COMPARATIVE EFFICACY STUDY OF DIMINAZINE ACETURATE, KAEMPFEROL AND THEIR COMBINATION ON EXPERIMENTAL *Trypanosoma brucei brucei* INFECTION IN MICE

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLLO UNIVERSITY ZARIA, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN VETERINARY PHARMACOLGY

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JANUARY, 2018

DECLARATION

I declare that the work in this dissertation titled "Comparative efficacy study of diminazene aceturate, kaempferol and their combination on experimental *Trypanosoma brucei brucei* infection in mice" has been performed by me in the Department of Veterinary Pharmacology and Toxicology. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this dissertation was previously submitted for another degree, diploma or certificate at this or any other institution.

Ya'u MUHAMMAD	**Signature**	**Date**

CERTIFICATION

This dissertation titled "Comparative efficacy study of diminazene aceturate, kaempferol and their combination on experimental *Trypanosoma brucei brucei* infection in mice" by Ya'u MUHAMMAD meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my beloved mother, Hajiya Khadijah Musa Isah (Mama majali).

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Glory is to Almighty Allah, Who gave me the strength, wisdom and sound health to carry out this work. May His peace and blessings be on Prophet Muhammad (S.A.W), his household and his companions.

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Abstract

In this study, the comparative efficacy study of kaempferol, diminazene aceturate and their combination against experimental *Trypanosoma brucei brucei* infection in mice was determined. Thirty six adult swiss albino mice both sexes weighing between 18 and 22 grams were randomly divided into six groups (I, II, III, IV, V and VI) of six mice each. Mice in group I were neither treated nor infected (un-infected, normal control). Mice in group II were pre-treated prophylactically with kaempferol (1 mg/kg *per os*) for 14 consecutive days prior to infection. Mice in groups II to VI each were inoculated with blood containing *Trypanosoma brucei brucei* (10^6 trypanosomes/ml of blood/animal) intraperitoneally (I.P). Following detection of parasitaemia, mice in group III were treated once with diminazene aceturate (3.5 mg/kg) I.P only. Mice in group IV were treated with diminazene aceturate (3.5 mg/kg) once I.P, and then continued with kaempferol (1 mg/kg *per os*) for nine consecutive days. Mice in group V were treated with kaempferol (1 mg/kg *per os*) only for nine consecutive days. Mice in group VI were given normal saline (5 ml/kg *per os*) only for nine consecutive days. Nine days post-infection, all mice were sacrificed by severing their jugular veins, blood samples were collected for haematological and biochemical analyses, while tissue samples were collected for histopathological examination. The results obtained showed significant differences between the levels of parasitaemia and survival rate of mice in groups II and VI. Following infection with *Trypanosoma brucei brucei* mean packed cell volume, haemoglobin concentration and red blood cell count significantly ($P < 0.05$) decreased in groups II and VI when compared to groups III, IV and V. Similarly, Mean corpuscular volume and mean corpuscular haemoglobin concentration significantly ($P < 0.05$) decreased in groups II and VI when compared to groups III, IV and V. The percentage of red blood cell haemolysis at 0.5%, 0.7% and 0.9% NaCl concentrations was significantly ($P < 0.05$) higher in groups II and VI when compared to groups III, IV and V.

Total leucocyte count significantly ($P < 0.05$) decreased in groups II and VI when compared to groups III, IV and V. The mean lymphocytes and neutrophil count reduced significantly ($P < 0.05$) in groups II and VI when compared to groups III, IV and V. There were significant ($P < 0.05$) increases in the mean alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase in groups II and VI when compared to groups III, IV and V. Antioxidant enzymes; superoxide dismutase, catalase and glutathione peroxidase increased significantly ($P < 0.05$) in groups III, IV and V when compared to groups II and VI. Serum level of malondialdehyde significantly ($P < 0.05$) increased in groups II and VI when compared to groups III, IV and V. In conclusion, kaempferol possesses antitrypanosomal activity and probably stimulate host immunity to control the proliferation of the parasite in the blood.

