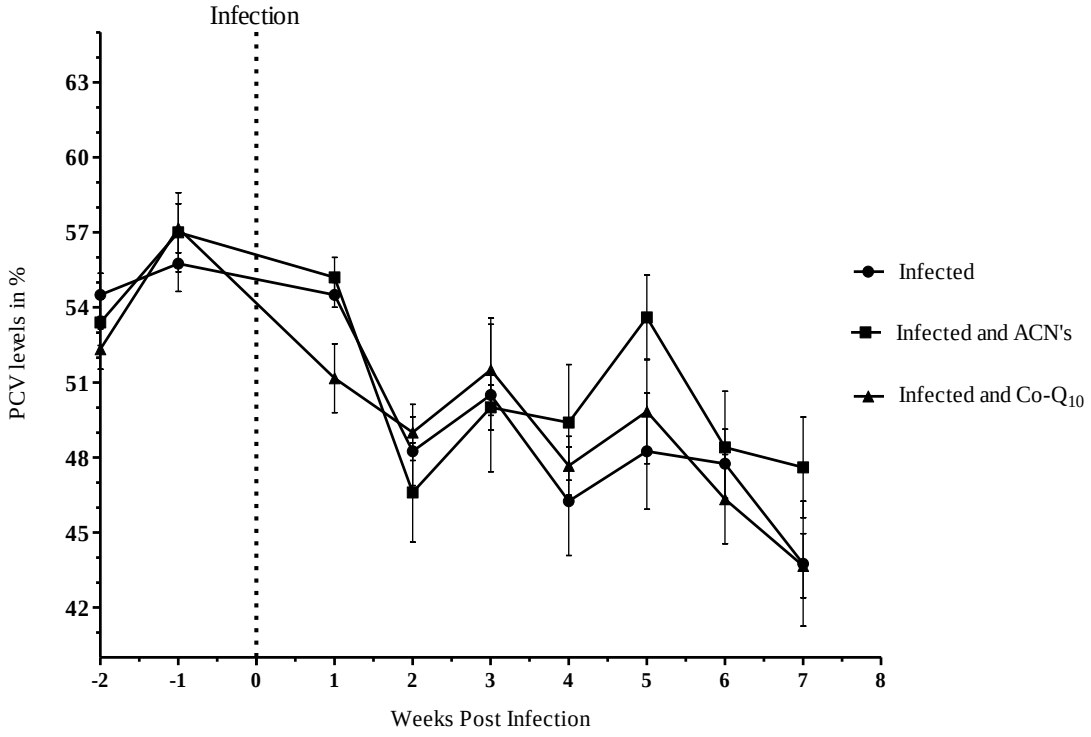


Figure 25: Changes in PCV levels (means±S.E.M) of *T. b. rhodesiense* infected mice supplemented with ACN's, Co-Q_{10} or water only.

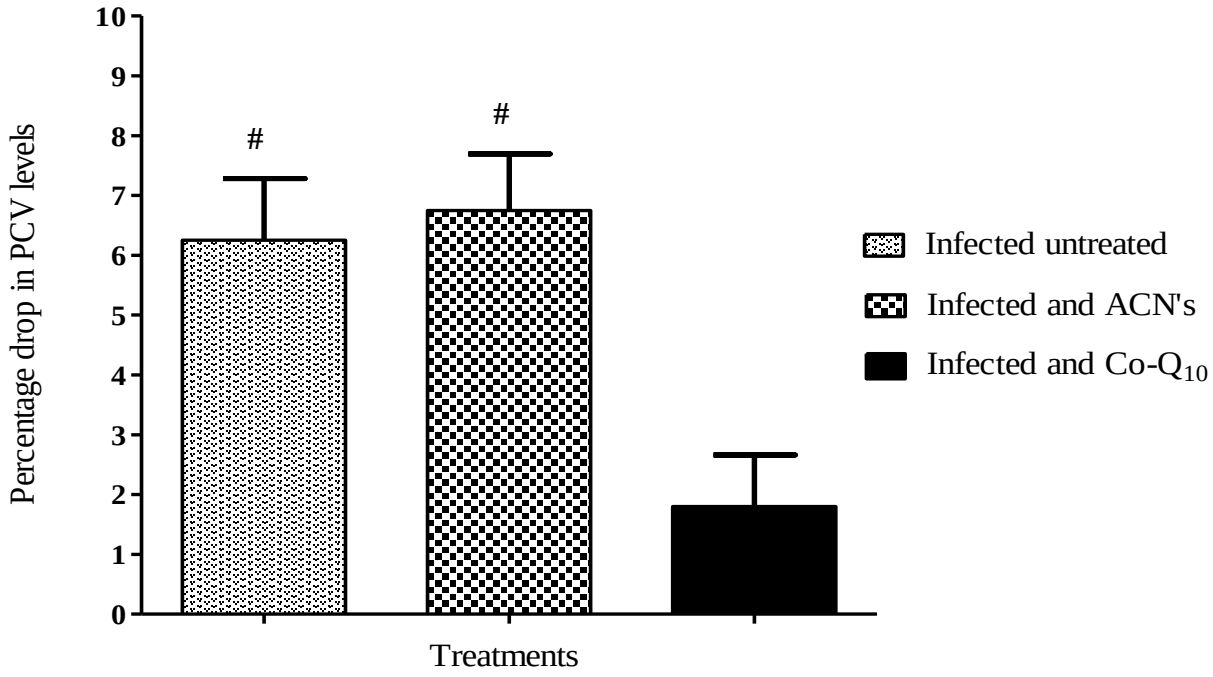


Figure 26: Percentage drop in PCV levels 7 dpi in *T. b. rhodesiense* infected mice supplemented with ACN's, Co-Q_{10} or water only. $^{\#}p<0.05$, statistically significant versus infected Co-Q_{10} group.

In relation to body weight, similar effects were observed between uninfected and infected animals supplemented with antioxidants (figure 27). Immediately following start of antioxidant administration there was an initial reduction in body weight, dropping from 26.62±1.91g and 24.07±1.66g to 24.56±1.66g and 23.04±0.60g in infected ACN's and infected Co-Q_{10} groups, respectively. This was followed by a steady increase observed up to the last day of the experiment, reaching 27.50±2.17g and 27.95±1.70g in infected ACN's and infected Co-Q_{10} groups, respectively. All infected untreated animals showed weight gain following infection consistently up to the 29th dpi, rising from 26.26±0.97g to 29.24±1.13g during this period. These animals then lost weight continuously to the last day of the experiment, dropping from 29.24±1.13g to 27.28g.

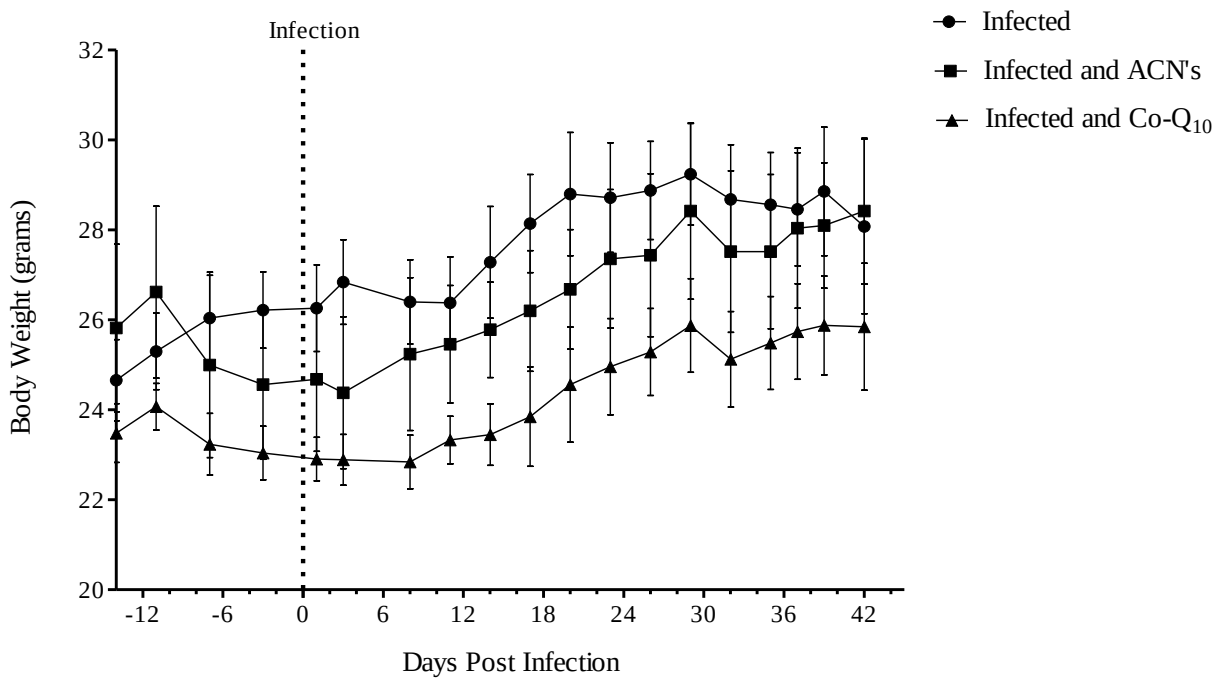


Figure 27: Changes in body weight (means±S.E.M) of *T. b. rhodesiense* infected mice treated with ACN's, Co-Q_{10} or water only.

4.5.4 Glutathione assay

Infection of mice with *T. b. rhodesiense* caused a significant drop (p=0.0006) in brain GSH (0.56±0.05µM) when compared to uninfected animals (1.27±0.12µM) (figure 28). However, the drop in GSH in the brain tissue was significantly ameliorated by supplementing with Co-Q_{10}. Co-Q_{10} raised brain GSH levels in infected animals to a concentration of 1.31±0.088µM. Animals supplemented with purple tea anthocyanins had a brain GSH concentration of 0.55±0.04µM and no significant difference in brain GSH concentration was observed between these animals and infected animals supplemented with water only (p>0.05).

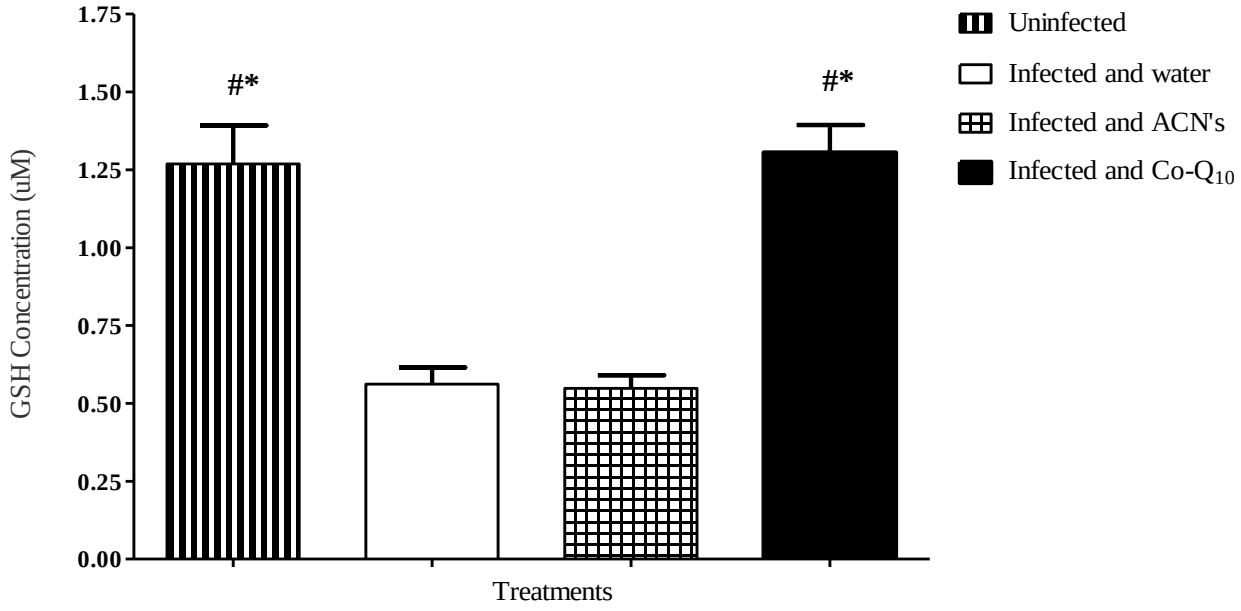
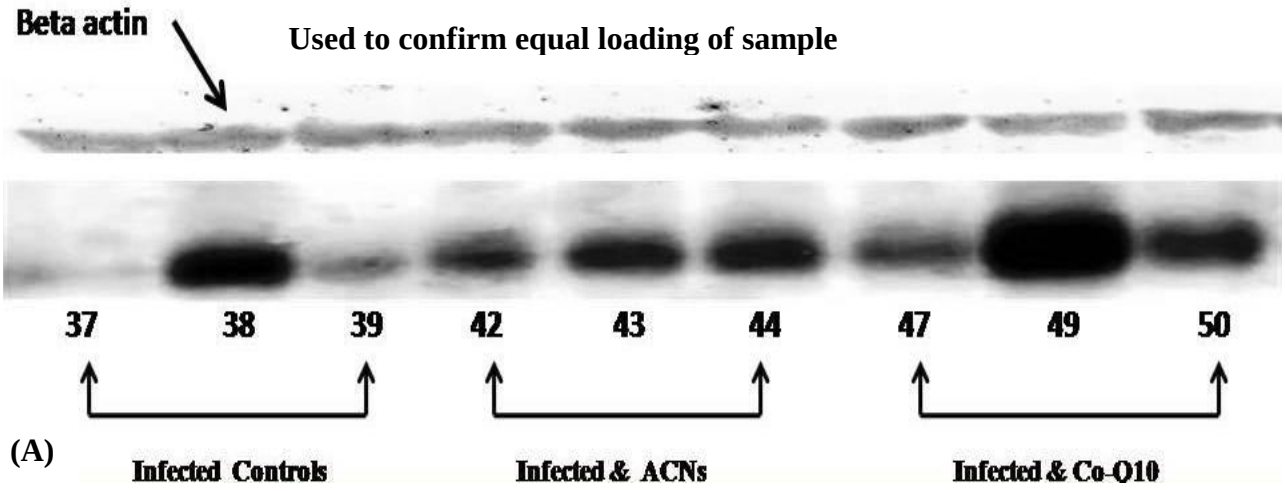


Figure 28: Total GSH levels in *T. b. rhodesiense* infected mice supplemented with ACN's, Co-Q_{10} or water only. $^{\#}p<0.05$, statistically significant versus infected untreated groups. $^{*}p<0.05$, statistically significant versus infected ACN's group.

4.5.5 Aconitase 1 (IRP-1) levels

One animal (number 38) remained uninfected throughout the experiment. *T. b. rhodesiense* KETRI strain 2537 caused a reduction in IRP-1 protein concentrations (figure 29A and 29B). This effect was reversed significantly by supplementing infected animals with Co-Q_{10} or purple tea ACN's ($p=0.0176$). However, Co-Q_{10} had stronger effects in raising IRP-1 concentrations. The difference observed between the two antioxidant supplements in raising the levels of IRP-1 was however not statistically significant ($p>0.05$).



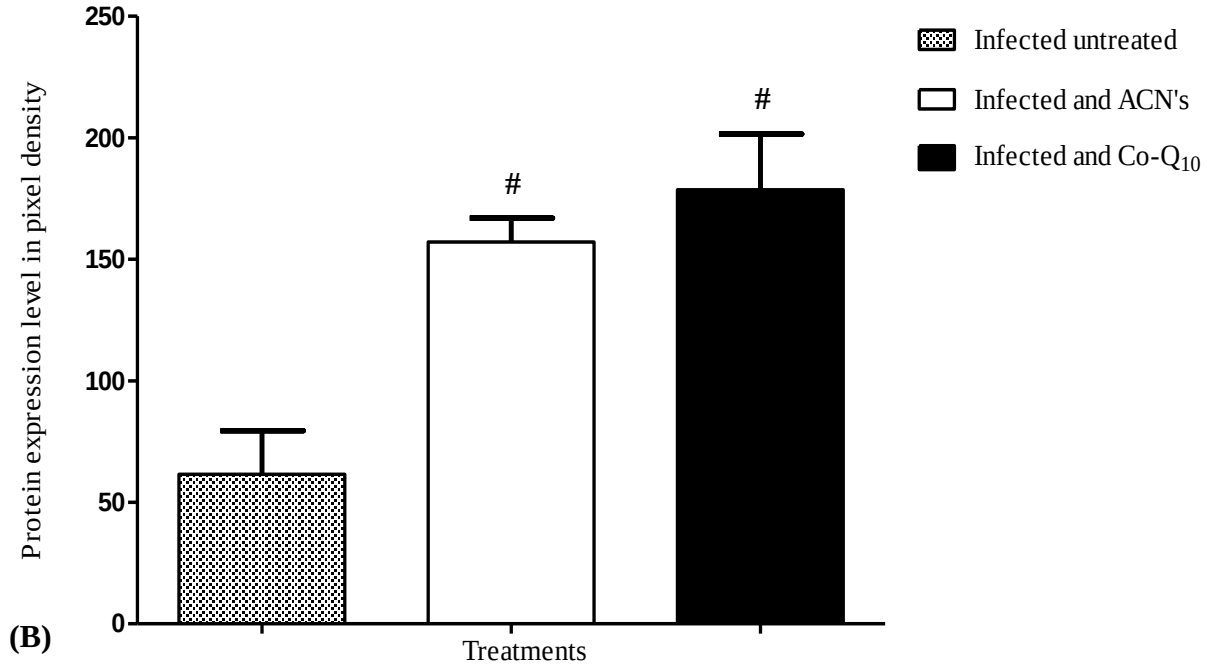


Figure 29: Immunoblotting for aconitase-1 expression. (A) The photomicrograph shows a representative of IRP-1 protein expression in three independent homogenates from brains of *T. b. rhodesiense* KETRI 2537 infected mice treated with ACN's, Co-Q_{10} or water only for the placebo group. (B) Pixel intensities of brain IRP-1 protein in *T. b. rhodesiense* KETRI 2537 infected mice treated with ACN's, Co-Q_{10} or water only for the placebo group. $^{\#}p<0.05$, statistically significant versus infected untreated groups.

4.5.6 Neuropathological response in infected mice

In the present study, infected animals revealed pathological changes in the brain but these effects were more pronounced in the untreated groups. Compared to the brains from infected and treated mice (ACN's or Co-Q_{10}), infected untreated mice displayed increased inflammation mostly resulting from influx of inflammatory cells (figures 30 and 31). Infected mice showed slight infiltrates of lymphocytes around blood vessels. In addition, there was a marked activation of microglia cell in the brain parenchyma. The microglia cells displayed features of hypertrophy that was reduced in the infected treated groups. Overall, microglial activation was most marked in brain sections from infected untreated animals when compared to infected animals treated with test antioxidants (figures 30 and 31). However, brains from infected mice treated with Co-Q_{10} displayed less pronounced microglial activation response compared to the infected ACN's treated groups.

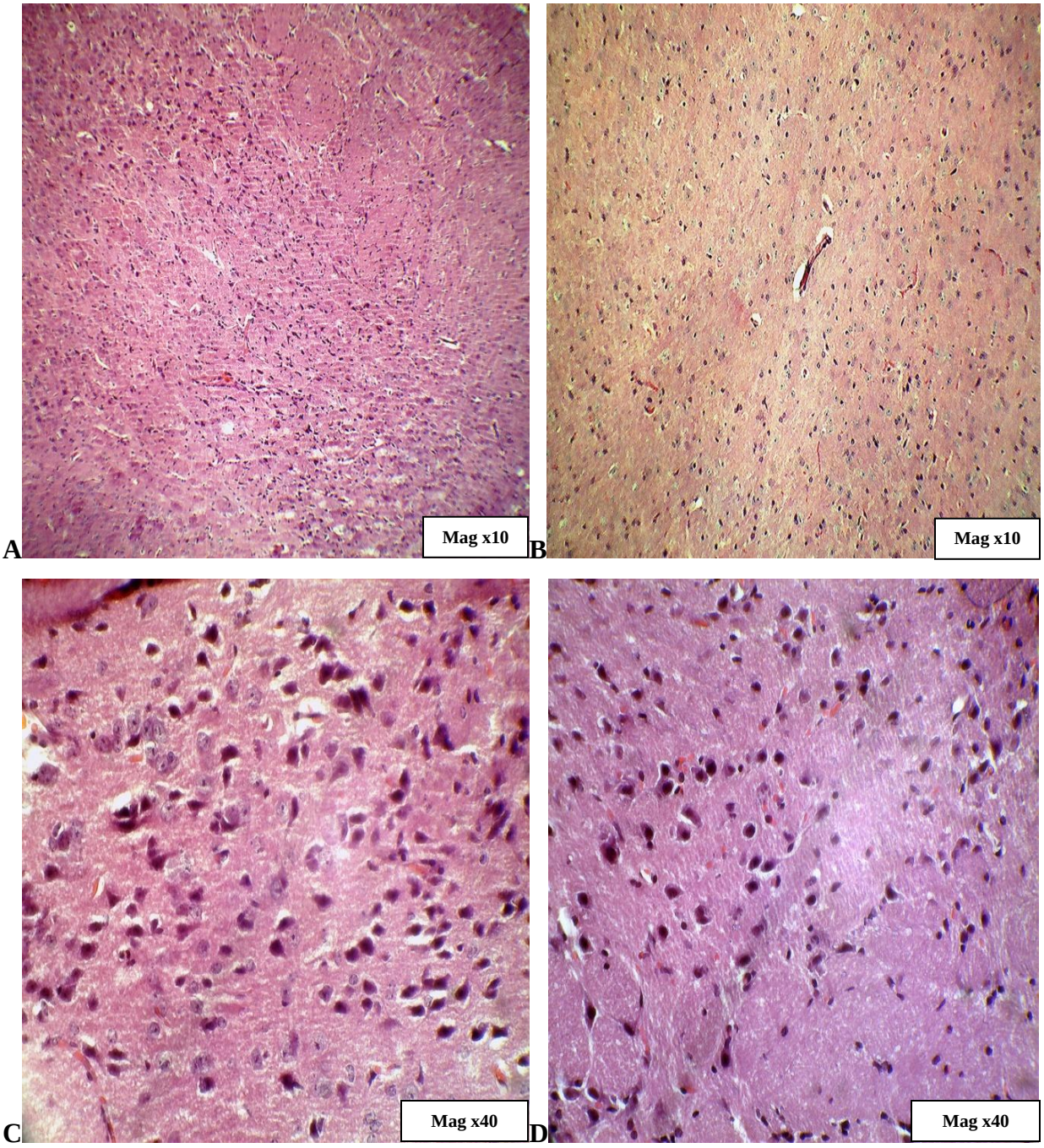


Figure 30: Sections from the forebrain showing pathology in. *T. b. rhodesiense* KETRI 2537 infected mice treated with ACN's (B and D) or water only (A and C). Moderate reduction in microglial activation in infected animals supplemented with ACN's.

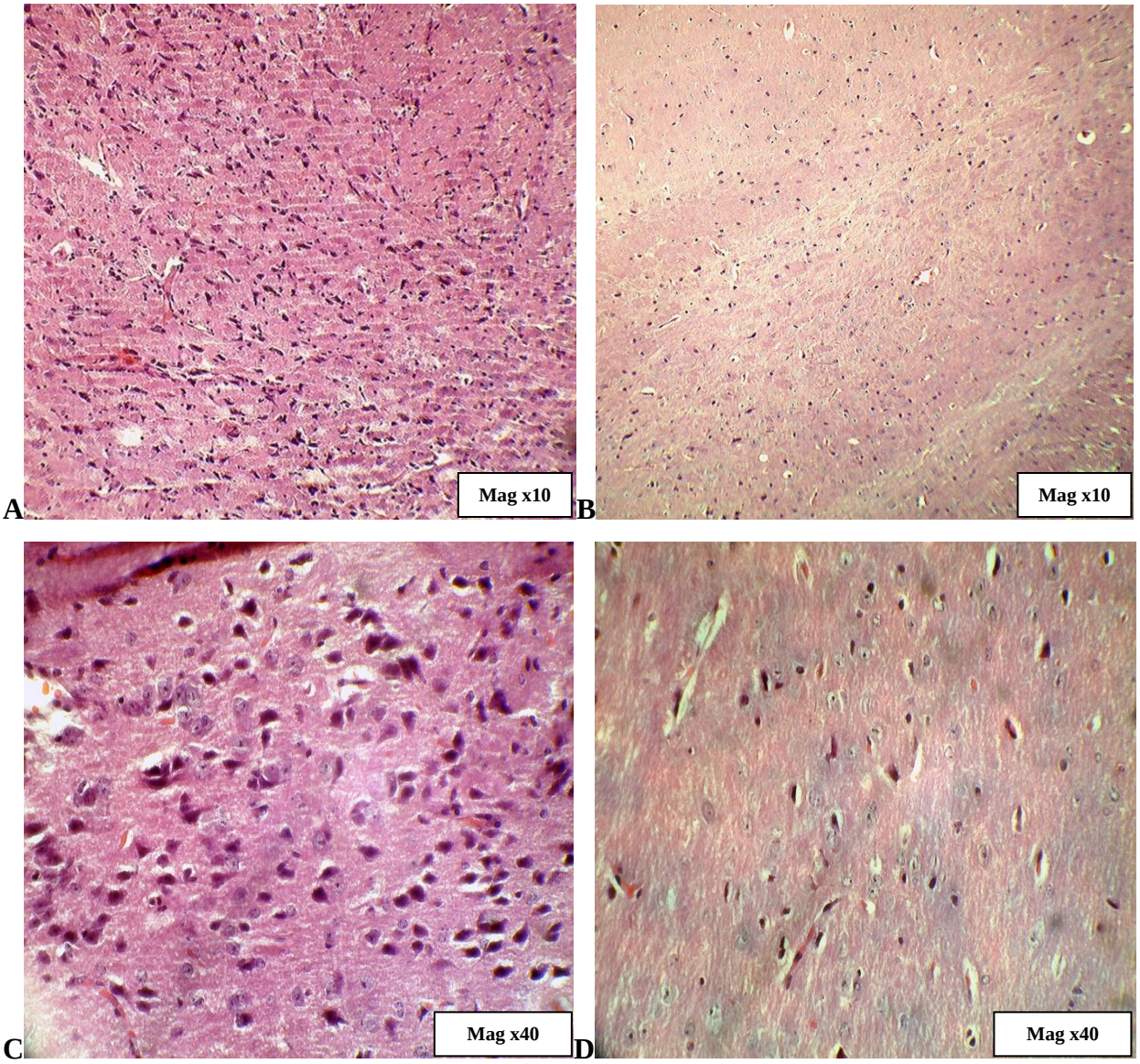


Figure 31: Sections from the forebrain showing pathology in *T. b. rhodesiense* KETRI 2537 infected mice treated with Co-Q_{10} (B and D) or water only (A and C). Note the prominent reduction in microglial cell activation in brain parenchyma in infected animals supplemented with Co-$Q_{10.}$

4.6 Effects of Antioxidants on PTRE Groups

4.6.1 Clinical symptoms and survival rate

All animals used for PTRE studies survived to the end of the experiment and portrayed clinical symptoms similar to those described in section (4.4.1). In addition, swellings on the lower right ear developed in two animals; one supplemented with ACN's only and the other

supplemented with both ACN's and Co-Q_{10} (figure 32). The swellings lasted for about one week after which they opened up releasing pus. A geimsa staining of the swelling's contents revealed only normal pus cells, trypanosomes being absent.