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LIST OF ABBREVIATIONS

ALP = Alkaline phosphatase

ALT = Alanine aminotransferase

AST = Aspartate aminotransferase

CAT = Catalase

DNA = Deoxyribonucleic acid

EOF = Erythrocyte osmotic fragility

GPx = Glutathione peroxidase

H_2O_2 = Hydrogen peroxide

Hb = Haemoglobin

Kf = Kaempferol

MCH = Mean corpuscular haemoglobin

MCHC = Mean corpuscular haemoglobin concentration

MCV = Mean corpuscular volume

MDA = Malondialdehyde

NO^- = Nitric oxide

PCV = Packed cell volume

RBC = Red blood cell

RNS= Reactive nitrogen species

ROS= Reactive oxygen species

SOD = Superoxide dismutase

TBA = Thiobarbituric acid

WBC =White blood cell

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Trypanosomosis is a disease caused by the protozoan parasite from genus Trypanosoma and transmitted by the tse- tse fly (Glossina species) and other biting flies (Masocha *et al.,* 2012), making the incidence of the disease to be of great concern in the tropics (Anosa *et al*., 1983). The trypanosomes which affect both man and animals have been subdivided into two, namely the haematic groups (*Trypanosoma congolense* and *T. vivax*) which always remain in the plasma of the host's blood and the tissue invading group (*T.brucei, T.evansi, T.rhodesiense, T.gambiense* and *T. equiperdum*) which are found extravascularly and intravascularly (Anosa *et al*.,1977).

African animal trypanosomosis or nagana is a disease caused by *T. congolense, T. vivax* and *T. brucei* spp. In wild animals, these parasites cause relatively mild infections while in domestic animals they cause a severe, often fatal disease (Molyneux *et al.,* 1996). All domestic animals can be affected by nagana and the symptoms are fever, listlessness, emaciation, hair loss, discharge from the eyes, oedema, anaemia, and paralysis (Masocha *et al.,* 2012). As the illness progresses the animals become progressively weak and eventually become unfit for work, hence the name of the disease "N'gana" which is a Zulu word that means "powerless/useless" (Molyneux *et al.,* 1996). Because of nagana, stock farming is very difficult within the tsetse belt.

The available means of control involves tsetse control, chemoprophylaxis, chemotherapy and use of trypanotolerant livestock. At present, control of trypanosomosis is chiefly done by chemotherapy and chemoprophylaxis using the following drugs: Diminazine, Homidium, and Isometamidium (Leach and Roberts, 1981; ILRAD Reports, 1990). Diminazene aceturate is probably the most commonly used therapeutic agent for trypanosomosis in livestock in Sub-

Saharan Africa (Geerts and Holmes, 1998), even in Nigeria. However, complete dependence on drugs in many situations of trypanosomosis has been hampered in many areas by their toxic effects, high cost and frequent development of resistance to these drugs by the parasites. This is considered a very serious problem in trypanosomosis control in Africa (Geerts and Holmes, 1998). This results from the fact that the drugs effectively eliminate the parasites from the blood stream and the animal appears recovered, and then undergoes relapse infection which may be characterized by severe neurological infection leading to the death of the affected animal. In Nigeria, the occurrence of drug resistance to available trypanocides has been attributed to the presence of fake drugs, abuse of the existing drugs and inadequate dosing of the drugs in trypanosomosis therapy (Ezeokonkwo *et al.,* 2010). Therefore, the current challenge to the majority of African pastoralists is to optimize the use of the relatively old existing drugs (Ezeokonkwo *et al.,* 2010). In view of this, the use of drug combinations, new therapeutic regimens and the use of Slow Release Devices (SRD) of existing trypanocides have been suggested (Geerts and Holmes, 1998).

Secondary metabolites in plants including flavonoids are responsible for a variety of pharmacological activities (Mahomoodally *et al.,* 2005; Pandey, 2007), recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds (Pandey, 2007). The beneficial health effects of flavonoids have also been related to their antioxidant, anti-inflammatory, antiestrogenic, cardioprotective, cancer chemopreventive, neuroprotective, antidepressant and anxiolytic effects (Butterweck *et al.,* 2000).

Kaempferol is a natural flavonol, a type of flavonoid, found in a variety of plants and plant-derived foods. Kaempferol acts as an antioxidant by reducing oxidative stress. Many studies suggest that kaempferol may reduce the risk of various cancers, and it is currently been studied for possible treatment of cancer (Leopoldini *et al.,* 2006).