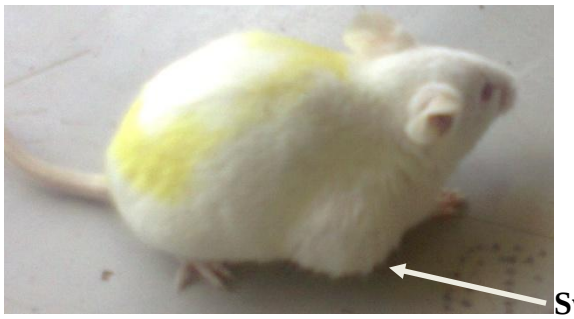


Figure 32: Mouse infected with *T. b. rhodesiense* having a swelling on the lower right ear. Animal marked with picric acid for easy identification.

4.6.2 Parasitaemia

Parasites were first detected in blood on the 4th dpi and all animals were positive by the 6th dpi, translating to a pre-patent period of 4-6 days. Significant differences in the pre-patent period were observed between PTRE controls and PTRE animals supplemented with both antioxidants, the latter group having a significantly shorter pre-patent period than the former (Table 4). First peak of parasitaemia occurred on the 6th dpi reaching parasite densities of antilog 7.26, 7.65, 7.85 and 7.52 trypanosomes/ml in PTRE controls, PTRE Co-Q_{10}, PTRE ACN's and PTRE, ACN's and Co-Q_{10} groups respectively. This was followed by a marked decrease for three consecutive days up to the 9th dpi with the parasitaemia levels dropping to antilog 6.64, 6.1, 5.8 and 5.82 trypanosomes/ml in PTRE controls, PTRE Co-Q_{10}, PTRE ACN's and PTRE, ACN's and Co-Q_{10} groups, respectively. The second peak of parasitaemia occurred on day 14 in PTRE controls (antilog 7.62 trypanosomes/ml), PTRE animals supplemented with ACN's (antilog 7.75 trypanosomes/ml) and PTRE animals supplemented with both antioxidants (antilog 7.8 trypanosomes/ml). However, PTRE animals supplemented with Co-Q_{10} reached their second peak on day 17 (antilog 8.25 trypanosomes/ml), having a higher parasite load than all the other groups.

Table 5: Values (Mean ± SEM) of pre-patent period in days in mice infected with *Trypanosoma brucei rhodesiense* employed for PTRE studies.

Mice Group	Mean Pre-patent period (days) ± SEM	Range
PTRE Controls	5.40 ± 0.25^{a}	4-5 days
PTRE and Co-Q_{10}	$4.67 \pm 0.21^{a\ b}$	4-5 days
PTRE and ACN's	$5.00 \pm 0.55^{a\ b}$	5-5 days
PTRE, ACN's and Co Q_{10}	$4.50 \pm 0.22^{b\ b}$	4-5 days

Treatment marked with the same letters are not significantly different at $p<0.05$

Following subcurative treatment with berenil (Diaminazine aceturate) 21 dpi, there was total parasite clearance from blood by 72 hours post treatment, only two animals from PTRE controls and PTRE animals supplemented with both antioxidants being positive for parasites by day three post treatment. It took 96 hours for parasites to be completely cleared from the bloodstream of these two animals.

Relapse of parasitaemia first occurred on day 21 post treatment with berenil in one animal in the PTRE control group, having parasitaemia levels of antilog 7.2 trypanosomes/ml. Parasitaemic waves were evident, but more pronounced in the secondary infection than in the primary infection (figure 33). Experimental animals were monitored for relapse of parasitaemia for 14 days following the first relapse case. After expiry of this period, parasites could still not be detected in the bloodstream of five animals. These five animals were distributed among the various groups as follows: 2 from PTRE controls, 2 from PTRE and ACN's group and 1 from PTRE and Co-Q_{10} group. All animals that were supplemented with both antioxidants had shown presence of parasites in their bloodstream by the end of the two week period. Melarsoprol treatment commenced immediately after for four consecutive days, at a dosage of 3.6mg/kg bwt. It took this organic arsenical 48hours to completely clear parasites from the bloodstream of infected mice.

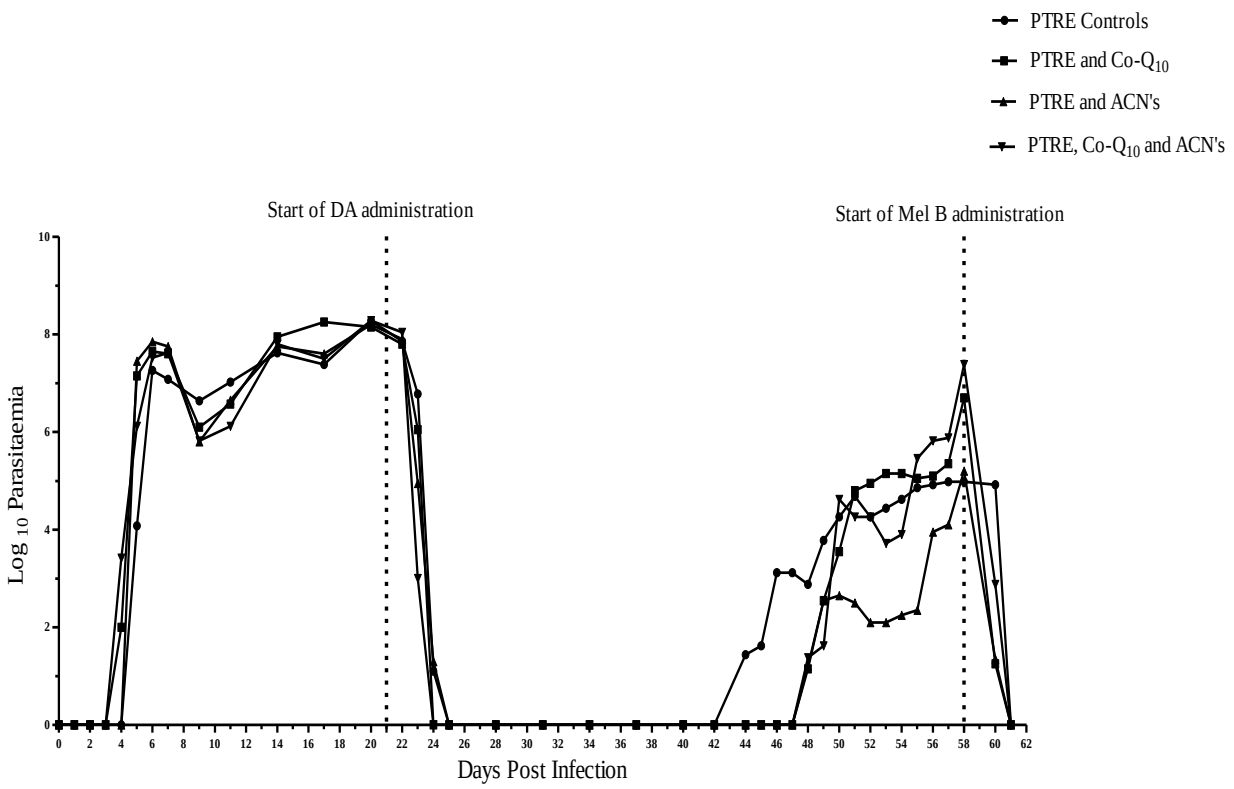


Figure 33: Parasitaemia of *T. b. rhodesiense* infected mice employed for PTRE studies and supplemented with ACN's, Co-Q_{10}, ACN's and Co-Q_{10} or water only.

Table 6: Values (Means±S.E.M) of relapse period in days in mice infected with *Trypanosoma brucei rhodesiense* employed for PTRE studies.

Mice Group	Mean Relapse period (days) ± SEM	Range
PTRE Controls	23.33 ± 1.453[a]	21-26
PTRE and Co-Q10	28.20 ± 1.770[a]	25-35
PTRE and ACN's	29.75 ± 2.500[a]	25-35
PTRE, ACN's and Co Q10	29.20 ± 1.855[a]	25-35

Treatment marked with the same letters are not significantly different at $p<0.05$

4.6.3 Packed cell volume and body weight

Decrease in PCV was evident immediately after infection and the drop was consistent until the second week post infection coinciding with the first wave of parasitaemia. PTRE groups

supplemented with both test antioxidants registered the highest decline in PCV levels from 55% to 43.4%, translating to a percentage drop of 21.09% drop within 7 dpi. PTRE ACN's, PTRE Co-Q_{10} and PTRE controls registered a percentage decline in PCV levels of 8.56%, 10.19% and 13.29%, respectively by the 7th dpi (figure 34).

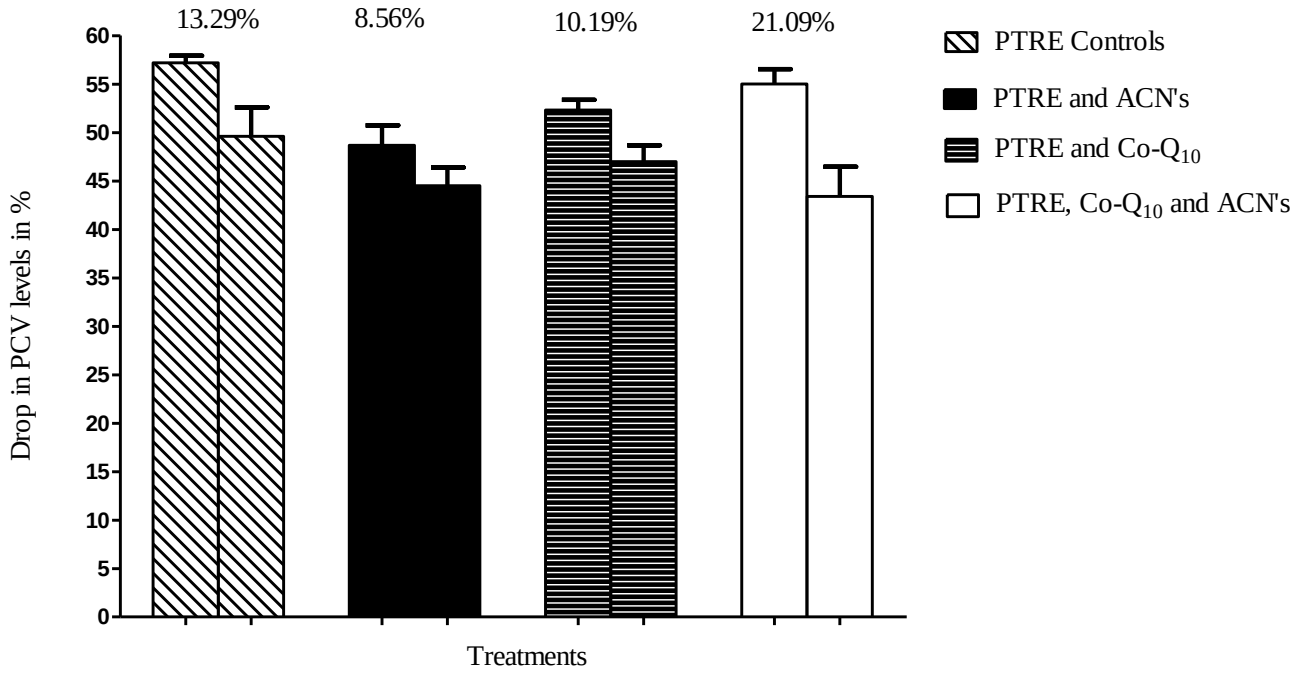


Figure 34: Percentage drop in PCV levels 7 dpi of *T. b. rhodesiense* infected mice employed for PTRE studies and supplemented with ACN's, Co-Q_{10}, ACN's and Co-Q_{10} or water only.

There was a general increase in PCV levels from start of week 4 post infection (figure 35) rising from 49± 1.14%, 44.167± 1.14%, 45.83± 0.98% and 47.8± 2.44% to 57.8± 1.07%, 57.83± 1.35%, 57.5± 1.69% and 60.4± 1.44% in PTRE controls, PTRE Co-Q_{10}, PTRE ACN's and PTRE, ACN's and Co-Q_{10} groups respectively. The rise in PCV levels coincided with the onset of berenil treatment and disappearance of parasites in blood and was persistent for about one week after which all animal groups registered a fall in PCV. Worth noting is the observation that both infected animals employed for PTRE studies and terminal stage studies had a marked decrease in PCV levels and live body weight following infection with *T. b. rhodesiense* KETRI strain 2537 on 29 dpi, signaling that this period could possibly indicate the beginning of, or stabilization the meningo-encephalitic phase. There was then a steady decrease in PCV levels from day 21 post treatment with berenil up to the last day of the experiment reaching 46.2± 2.31%, 48.83± 1.54%, 48.33± 1.69% and 47± 0.95% in PTRE controls, PTRE Co-Q_{10}, PTRE

ACN's and PTRE, ACN's and Co-Q_{10} groups, respectively. This drop in PCV coincided with the time the first animal was positive for parasites in the bloodstream after a secondary infection from relapsed parasites, and the decline became more pronounced as more animals experienced relapse of parasites back into the bloodstream.