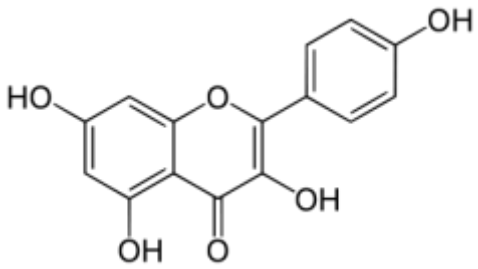


Figure 1.1: Chemical Structure of kaempferol, adopted from (Calderon-montano *et al.,* 2011)

Diminazene aceturate is an odourless complex compound (yellow powder) which is relatively soluble in water but slightly soluble in organic solvent. Diminazene aceturate is effective against infection with *T. vivax* and *T. congolense* but less effective against *T. brucei brucei,* necessitating an increased dose against this species. Diminazene aceturate is used at dose of 7 mg/kg against *T. brucei brucei* instead of 3.5 mg/kg against other species (Brander *et al.,* 1991; Kuriakose *et al.,* 2012). Treatment with diminazene aceturate in dogs infected with trypanosomes is often caused acute clinical signs including vomiting and diarrhoea (Mamman *et al.,* 1993). Diminazene aceturate overdose causes fatal nervous complications after 24-48 hours of administration in dogs. Clinical signs in dogs with diminazene aceturate toxicity that is due to overdose are depression or stupor, continuous vocalization, ataxia, opisthotonos, extensor rigidity, nystagmus and seizures (Boozer and Macintire, 2003).

1.2 Statement of Research Problems

Trypanosomosis is one of the neglected parasitic diseases that affects human health and also largely accounts for the low livestock productivity of the African continent (Welburn *et al.,* 2006). It is estimated that about 70 million people distributed over 1.55 million km^2 in Africa are at risk of the disease (Simarro *et al.,* 2012). On the other hand, animal trypanosomosis is distributed over approximately 25 million km^2 in Africa, where it reduces livestock productivity by up to 50 % (Umar *et al*., 2007).

Pathogenic trypanosome infections of domestic animals are still a major scourge in sub-Saharan Africa and they largely account for the low livestock productivity of the continent (Umar *et al*., 2001), thus making it an important priority for biomedical and public agencies, agricultural sector and the scientific community (Kuriakose *et al.,* 2012). The disease caused by the *Trypanosoma brucei* subgroup is associated with anaemia, hepatocellular degeneration and glomerulonephritis (Umar *et al*., 2008) which is largely attributed to the large amount of free radicals and superoxides generated by the trypanosomes that attack membrane polyunsaturated fatty acids and proteins, resulting in cellular injuries and consequently affecting vital tissues and organs of the infected animals (Umar *et al*., 2001; Igbokwe, 1994; Umar *et al*., 2008) There is little or no chance for production of antitrypanosomal vaccine in the near future because of antigenic variation exhibited by the parasites.

The search for vaccines against African trypanosomosis remains elusive due to antigenic variation in trypanosomes. Hence, prevention of the disease is by vector control which includes the use of targets and traps, bush clearing, spraying of insecticides and sterile insect technique.

However, these control measures though effective, are difficult to sustain (Rogers and Randolph, 1985).

Chemotherapy and chemoprophylaxis by trypanocides using diminazene aceturate and the salts of isometamidium and homidium formed the most important aspect of control and eradication of trypanosomoses (Leach and Roberts,1981; Kinabo, 1993; Anene, *et. al*. 2001). Reports of surveys in Eastern and Southern Africa (Ndung'u *et al*., 1999) and in West Africa (McDermott *et al.,* 2000; Maikai *et al.,* 2007) have shown that the rate of trypanocidal drug resistance to be very high due to the parasites and exhibition of antigenic variation which hampers vaccine production (Kuzoe, 1993). In addition, the use of these trypanocides is beset by numerous limitations, including toxicity and unaffordability of the drugs to the resource poor rural farmers (Kuzoe, 1993; Donald, 1994; Anene, *et. al*., 2001).

1.3 Justification of the Study

The negative impact of trypanosomosis on animals, human health, nutrition and economy is enormous, thereby necessitating continuous research for better ways of controlling the disease (Atawodi, 2005). Also in the absence of effective vaccination, control of trypanosomosis could principally be achieved by means of either chemoprophylactic or chemotherapeutic agents (Maser *et al.,* 2003; Antia *et al.,* 2009). Most of these drugs for the control of both animal and human trypanosomoses are chemically related (Bizimana *et al.,* 2006) and have been in use for decades (Maser *et al.,* 2003; Antia *et al.,* 2009). The repeated use of these trypanocides has led to the development of drug-resistant trypanosome populations. Also, most of these available trypanocides are bedevilled by many undesirable toxic side effects (Deterding *et al.,* 2005) that

are serious and even life-threatening and therefore the need for new, effective, safe, easy-to-administer and inexpensive trypanocides (Hoet *et al.*, 2007).