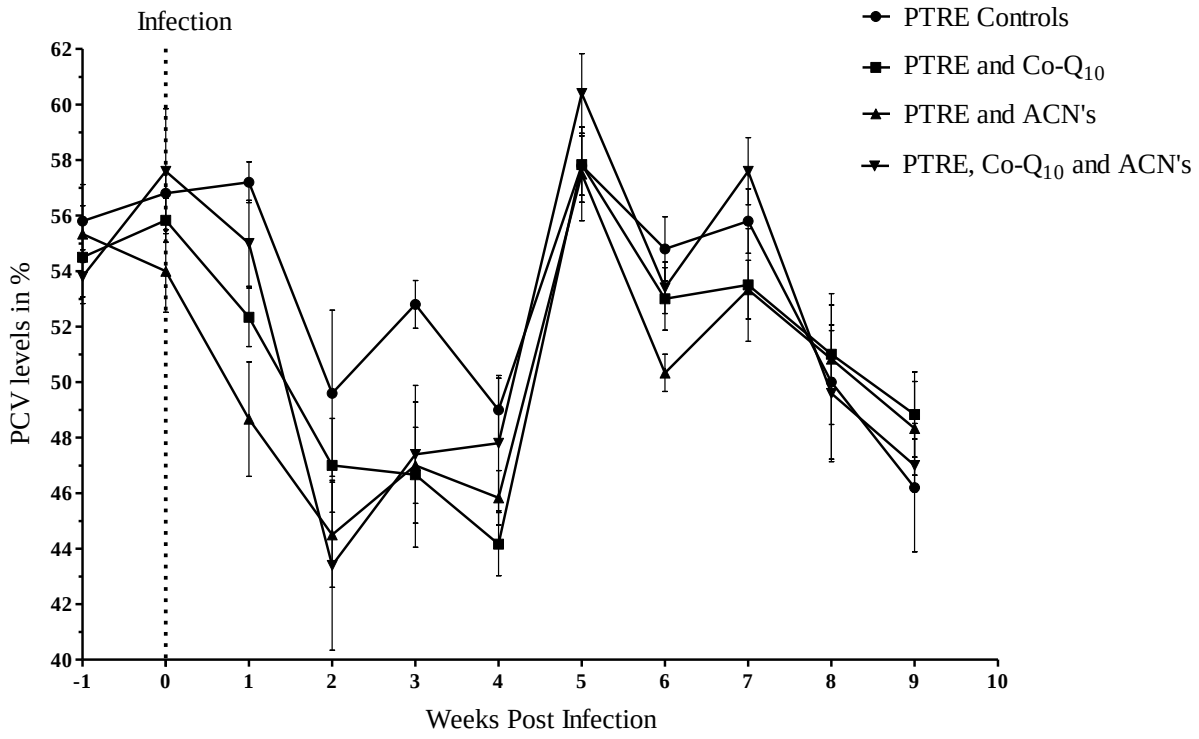


Figure 35: Changes in PCV levels of *T. b. rhodesiense* infected mice employed for PTRE studies and treated with ACN's, Co-Q_{10}, ACN's and Co-Q_{10} or water only.

Following start of antioxidant administration (figure 36), animals supplemented with ACN's registered a sharp drop in body weight consistently for two weeks, dropping from 24.85±1.14g and 24.04±0.66g to 22.57±0.86 and 21.65±0.91g in PTRE ACN's and PTRE, ACN's and Co-Q_{10} groups respectively. Co-Q_{10} only treated group also registered a drop in body weight 4 days post antioxidant administration dropping from 22.75±0.68g to 21.65±0.91g by 1st dpi PTRE animals not subjected to antioxidants gained weight consistently from the 1 dpi to the last day of the experiment, the increase being more pronounced from the 45th dpi (25.88±0.66g) to 49th dpi (29.86±0.85g). This gain in body weight was consistent with a sharp drop in parasitaemia levels during this period, dropping from antilog 3.12 to 2.88 trypanosomes/ml.

PTRE animals treated with ACN's, Co-Q_{10} or ACN's and Co-Q_{10} showed decreased body weights from about the 20th dpi to about the 32nd dpi, dropping from 26.18±1.23g, 29.35±0.90g and 24.70±1.89g to 23.99±0.89g, 26.48±0.57g and 23.03±0.94g respectively. Thereafter, there was a marginal increase in body weight in all antioxidant groups marked with regular fluctuations and reaching 25.35±0.95g, 27.8±0.73g and 24.31±0.91g in ACN's, Co-Q_{10} or ACN's and Co-Q_{10} groups respectively by the 55th dpi. A steady decrease was then observed in these groups until experiment was terminated.

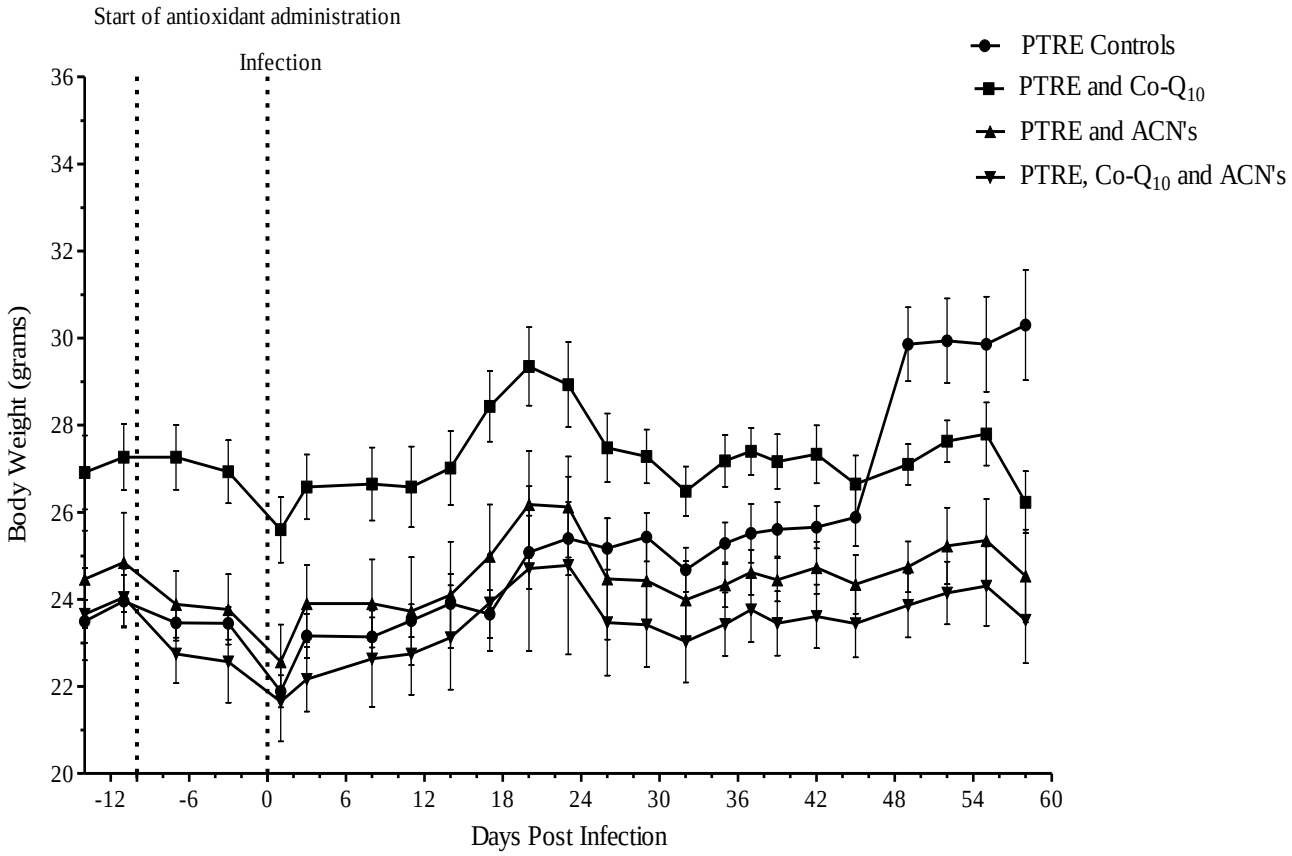


Figure 36: Changes in body weight of *T. b. rhodesiense* infected mice employed for PTRE studies and treated with ACN's, Co-Q_{10}, ACN's and Co-Q_{10} or water only.

4.6.4 Glutathione assay

Trypanosomal effects on GSH levels were not evident in PTRE groups probably due to the drugs administered to the animals at different time points to produce a PTRE model that mimics the PTRE observed in human patients during late stage of the disease. However, Co-Q_{10} had similar effects on GSH levels. Co-Q_{10} was able to raise brain GSH levels significantly ($p=0.0015$) to 2.82±0.17μM when compared to PTRE controls which had a concentration of

1.67±0.13µM. PTRE animals supplemented with ACN's only or ACN's and Co-Q_{10} had slightly higher GSH levels of 1.87±0.11µM and 1.94±0.13µM respectively when compared to PTRE animals not subjected to the test antioxidants (figure 37). However, no significant differences were observed between PTRE ACN's, PTRE, ACN's and Co-Q_{10} and PTRE controls groups ($p>0.05$).

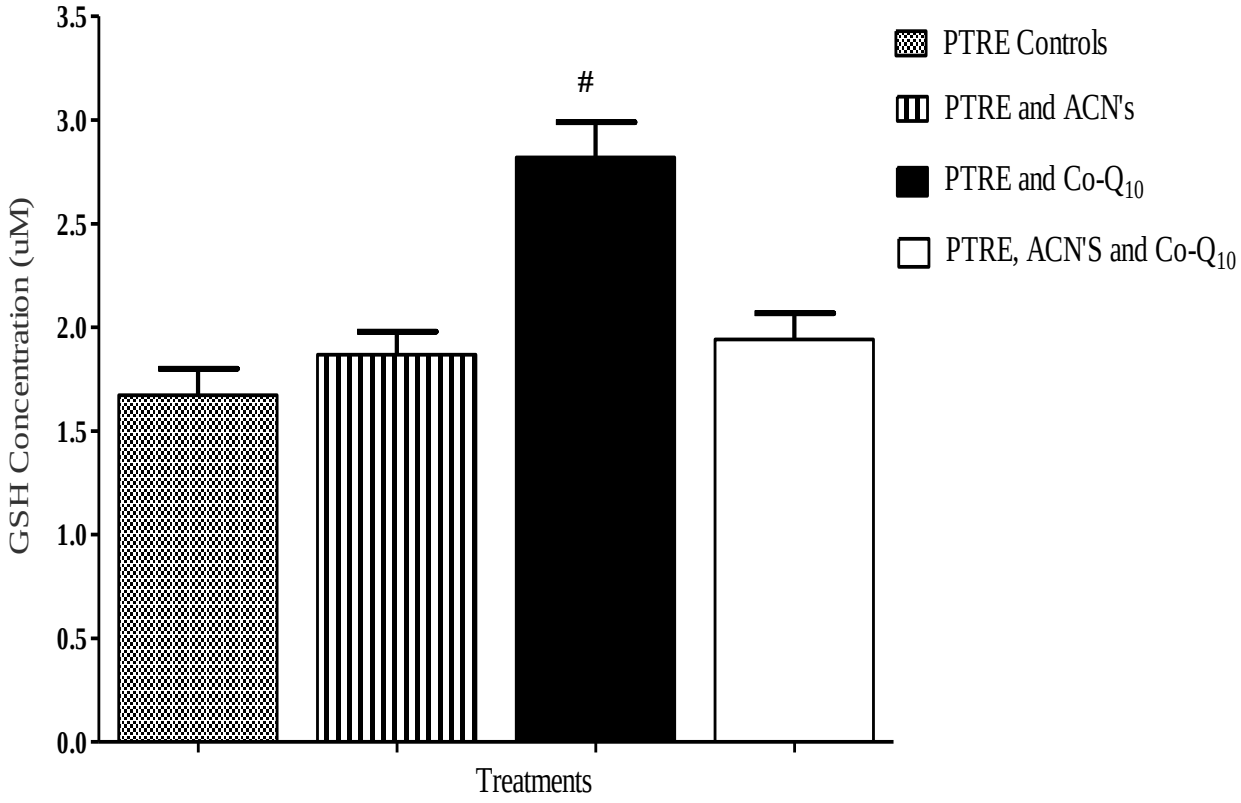


Figure 37: Total GSH levels in *T. b. rhodesiense* infected mice employed for PTRE studies and supplemented with ACN's, Co-Q_{10}, ACN's and Co-Q_{10} or water only. $^{\#}p<0.05$ statistically significant versus controls.

4.6.5 Aconitase (IRP) 1 levels

In animals employed for PTRE studies, test antioxidants potrayed similar effects as those observed in infected terminal groups (figure 38A and 38B). Co-Q_{10} significantly up-regulated IRP-1 levels when administered in isolation. However, a combination of the two test antioxidants marginally lowered IRP-1 levels when compared to the controls though not statistically significant. Purple tea anthocyanins were also able to marginally raise IRP-1 concentrations but the increase was not significant at $p>0.05$.

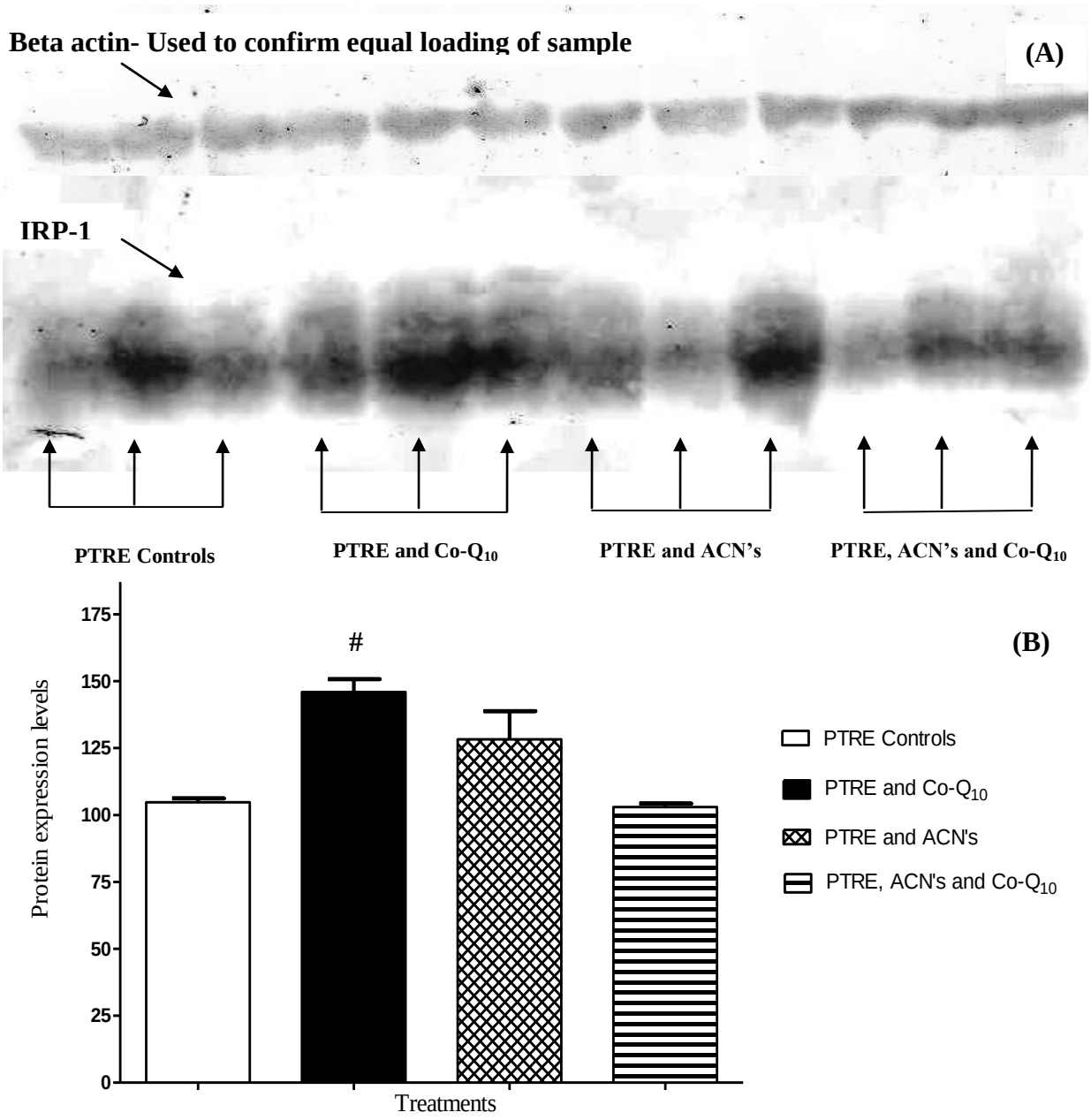


Figure 38: Immunoblotting for aconitase-1 expression. (A) The photomicrograph shows a representative of IRP-1 protein expression in three independent homogenates from brains of *T. b. rhodesiense* KETRI 2537 infected mice employed for PTRE studies and treated with ACN's, Co-Q_{10}, ACN's and Co-Q_{10} or water only. (**B)** Pixel intensities of brain IRP-1 protein in *T. b. rhodesiense* KETRI 2537 infected mice employed for PTRE studies and treated with ACN's, Co-Q_{10}, ACN's and Co-Q_{10} or water only. $^{\#}p<0.05$ statistically significant versus controls.

4.6.6 Neuropathological response in infected mice employed for PTRE

All animals employed for PTRE studies were treated with DA at a dose of 5mg/kg body weight from the 21st dpi for three days to exacerbate the inflammatory reaction seen during late stage of the disease. Infected mice showed infiltrates of lymphocytes and macrophages at the blood vessel regions and in severe cases the lymphocytes were also found within the brain parenchyma associated with disruption of the brain tissue. In addition, there was a marked activation of microglia in the brain parenchyma (figure 39). Brain sections from PTRE induced mice without any antioxidant treatment showed a more marked presence of inflammatory cells, microglial activation and disruption of the brain parenchyma when compared to PTRE mice supplemented with either one or both test antioxidants. In-comparison the Co-Q_{10} treated mice showed a more marked reduction in the inflammatory response of cellular infiltration, brain tissue disruption and microglial activation compared to the ACN's treated group of mice (figures 40 and 41).

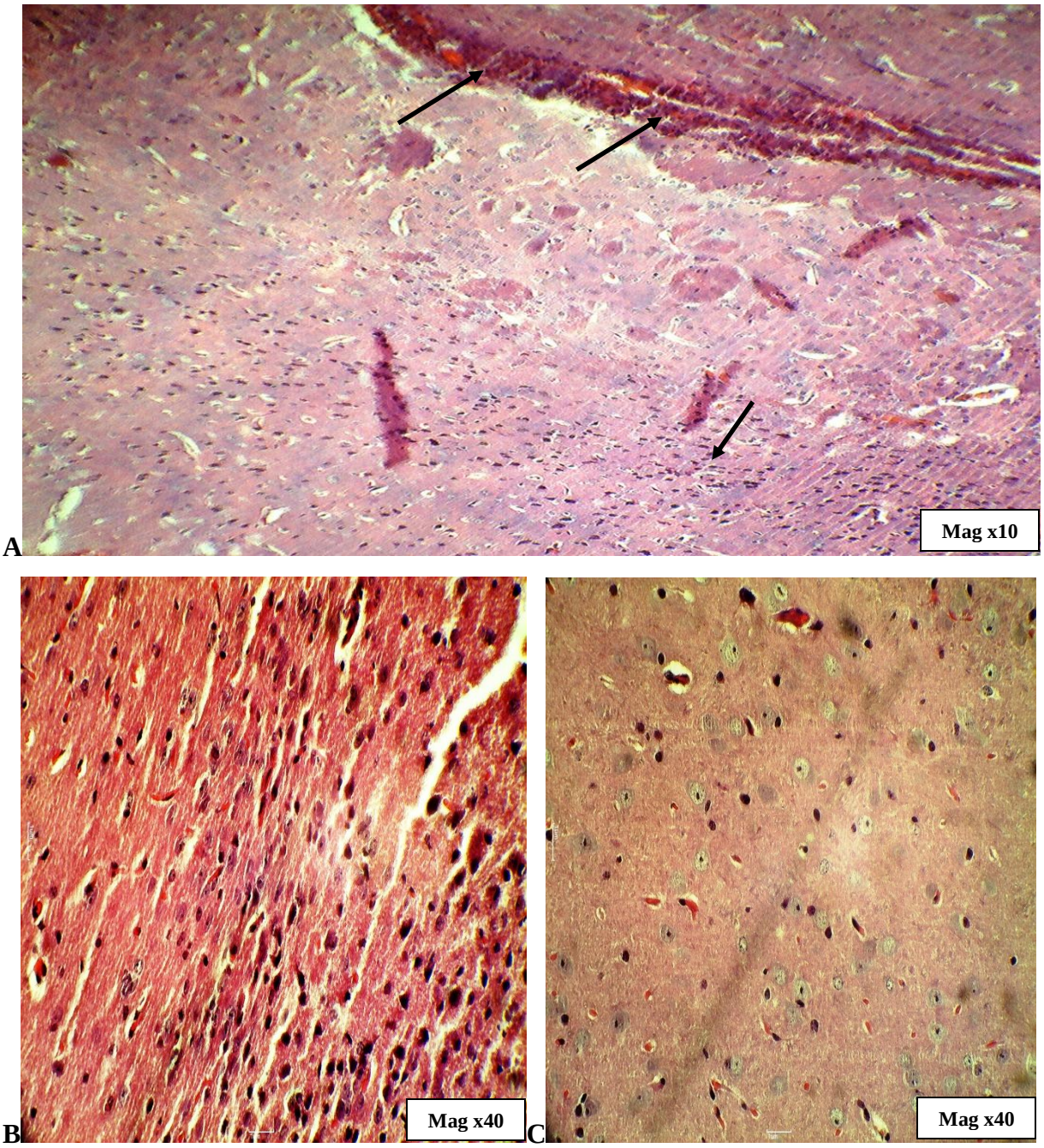


Figure 39: Sections from the forebrain Differences in the neuroinflammatory response in *T. b. rhodesiense* KETRI 2537 infected mice employed for PTRE studies and treated with ACN's (C) or water only (A and B). Arrows (on the upper side of slide A) indicate perivascular cuffs of inflammation which are several cells deep around blood vessels.

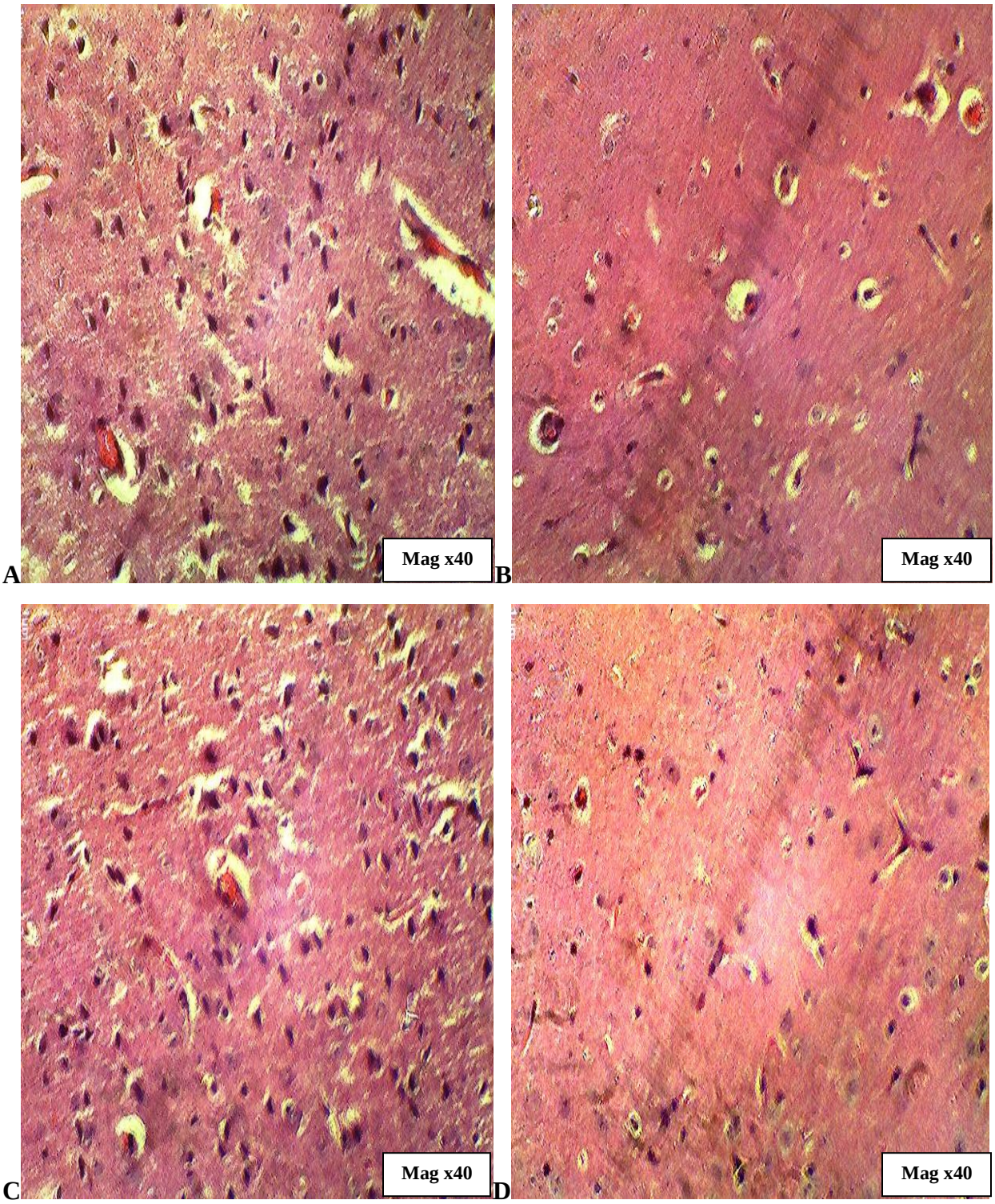


Figure 40: Sections from the forebrain showing differences in the neuroinflammatory response in *T. b. rhodesiense* KETRI 2537 infected mice employed for PTRE studies and treated with Co-Q_{10} (B) or water only (A).