Unfortunately, the high cost of drugs with increasing adverse effects and the emergence of resistance strains have rendered existing chemotherapy inadequate (Atouguia and Costa, 1999). Therefore, there is need to explore other agents, especially of plant origin for use as new generations of anti-trypanocidal agents that could be more effective, less toxic, and readily available.

Antioxidants are important inhibitors of oxidative species generation and scavengers of free radicals. Antioxidants provide protection to humans and animals against infections and degenerative diseases with oxidative stress aetiology. Kaempferol is a flavonoid and antioxidant, has good peroxynitrite scavenging activity when compared with other phenols and flavonoids (Heijnen *et al.*, 2001; Santos and Mira, 2004). It has been shown to to have neuroprotective effect against L-glutamate toxicity to mouse hippocampal neurons in culture (Li and Pu, 2011), and it also blocked the apoptosis induced by K^+ deprivation of rat cerebellar granule neurons in culture, in parallel to the blockade of the cellular oxidative stress caused by the reactive oxygen species burst observed at the very early stages of this apoptosis just before it becomes an irreversible process (Samhan-Arias *et al*., 2004). Melisa *et al* (2014) reported that kaempferol isolated from the leaves of *Schima wallichii* at a concentration of 250 μM inhibited the growth of *Plasmodium falciparum* in a time-dependent manner both *in vivo* and *in vitro.* Kaemperol is also reported to be effective against hepatic fibrosis induced by schistosoma egg (Zhou *et al.*, 2013)

1.4 Aim of the Study

The general aim of the study was to determine the comparative efficacy of kaempferol, diminazene aceturate and their combination against experimental *Trypanosoma brucei brucei* infection in mice"

1.5 Specific Objectives of the Study

The objectives of the study were to determine the:

- Prophylactic effect of kaempferol against experimental *Trypanosoma brucei brucei* infection in mice.
- Therapeutic effect of kaempferol in mice experimentally infected with *Trypanosoma brucei brucei.*
- Effect of kaempferol in combination with diminazene aceturate in mice infected with *Trypanosoma brucei brucei.*

1.6 Research Questions

- Does kaempferol does possess anti-trypanosomal and antioxidant activity against *T. brucei brucei* infection in mice?
- Does combination of kaempferol and diminazene aceturate have superior anti-trypanosomal activity than diminazene aceturate alone in mice?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Trypanosomes

Trypanosomes are haemo-flagellated protozoan parasites that inhabit the blood plasma, the lymph and various tissues of their hosts. These parasites cause diseases that affect man and animals, affecting cattle rearing and agricultural development (Ikede and Losos, 1972), horses (Neitz and McCully, 1971), dogs (Adewunmi and Uzoukwu, 1979; Omamegbe *et al*., 1984; Ezeokonkwo *et al*., 2010) and laboratory animals (Anosa, 1983). Most species are transmitted by insects. Trypanosomosis is the name of several diseases in vertebrates caused by parasitic protozoan, trypanosomes from the genus *Trypanosoma* (Molyneux *et al*., 1996).

2.2 Human African trypanosomosis

Human African trypanosomiasis (HAT) or sleeping sickness occurs only in Sub-Saharan Africa (WHO, 2006). It is caused by two subspecies of *Trypanosoma brucei*, namely *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* (Uzoukwu, 1970). Wild and domestic animals can host these parasites and may represent under particular conditions an important reservoir of infection for the tsetse flies (WHO, 2000). *Trypanosoma brucei gambiense* causes over 98% of reported cases (WHO, 2000). In human, the first signs of the disease, were fevers, headaches, itchiness, and joint pains which begins one to three weeks post bite (Uzoukwu, 1979), Weeks to months later the second stage begins with confusion, poor coordination, numbness and trouble sleeping (Uzoukwu, 1979). Diagnosis is via finding the parasite in a blood smear or in the fluid of lymph nodes (Uzoukwu, 1979).

2.3 African Animal Trypanosomosis

African animal trypanosomosis or *nagana* is a disease caused by *T. congolense, T. vivax* and *T. brucei* spp. In wild animals, these parasites cause relatively mild infections while in domestic animals they cause a severe, often fatal disease (Molyneux *et al.*, 1996). All domestic animals can be affected by nagana and the symptoms are fever, listlessness, emaciation, hair loss, discharge from the eyes, oedema, anaemia, and paralysis. As the illness progresses the animals become progressively weak and eventually become unfit for work, hence the name of the disease "N'gana" which is a Zulu word that means "powerless/useless" (Molyneux *et al.*, 1996). Because of *nagana*, stock farming is very difficult within the tsetse belt.