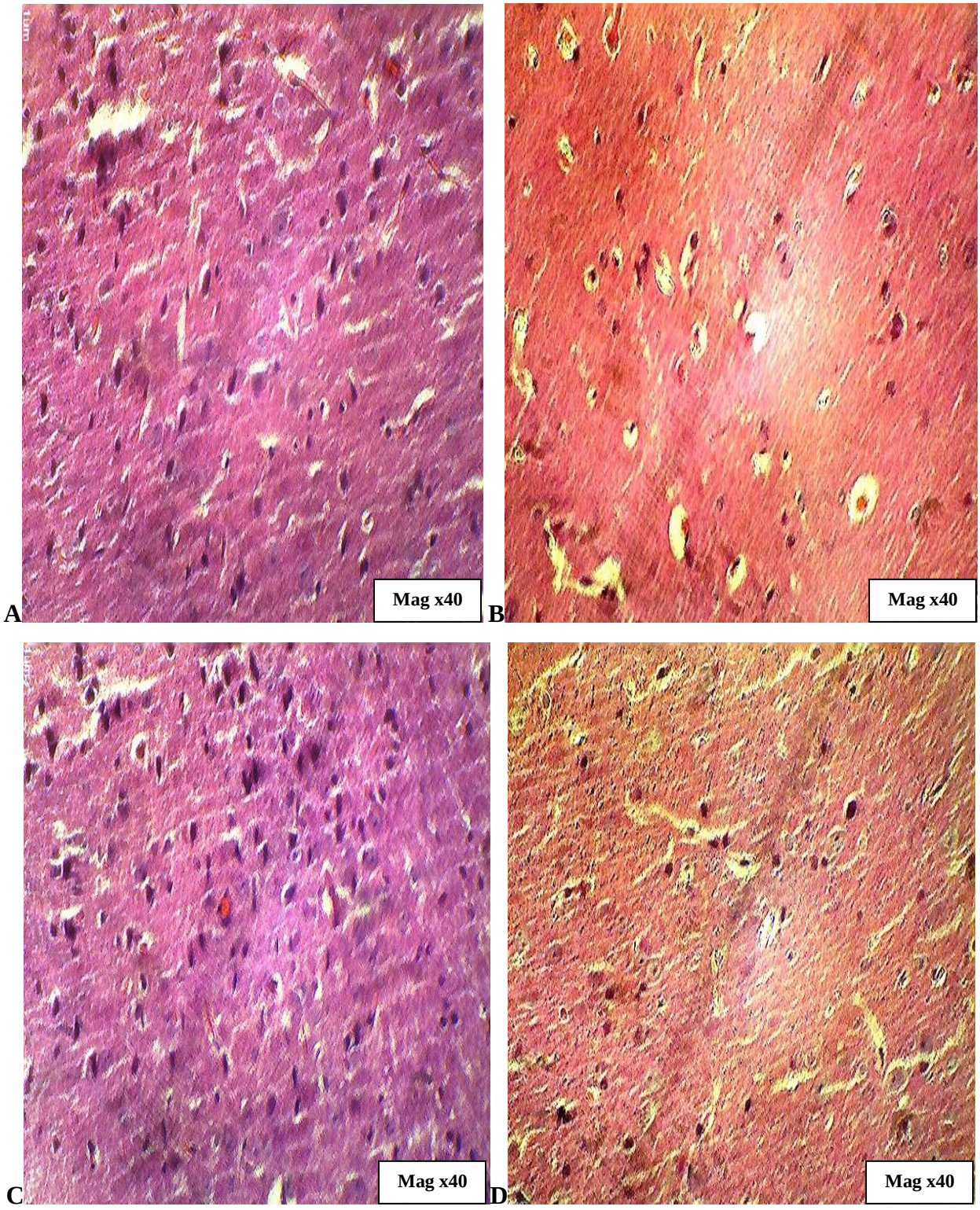


Figure 41: Sections from the forebrain showing differences in the neuroinflammatory response in *T. b. rhodesiense* KETRI 2537 infected mice employed for PTRE studies and treated with ACN's and Co-Q_{10} (B and D) or water only (A and C).

4.7 Discussion

4.7.1 Kenyan purple tea anthocyanins in brain tissue

Polyphenols and other phytochemicals in plants must cross the blood brain barrier to increase their bioavailability and effectiveness in the central nervous system. Otherwise, the multitude of health benefits associated with these flavonoids such as strong antioxidant and anti-inflammatory properties would be excluded from the brain and the CNS in general. Despite their proven potential health benefits, studies on the bioavailability of ACN's in the brain tissue have received much less attention than those for other flavonoids in tea. Most studies have concentrated on the bioavailability and neuroprotective effects of catechins, the most abundant polyphenols in tea. EGCG, the most potent member in the catechin family has previously been shown to cross the blood brain barrier (Nakagawa and Mizawa, 1997). Mandel *et al*. (2006) reported that tea catechins are brain permeable and protect against neuronal death in a multitude of neurological diseases. Despite this remarkable observation, there exists no data on the bioavailability of Kenyan purple tea ACN's hence this study endeavored to evaluate the bioavailability of purple tea ACN's in the brain tissue of rodents.

The amplification of endogenous antioxidants in the brain tissue of animals supplemented with ACN's strongly hinted the presence of these flavonoids in the brain since compounds are known to act directly at the sites where they localize. The presence of these flavonoids in the brain was later confirmed by the detection of metabolites in ACN's fed animals but absent in the placebo group. Most of the metabolites detected had retention times in the region of 20-25 minutes, coinciding with the retention times of the three major anthocyanidins present in the Kenyan purple tea cultivars namely cyanidin (21.580), pelargonidin (24.671) and peonidin (25.630) (Kerio *et al.,* 2012). However, it was not possible to detect intact ACN's from the brain homogenates. Considering that approximately two-thirds of ACN's are highly biotransformed and end up as methylated and glucuronidated metabolites (Kay *et al.,* 2006), absence of intact anthocyanins in the brain homogenates was not surprising.

Although the study reports for the first time that ACN's from Kenyan purple tea cultivars are bioavailable in the brain, several other authors have previously reported presence of anthocyanins from other plant sources in the brain and the CNS in general. Andres-Lacueva *et al.* (2006) reported that ACN's from blueberries are able to transverse the blood brain barrier and their concentrations correlated with cognitive performance. Kalt *et al*. (2008) concurred with this

finding and reported presence of intact anthocyanins in the eyes and brain tissue of blueberry fed pigs. Findings from the present study provide new information on the ability of Kenyan purple tea ACN's to cross the BBB and possibly provide neuroprotection.

4.7.2 Effects of Kenyan purple tea ACN's and Co-Q_{10} on brain antioxidant capacity in uninfected mice

Progress in the treatment of HAT has not been impressive to say the least. Treatment of HAT which is stage specific relies on drugs that were discovered decades ago and are characterized with high levels of toxicity and undesirable side effects (Kennedy, 2004). The situation is further compounded by the fact that drugs used to treat the early stage of the disease are unable or poorly able to penetrate the BBB and are thus rendered ineffective in the late stages of the disease. Needless to say, the need for safer drugs is of vital importance. It is therefore paramount that additional research and development efforts be geared towards the search of novel drugs and enhancement of current drugs by making them less toxic and more effective.

Trypanocidals such as melarsoprol, nifurtimox and benznidazole have been implicated in the production of intracellular reactive oxygen molecules resulting in oxidative stress (Cavigelli *et al.,* 1996; Maya *et al.,* 2006). ROS and other free radicals have been associated with a plethora of cellular alterations resulting in loss of GSH, oxidation of thiols to disulphides, impairment in energy generation and DNA cleavage eventually leading to cell death (Parke, 1994). The antioxidant system therefore potentially presents a critical pathway which could be targeted with an aim of improving treatment outcome in late stage HAT. Anthocyanins, members of the flavonoid group and powerful antioxidants were extracted from the leaves of the purple leaf colored *Camellia sinensis* and characterized using HPLC before being used in mice model for HAT and PTRE. Anthocyanin fractions identified by using pure anthocyanin standards revealed different fractions from those established by Kerio *et al.* (2012) despite using the same purple tea variety in both experiments. However, tea cultivars used in this experiment were obtained from the Kericho station as opposed to the cultivars used by Kerio *et al.* (2012) which were obtained from the Kangaita substation. Kericho and Kangaita stations have different altitudes and tea growing conditions. The different environmental conditions including temperature, humidity, mineral nutrient availability, light intensity among others could result in markedly different flavonoid profiles. Indeed, these differences may have contributed to the different anthocyanin profiles observed between the two tea cultivars. Overall, higher altitudes are generally

considered advantageous and are associated with higher anthocyanin concentrations than their lower counterparts (Mateus *et al.*, 2002).

Melarsoprol, a highly toxic drug associated with lethal side effects and in use since 1949 (Friedheim, 1949; Pepin and Milord, 1994), is the only available drug used to treat the acute form of HAT once the CNS is involved. Molecular mechanisms that lead to melarsoprol toxicity still remain elusive. In the current study, melarsoprol was shown to decrease the packed cell volume of mice in the absence of trypanosomes. Indeed, melarsoprol has been implicated in induction of apoptosis through decrease/loss of bcl-2 proto-oncogene expression leading to the reduction of growth and survival of cells (Konig *et al.,* 1997), making this organic arsenical a viable candidate in the fight against malignancies. Since arsenicals have a broad range of activity, it is possible that erythrocytes also suffer the same fate as malignant cells with the arsenical being unable to distinguish normal from abnormal cells in its mode of action.

Moreover, GSH levels in brain tissue of mice challenged with melarsoprol were significantly lower than in brain tissue of untreated animals in this study. Other arsenicals have also been implicated in the reduction of glutathione resulting in oxidative stress (Cavigelli *et al.,* 1996). This phenomenon can be explained by the reversible inhibition of the cellular enzyme glutathione reductase by the organic arsenical melarsoprol (Cunningham *et al.,* 1994). Melarsen oxide, a metabolite of melarsoprol has been shown to interact with glutathione forming a stable adduct which is a competitive inhibitor of glutathione reductase, an enzyme responsible for maintaining intracellular glutathione in its reduced form (Cunningham *et al.,* 1994). Therefore the low GSH levels observed in animals challenged with melarsoprol in this study can be attributed to the inhibition of glutathione reductase, enhancing conversion of reduced glutathione (GSH) to the oxidized form (GSSG) leading to conditions of oxidative stress. This reduction in GSH by melarsoprol indicates a primary reduction of protective mechanisms against ROS and could make neuronal cells susceptible to oxidative stress and ultimate cell death (Dauer and Przedborski, 2003). Since GSH reduction has been reported in patients and animals of various neurodegenerative disorders such as Parkinson's and Alzheimer's disease (Ballatori *et al.,* 2009; Solano *et al.,* 2008), this phenomenon could play a major role in the induction of PTRE.

Results also indicate that melarsoprol significantly reduced aconitase-1 concentration in the brain. Aconitase contains an iron sulphur cluster that renders this metalloprotein extremely sensitive to oxidative stress (Bota and Davies, 2002). Since melarsoprol has been implicated in

the production of ROS and other free radicals (Barchowsky *et al*., 1996; Wang *et al*., 1996; Chen *et al*., 1998), then it is possible that this trypanocidal drug potently oxidizes aconitase-1 rendering the endogenous antioxidant inactive. Consequently, the low concentrations of aconitase-1 observed can be attributed to the degradation mechanism of oxidised proteins in the cell via the lysosomal and proteosome pathway (Bota and Davies, 2002). Apparently, if oxidised proteins are not efficiently eliminated from the cell and are instead left to accummulate, the end result is enhanced toxicity levels and alteration of cell function (Szweda *et al.,* 2002). This degradation mechanism is therefore an important component of cellular defence against ROS and other free radicals.

Consequences of oxidative inactivation of aconitase-1 are detrimental in the brain. First, oxidative inactivation of aconitase would result in the formation of fenton reactants H_2O_2 and Fe^{2+} leading to the vicious formation of the hydroxyl radical ($^{\cdot}OH$) (Cantu *et al.,* 2009). Such phenomenon would further aggravate melarsoprol induced oxidative stress. Secondly, oxidative modification of aconitase would interfere with the krebs cycle ultimately leading to reduced ATP levels and a decreased production of cytosolic nicotinamide adenine dinucleotide phosphate reduced (NADPH), a major source of reducing equivalents and an important defense against oxidative stress (Tong and Rouault, 2006). Depletion of ATP in the brain and a decrease in cellular reducing potential can in turn interfere with one of the major functions of astrocytes in the brain such as neuroprotection and maintenance of a robust antioxidant system, making nerve cells vulnerable and contributing to neuronal death and resultant encephalopathy witnessed in post melarsoprol treatment in late stage HAT. Indeed, impaired bioenergetics is a commonality underlying several neurodegenerative disorders such as Alzheimer's, Parkinson and Huntington diseases (Galino *et al.*, 2011).

In the presence of antioxidants, most adverse side effects associated with melarsoprol in this study were ameliorated. This was not surprising as previous studies have also demonstrated that increasing antioxidant levels may protect against toxicity induced by other arsenicals (Bongiovanni *et al.,* 2007; Sharma *et al.,* 2007). Co-enzyme Q_{10}, an endogenous antioxidant and an essential cofactor in the electron transport chain, was able to protect animals against the observed decline in PCV levels following melarsoprol treatment. Co-Q_{10} scavenges free radicals and enhances membrane integrity by preventing oxidation of lipids within biological membranes (Spindler *et al.,* 2009). Since lipid oxidation is correlated with membrane disintegration and

ultimate cell death (Avery, 2011), then it is possible that Co-Q_{10} protected animals against decline in PCV levels by keeping levels of free radicals in blood low shielding the red blood cells membrane against oxidative damage. Moreover, Co-Q_{10} has been shown to exhibit antiapoptotic effects by activating and increasing expression of mitochondrial uncoupling proteins (Spindler *et al.,* 2009), a phenomenon which could have counteracted apoptotic effects exhibited by the organic arsenical melarsoprol thus protecting the red blood cells against the programmed cell death.

The same results were however not replicated using purple tea anthocyanins, which enhanced the decline in PCV levels following melarsoprol administration. This drop in PCV level was also observed in the absence of the trypanocidal melarsoprol. Tea flavonoids have been credited with the ability to affect the absorption and metabolism of ions due to their capacity to interact with a variety of metal ions. Specifically, tea flavonoids have the ability to interact with iron forming insoluble complexes that strongly inhibit iron absorption with a concomitant decrease in haemoglobin levels (Hunt and Roughead, 2000). A previous study had indeed established that consumption of black tea by young women in India posed strong inhibitory effects on iron absorption, with 20mg of polyphenols from black tea reducing iron absorption by as much as 66% (Thankachan *et al.,* 2008). Imai and Nakachi (1995) reported similar findings showing that increased consumption of tea is associated with decreased haemoglobin (Hb) concentrations. It is therefore hypothesised that a decrease in Hb due to presence of ACN's in the test animals treated with ACN's resulted in insufficient Hb in the body causing a reduction in the synthesis of red blood cells and thus translating to low PCV levels.

Results also indicate that GSH levels were significantly higher in animals supplemented with both antioxidants, Co-Q_{10} and ACN's when administrered separately. Indeed, antioxidants have been shown in several instances to protect against depletion of endogenous antioxidants and oxidative damage of cellular components. Kerio *et al.,* (2012), working with the same variety of purple tea reported an increase in cellular GSH content in cells exposed to anthocyanins. Berry juice rich in anthocyanins has been shown to decrease oxidative DNA damage and increase GSH levels in healthy human volunteers (Weisel, 2006). Moreover, Co-Q_{10} has been reported to restore GSH to normal levels in rats challenged with rotenone, a highly toxic natural product from the *Leguminosae* plant family employed as an insecticide (Abd-El-Gawad *et al.,* 2004). It is therefore clear that antioxidants protect against glutathione depletion decreasing vulnerability of

cells to oxidative stress. In the present study, Co-Q_{10} and Kenyan purple tea ACN's prevented against melarsoprol-induced decreases in GSH suggesting that these nutraceuticals had a sparing effect on endogenous antioxidants.

In addition, Kenyan purple tea anthocyanins significantly raised aconitase-1 concentrations, an endogenous antioxidant protein functioning both as an enzyme and as an iron regulatory protein. Aconitase and other iron sulfur clusters in general are highly sensitive to oxidative stress (Pantopoulos *et al.,* 1997). The susceptibility of aconitase to oxidative inactivation is owed to the presence of an acid labile iron atom in the active site of this metalloprotein (Flint *et al.,* 1993). Since aconitase is involved in shuttling of electrons in the electron transport chain, its inactivation leads to accumulation of reduced metabolites and causes an increase in the production of free radicals further aggravating the oxidative stress conditions (Van den Enden *et al.,* 1995; Yan *et al.,* 1997). Therefore, the presence of antioxidants were beneficial to this iron sulfer cluster protein by squelching free radicals produced either through metabolism or by the organic arsenical melarsoprol. However, a combination of the test antioxidants namely Co-Q_{10} and tea anthocyanins were only partially able to raise aconitase-1 concentrations both in the presence and absence of the trypanocidal melarsoprol. Similar results were also obtained while assaying for GSH, with the individual antioxidants having greater effects in raising GSH levels than in combination suggesting an antagonism of the two antioxidants when administered together. Antagonistic effects between the two antioxidants was unexpected as it was thought that they would have had a more pronounced effect in combination rather than in isolation.

Anthocyanins have been credited with lipid lowering effects, especially for cholesterol and triglycerides (Han *et al*., 2008). This lipid lowering effect is associated with an increase in hydroxymethly glutarly CoA (HMG-CoA) with concomitant degradation of HMG-CoA reductase via negative feedback mediated by intracellular cholesterol levels and the hormone glucagon (Han *et al*., 2008). It is postulated that among others, this effect may be accountable for the negative interaction between ACN's and Co-Q_{10}. Indeed Co-$Q_{10,}$ an endogenously synthesized lipid, is produced from a common pathway as cholesterol (Dallner and Sindelar, 2000). Degradation of HMG-CoA reductase by anthocyanins also causes a decrease in the synthesis of farnesyl pyrophosphate, an intermediate in the synthesis of Co-Q_{10} (Liang *et al.,* 2012). Therefore, anthocyanins and other substances known to lower cholesterol levels or

prevent its absorption are expected to have adverse effects on non sterol compounds such as Co-Q_{10} (Bliznakov and Wilkins, 1998). The negative interactions have also been observed while employing other lipid lowering substances such HMG-CoA competitive inhibitors known as statins (Bliznakov and Wilkins, 1998). In fact, several authors including Langsjoen and Langsjoen (2003) and Passi *et al.* (2003) have reported the ability of Co-Q_{10} to reverse the detrimental side effects associated with statins including muscle myopathy and rhabdomyolysis.

4.7.3 Effects of Kenyan purple tea ACN's and Co-Q_{10} on *Trypanosoma brucei rhodesiense* infection in mice

Disease severity in human African trypanosomiasis is associated with oxidative stress and inflammatory responses. Several authors have indeed been able to establish that the body's innate antioxidant capacity is highly compromised during infection with trypanosomes. Depletion of glutathione, ascorbic acid (Umar *et al*., 2010) and production of free radicals from activated macrophages and trypanosome parasites are just some of the mechanisms that enhance oxidative stress during disease progression (Meshnick *et al.,* 1978; Askonas, 1985). In addition, once the CNS is involved during late stage of the disease, widespread meningeal inflammation characterized by an infiltration of inflammatory cells and perivascular cuffings occurs in the brain. Therefore, in view of the prominent inflammatory responses and antioxidant system failure due to trypanosome infections, employing compounds known to possess potent antioxidant and anti-inflammatory properties in ameliorating severity of the disease seemed logical.

In the present study, infected animals supplemented with ACN's in both the terminal groups and the PTRE groups had the highest pre-patent periods than their counterparts. This can be attributed to the toxic properties of these tea polyphenols thus delaying the establishment of trypanosome parasites in the mice. Parasitaemic waves were evident, a phenomenon attributed to the change in trypanosome's surface coat which renders the parasite elusive to the host's immune system (Vickerman, 1985). In animals employed for PTRE studies, parasitaemic waves were also evident but more pronounced in the secondary infection than in the primary infection. This finding is consistent with findings of earlier researchers such as Ndung'u *et al.* (2008) who reported that mice infected with bloodstream forms of *T. b. rhodesiense* demonstrated fewer waves of parasitaemia than mice infected with cerebrospinal fluid forms. Indeed, other studies have even demonstrated a morphological difference between bloodstream and CNS forms of

trypanosomes, the latter being highly motile and very slim as compared to the bloodstream forms (Wolburg *et al.,* 2012). These differences between primary and secondary trypanosome infections could be attributed to the hostility of the cerebrospinal fluid generating genetic mutants with different degrees of virulence than bloodstream forms (Pentreath *et al.,* 1992).

However, antioxidants purple tea ACN's and Co-Q_{10} were unable to reduce parasitaemia levels which remained comparable to parasitaemia levels in the infected placebo group. This finding was not surprising as compounds with trypanocidal activity have been reported to possess the ability to initiate free radical reactions causing oxidative stress rather than reinforcing the body's innate antioxidant capacity (Meshnick *et al.,* 1978). This, in part, is probably due to the fact that trypanosome parasites are highly sensitive to the cytotoxic effects of reactive molecules (Vincendeau *et al.,* 1992). Therefore, presence of antioxidants during trypanosome infection would scavenge and reduce levels of reactive oxygen and nitrogen species produced by macrophages shielding trypanosomes from oxidative attack.

Indeed, pre-treatment of trypanosomes with antioxidants N-acetyl-L-cysteine (NAC) or GSH resulted in significant reduction in trypanosome death (Figarella *et al.,* 2006). Moreover, presence of antioxidants has also been shown to enhance trypanosome parasite survival not only in the host, but also in the tsetse fly vector. The addition of antioxidants to the blood meal of *Glossina morsitans morsitans* significantly increases trypanosome midgut infections clearly depicting ROS involment in trypanosome death (MacLeod *et al.,* 2006). In addition, Co-Q_{10}, an endogenously synthesized lipid that shuttles electrons from complex 1 and II to complex III of the electron transport chain, is known to enhance cellular energy production via oxidative phosphorylation. Since bloodstream trypanosomes almost entirely rely on energy from glycolysis for their survival in the host (Brohn and Clarkson, 1980), it is possible that the enhanced energy production by infected animals supplemented with Co-Q_{10} favored the very existence of trypanosome parasites within the host. Anthocyanins on the other hand have been implicated in the inhibition of cycloxygenase-1(COX-1) and cycloxygenase-2 (COX-2), enzymes involved in the regulation of inflammatory mediators known collectively as eicosanoids (Seeram *et al*., 2003). Inflammatory mediators such as cytokines, chemokines, nitric oxide and eicosanoids have previously been reported to suppress proliferation of trypanosome parasites and act as immunomodulators during infection (Mukherjee *et al*., 2011). Moreover, prostaglandins especially of the D and J series have been reported to induce programmed cell death in the

bloodstream forms of trypanosomes diminishing parasitaemia levels by elimination of non-dividing stumpy forms of trypanosomes (Figarella *et al.,* 2006). Therefore, it is possible that presence of anthocyanins during infection of mice in this study may have aided parasite survival by suppressing the production of eicosanoids and other inflammatory mediators. Nevertheless, Karori *et al.* (2007) using different types of Kenyan tea infusions reported that tea had the ability to lower blood parasitaemia levels during trypanosomiasis infection and attributed this trypanocidal activity to the toxicity of tea polyphenols which have the ability to bind the parasite cell wall and consequently disrupt its cell membrane. It is therefore possible that polyphenols present in tea act in synergy to exert their trypanocidal properties rather than independently as is the case in this study.

Anemia developed similarly in infected untreated animals and infected animals supplemented with antioxidants. Anemia, previously pronounced as the primary cause of death during trypanosomiasis infection (Jenkins and Facer, 1985), was observed as rapid decline in PCV levels and was evident immediately after infection coinciding with the first wave of parasitaemia in both infected terminal groups and infected PTRE groups. This rapid decrease in PCV levels during the first wave of parasitaemia has been reported in previous studies (Murray and Dexter, 1988; Ndung'u *et al.,* 2008). It is clear therefore that an increase in parasitaemia levels is associated with a plethora of mechanisms which induce anemia such as entry of trypanosome surface coat VSG into erythrocytes enhancing complement mediated lysis (Rifkin and Landsberger, 1990), binding of autologous antibodies of the IgG and IgM type and C_3 complement protein to erythrocytes (Kobayashi *et al.,* 1976), sensitization of erythrocytes with immunoglobulin M-antigen complexes (Amole *et al.,* 1992) and retention and accumulation of erythrocytes in the spleen leading to massive destruction via erythrophagocytosis in the spleen and liver (Murray and Dexter, 1988). In infected PTRE groups, there was a rise in PCV levels 22 dpi and this correlated with berenil treatment and subsequent disappearance of trypanosome parasites in mice. This phenomenon indicated that the trypanosome strain used in this experiment was highly susceptible to this trypanocide and that anemia in infected animals is positively correlated with parasite load in blood. This was further validated by the decrease in PCV levels as from the seventh week post infection coinciding with the relapse of parasites back into the bloodstream to cause a secondary infection. However, relapse of parasites into the bloodstream was delayed in the antioxidant groups though not significantly. This can be attributed to the

neuroprotective effects of flavonoids known to down regulate astrocytosis and microgliosis and the resultant neuroinflammatory condition thereby maintaining the integrity of the blood brain barrier (Rrapo *et al.,* 2009). Similarly, Co-Q_{10} which is an extremely lipophilic compound is also known to inhibit the production of inflammatory mediators responsible for the breakdown of the blood brain barrier (Sharma *et al.,* 2006).

Results from this study have shown that trypanosomiasis infection in mice significantly reduced the levels of brain GSH. Previous findings have associated trypanosome infection with drops in GSH presumably due to the marked oxidative stress with concomitant utilization of GSH (Igbokwe *et al.,* 1996; Igbokwe *et al.,* 1998; Ranjithkumar *et al.,* 2011). Furthermore, trypanosome parasites contain a unique GSH analogue, trypanothione (TSH) that is present almost entirely in the dithiol-reduced form and which serves to reduce free hydroxyl radicals in the parasites protecting them against oxidative stress (Shames *et al.,* 1986; Smith *et al.,* 1992). TSH differs from GSH by the covalent linkage of two GSH tri-peptides by a spermidine molecule via glycine carboxylates (Smith *et al.,* 1992). Trypanothione synthetase, an enzyme responsible for the synthesis of TSH is highly specific for glutathione and the parasite antioxidant TSH is consequently synthesized from its mammalian equivalent, glutathione (Smith *et al.,* 1992: Comini *et al.,* 2003). This implies that the trypanosome parasites shield themselves from oxidative stress at the expense of the mammalian host and this could explain the reduced levels of GSH in the brain tissue. However, the presence of antioxidant Co-Q_{10} reversed the trypanosomal decrease in GSH and restored the levels of this endogenous thiol to normal levels in the brain tissue compared to uninfected healthy animals. Indeed, antioxidants have been shown on several instances to protect against decreases in GSH following infection with trypanosome parasites. Omar *et al.* (2010) proved that antioxidants are able to significantly ameliorate trypanosomiasis induced drop in GSH by elevating the amounts of GSH above even the levels recorded in healthy uninfected animals. In addition, the aqueous extracts of *Hibiscus sabdariffa*, known for its high content of ascorbic acid has been shown to have a sparing effect on systemic antioxidant reserves in *Trypanosoma congolense*–infected rats (Umar *et al.,* 2009).

Infected animals treated with the test antioxidants in this study had significantly higher levels of aconitase-1 than infected untreated animals. This can be attributed to the extreme oxygen lability of iron-sulphur clusters in aconitase that renders this metalloprotein extremely sensitive to oxidative stress, a condition highly associated with pathogenesis of trypanosomiasis.

Oxidative stress is thought to arise from the oxidative burst of macrophages, depletion of endogenous antioxidants and production of enormous amounts of ROS by bloodstream forms of trypanosomes (Meshnick *et al*., 1977). Once oxidized, aconitase-1 assumes an abnormal structure characterized by increased exposure of hydrophobic patches and loss of antigen-recognition sites with concomitant loss or decrease in enzymatic activity (Bota and Davies, 2002). Consequently due to its vital role in the generation of NADPH, an essential cofactor in the reduction of oxidized glutathione GSSG to the reduced form GSH, loss or decrease in activity of aconitase-1 would further aggravate oxidative stress due to the disease. Oxidized aconitase is then preferentially degraded via the proteolytic activity of Lon protease, a homologue of the bacterial Lon protease (Wang *et al.*, 1993; Bota and Davies, 2002). Therefore, higher concentrations of aconitase are expected in the presence of antioxidants such as those used in this study which potently scavenge for free radicals sparing this metalloprotein from oxidative damage.

Additionally, inflammatory response in CNS pathology due to the parasites and post melarsoprol treatment was investigated in the present study. Inflammation has highly been implicated in the pathogenesis of late stage CNS HAT and PTRE complication (Pepin and Milrod, 1994). Following the development of PTRE in an experimental mouse model that mirrors many of the pathological features of PTRE in human subjects, a variety of inflammatory mediators are produced such as TNF-α, IL-1, IL-4, IL-6 and macrophage inflammatory protein (MIP)-1 (Kennedy, 1999). This inflammatory condition, highly amplified by use of trypanocidals such as diaminazine aceturate which does not cross the blood brain barrier sparing trypanosomes present in the CNS, is characterized by chronic meningoencephalitis. Previous studies have suggested that the inflammatory response observed can be attributed to astrocytes which are brain cells with the capability of producing pro-inflammatory cytokines such as TNF-α and IL-1 when activated, contributing to the apoptotic cell death of neurons observed in many neurodegenerative diseases (Kennedy, 1999; Vauzour *et al.,* 2008). This phenomenon is validated by the fact that production of several cytokine transcripts within the CNS correlate with the onset of astrocyte activation (Hunter *et al.,* 1991; Hunter *et al.,* 1992b). However, other brain cells such as the microglia are also capable of producing inflammatory cytokines exacerbating the neuroinflammatory condition (Hanisch, 2002). As a result, several anti-inflammatory agents have been evaluated with an aim of ameliorating the severity of late CNS stage HAT and PTRE

complication. Hunter *et al.* (1992c) was able to establish that azathioprine, a potent anti-inflammatory agent and immunosuppressant significantly reduced the degree of CNS inflammation prior to the induction of PTRE but had no effect in an established PTRE. Kennedy *et al.,* (1997) also demonstrated that RP-67 580, an SP antagonist significantly reduced severity of an established meningoencephalitis as well as degree of astrocyte activation in a PTRE mouse model. Steroidal anti-inflammatory drugs such as prednisolone have also been tested clinically and proved efficacious to some extent in ameliorating PTRE (Pepin *et al.,* 1989).

These observations raised the possibility that oral administration of ACN's and/or Co-Q_{10}, compounds whose protective effects have widely been ascribed to their anti-inflammatory properties, may have positive effects in preventing and/or reducing the severity of the meningoencephalitis in late stage CNS HAT and PTRE complication. Consistent with this hypothesis, results in the present study have demonstrated that Kenyan purple tea ACN's and Co-Q_{10} can be used in an experimental mouse model to ameliorate CNS inflammation highly implicated in late stage HAT and PTRE complication. These compounds, both characterized as being powerful anti-oxidants and anti-inflammatory agents, might be mediating their effect by inhibiting the production of inflammatory cytokines and antigen presentation by astrocytes and microglia. Indeed, anthocyanin rich extracts have been shown to inhibit production of inflammatory mediators such NO, IL-1β and TNF-α in activated microglia cells (Lau *et al.,* 2007). Moreover, ACN's have been shown to inhibit COX-2 expression, an isoenzyme highly up-regulated in inflamed cells and involved in inflammatory processes by blocking the mitogen-activated protein kinase (MAPK) pathway, with simultaneous modulation of the NF-κB pathway and activator protein-1 (AP-1) (Hou, 2005). Furthermore, ACN's extracts have been shown to down regulate a wide array of inflammatory mediators' including inducible nitric oxide synthase (iNOS), TNF-α, IL-1β, IL-6, and the cytokine-induced neutrophil chemoattractant-1 (CINC-1) in a zymosan-induced inflammatory mouse model (Tsuda *et al.,* 2002). On the other hand, Co-Q_{10} has also been observed to inhibit the expression of IL-6, TNF-α and NF-κB, an anti-inflammatory effect mediated via gene expression modification and/or antioxidant/radical-scavenging activity of CoQ_{10} (Sharma *et al.,* 2006; Schmelzer *et al.,* 2008). Nevertheless, the possibility that these nutraceuticals might be mediating their effect directly by reducing the influx of inflammatory cells in the brain with concomitant reduction in inflammatory parameters associated with the infection and PTRE complication cannot be overruled.

In conclusion, findings of this study provide further compelling evidence for the role of antioxidant failure in CNS pathology due to *T. b. rhodesiense* parasites and post melarsoprol treatment. Moreover, it was observed that consumption of Co-Q_{10} and/or Kenyan purple tea ACN's resulted in a significant reduction in the clinical features associated with late stage CNS HAT and PTRE complication. Therefore, the test antioxidants used in this study have potential application for use as adjuncts to current trypanocidal therapy in CNS-stage disease to help prevent or modulate PTRE complication that kills about 5% of all patients who are treated with the organic arsenical melarsoprol.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The following conclusions are drawn from this study:

i) Invasion of *T. b. rhodesiense* parasites in the brain cause depletion of aconitase-1 and GSH impairing the brain's antioxidant defenses. The condition is further exacerbated by the use of melarsoprol which also causes a decrease in endogenous antioxidant reserves in the brain. Decreased aconitase-1 and GSH levels could result in oxidative stress in the brain. This observation suggests that oxidative stress probably plays a significant role in the pathogenesis of *T. b. rhodesiense* infection and/or melarsoprol induced reactive encephalopathy.

ii) Kenyan purple tea anthocyanins are able to cross the blood brain barrier and exert their physiological effects by up-regulating endogenous antioxidant reserves.

iii) Co-Q_{10} and Kenyan purple tea anthocyanins restore aconitase-1 and GSH to adequate levels in the brain following *T. b. rhodesiense* infection and/or melarsoprol treatment thereby preventing fall in systemic antioxidant reserves. Restoration of endogenous antioxidants implies that Co-Q_{10} and Kenyan purple tea anthocyanins boost the antioxidant capacity in the brain conferring neuroprotection in mice model of HAT and/or PTRE.

iv) Co-Q_{10} and/or Kenyan purple tea anthocyanins assuage inflammation due to parasites and/or post melarsoprol treatment in the brain resulting in a significant reduction of clinical features associated with *T. b. rhodesiense* infection and PTRE complication in an experimental mouse model.

5.2 Recommendations

The following recommendations are drawn from this study:

i) Antioxidants Co-Q_{10} and Kenyan purple tea anthocyanins should be pursued as supplement to the therapeutic effects of existing medications in late stage HAT enhancing treatment outcome and reducing PTRE occurrence.

ii) Co-Q_{10} and Kenyan purple tea anthocyanins should be studied as suitable candidates for consideration as dietary supplements to modulate conditions associated with oxidative stress in the brain such as Alzheimer's and Parkinson's disease, Amyotrophic lateral sclerosis (ALS) and Multiple sclerosis.

iii) Anthocyanins extracted from the Kenyan purple tea produced a free flowing powder that was bright red in colour with a characteristic smell of berries. These pigments can therefore be targeted in the production of natural food colorants and capsules for use in the pharmaceutical industry. Moreover, ACN's from Kenyan purple tea can be used to enhance the health benefits of ready to drink beverages in the Kenyan market.

iv) Tea anthocyanins were shown in this study to cause a drop in PCV levels. Studies should be carried out to decipher the mechanisms involved and thus develop ways of counteracting the effect.

v) Further work should be carried out to extend these findings and to unravel other possible mechanisms under which Co-Q_{10} and Kenyan purple tea anthocyanins mediate their effect.

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APPENDICES

APPENDIX I: Formula for determination of individual anthocyanin

$$\frac{(\mathbf{A_{sample}} - \mathbf{A_{intercept}}) \times \mathbf{V} \times \mathbf{d} \times \mathbf{100}}{\mathbf{Slope}_{\mathbf{anthocyanin}} \times \mathbf{m} \times \mathbf{DM}}$$

Where:

$\mathbf{A_{sample}}$	is the peak of the individual anthocyanin in the test sample
$\mathbf{A_{intercept}}$	is the peak area at the point the individual anthocyanin calibration line intercepts the y-axis
$\mathbf{Slope}_{\mathbf{anthocyanin}}$	is the individual anthocyanin calibration line slope
V	is the sample extraction volume
d	is the dilution factor
m	is the mass, in grams of the sample test portion
DM	is the dry matter content in percentage

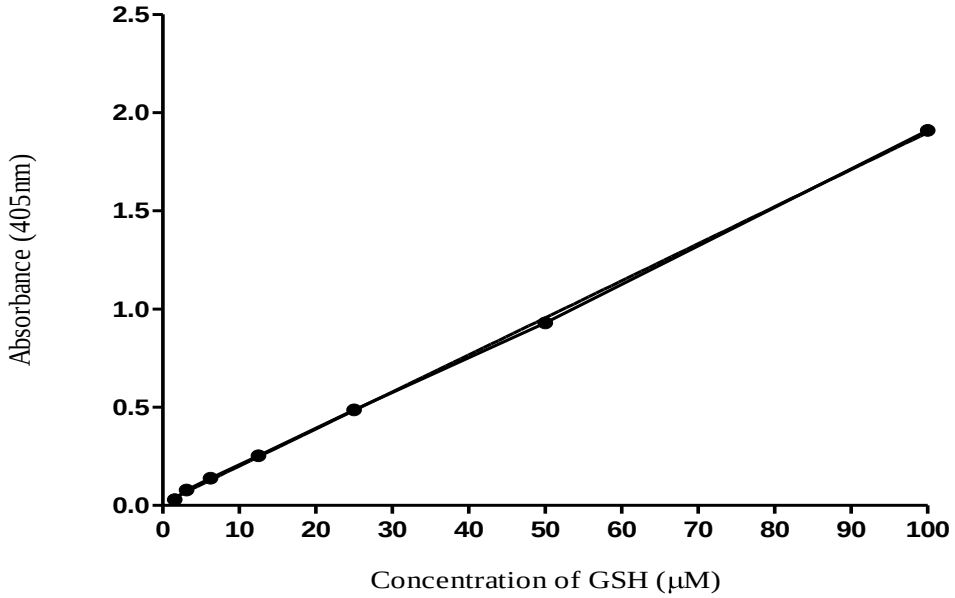
APPENDIX II: SDS-PAGE gel reagents

Phosphate buffered saline (PBS) pH 7.4	0.14M NaCl 8.00g/l 2.7 mM KCl 0.2g/l 1.5mM KH_2O_4 0.2g/l 8.1 mM Na_2HPO_4 1.15g/l
Acrylamide/bisacrylamide stock solution 30%: 1%	30g acrylamide and 1g bisacrylamide dissolved in about 50mls distilled water. The volume was then adjusted to 1000mls, filtered and stored at 4^oC.
1.5M Tris-HCl pH 8.8	18.16g of Trizma base dissolved in 70mls, pH adjusted to 8.8 using 3M HCl and final volume adjusted to 100mls using distilled water.
0.5M Tris-HCl pH 6.8	6.06g of Tris-HCl dissolved in 70mls, pH adjusted to 6.8 using 3M HCl and final volume adjusted to 100mls using distilled water.
10% Sodium dodecyl sulphate (SDS)	10g SDS dissolved in 100mls distilled water and stored at room temperature
10% Ammonium persulphate (APS)	0.1g dissolved in 1ml distilled water and stored at 4^oC. This was made fresh for each use

APPENDIX III: SDS-PAGE setup

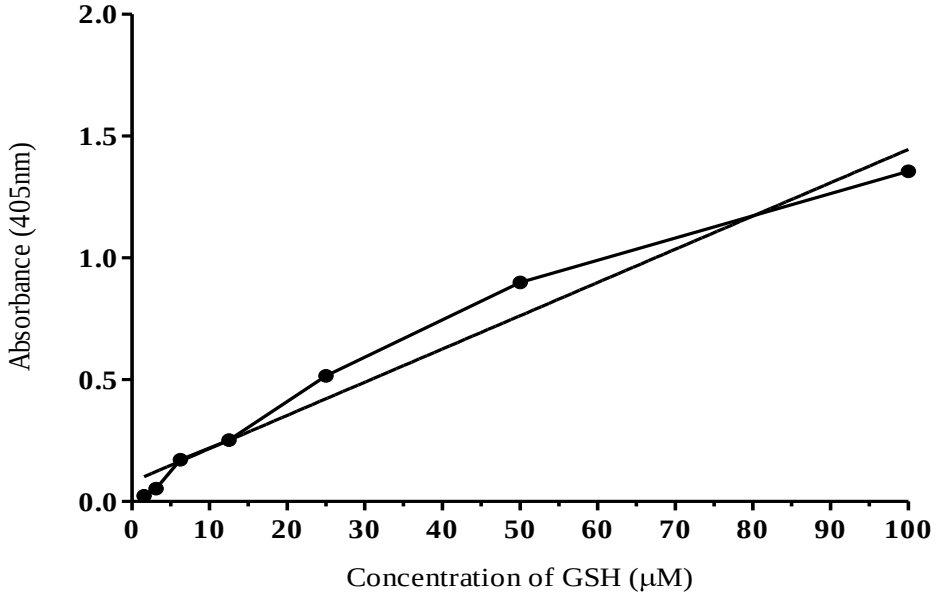
Recipe	Resolving gel 10%	Stacking gel 5%
Acrylamide/Bis-acrylamide stock solution (mls)	10	1
1.5M Tris-HCl pH 8.8 (mls)	7.5	-
Distilled water (mls)	12.15	3.42
0.5M Tris- HCl	-	1.5
10% SDS (µl)	600	60
TEMED (µl)	20	15
APS (µl)	100	50

APPENDIX IV: Total GSH calibration curve-1



Goodness of fit: r^2= 0.9996

APPENDIX V: Total GSH calibration curve-2



Goodness of fit: r^2= 0.9686