2.4 Discovery of the Tsetse fly-Trypanosome

It was the Scottish missionary and explorer David Livingston (1813–1875) who first suggested that *nagana* is caused by the bite of tsetse flies. In 1852, he reported the occurrence of a disease in the valleys of the Limpopo and Zambezi rivers as well as at the banks of the lakes Nyasa and Tanganyika from which all the cattle he carried died after they have been bitten by tsetse flies (WHO, 2006). However, it took another 40–50 years until trypanosomes were identified as the causative agents of *nagana* and sleeping sickness. In 1895, the Scottish pathologist and microbiologist David Bruce (1855–1931) discovered *T. brucei* as the cause of cattle trypanosomosis (*nagana*) (WHO, 2006).

2.5 Transmission and Symptoms

Trypanosomosis is a disease complex caused by several *Trypanosoma* species, transmitted cyclically by the *Glossina species* (tsetse flies), and mechanically by biting flies (*Tabanids* and

Stomoxys) (Luckins and Dwinger, 2004). The disease is characterized by pyrexia, fluctuating parasitaemia, anaemia and immuno-suppression (Ikede and Losos, 1972; Anosa, 1983), and has been described as a complex debilitating and often fatal condition caused by infection with one or more of the pathogenic tsetse-transmitted haemoflagellate protozoan parasites of the genus *Trypanosoma* (Anene *et al*., 2001).

2.6 Pathogenesis

Infected tsetse fly innoculate metacyclic trypanosomes into the skin of animals, where the trypanosomes reside for a few days and cause localized inflammation (chancres) (Pan *et al.,* 2006). The metacyclic trypanosome then enters the lymph and lymph nodes, then the bloodstream, where they divide rapidly by binary fission (Poulnock *et al.,* 2010). *T brucei* species invade tissues and cause tissue damage in several organs. The immune response is vigorous, and immune complexes cause inflammation which contributes to fever and other signs and lesions of the disease (Magez *et al.,* 2008). Antibodies against the surface-coat glycoproteins kill the trypanosomes (Merck, 2015). However, trypanosomes have a large family of genes that code for variable surface-coat glycoproteins that are switched in response to the antibody response, evading immunity (antigenic variation). This antigenic variation results in persistence of the organism. Antigenic variation has prevented development of a protective vaccine and permits reinfections when animals are exposed to a new antigenic type (Merck, 2015; Verckman *et al.,* 1988).

2.7 Pathophysiology of Anaemia in Trypanosomosis

The pathophysiology of anaemia in trypanosomosis is complex and multifactorial in origin (Naessens *et al.,* 2005). It initiates a cascade of events leading to haemolytic anaemia and

cardiovascular collapse (Anosa, 1988). In human trypanosomosis, disseminated intravascular coagulation has been reported (Barret-Connor *et al.,* 1973). Among the complex and multifactorial aetiologies associated with the anaemia is haemolysin, a sensory/excretory product of living trypanosomes. This product is known to lyse red blood cells in the absence of antibodies (*in-vitro*) and haemodilution (*in-vivo*). This mechanism has been adequately described in gold fish (*Carassius auratus*) infected with *Trypanosoma dahilewskyi* (Nazrul-Islam and Woo, 1991) and in murine models infected with *T. b. rhodesiense* (Naessens *et al.,* 2005).

2.7.1 Haemolytic anaemia caused by animal and human trypanosomes

Haemolytic anaemia has been reported in *T. brucei* infection of red fronted gazelles (*Gazella rufifrons*) (Mbaya *et al.,* 2009a), sheep and goats (Edward *et al.,* 1956; Ikede & Losos, 1972), *T.congolense* infection of sheep and goats (Edwards *et al*., 1956), *T. vivax* infection of sheep and goats (Anosa, 1977). Similarly, it was reported in vervet monkeys (*Cercopethicus aethiopes*)(Abenga and Anosa, 2006), and baboons (*Papio anubis*) (Mbaya *et al.,* 2009a) infected with the West African human sleeping sickness trypanosome; *T.b. gambiense*.

2.7.2 Various stages of the anaemia in trypanosomosis

Three phases of anaemia have been reported to occur in trypanosomosis. They are, phase I (acute), phase II (chronic) and phase III (recovery) (Anosa, 1988).

2.7.2.1 Phase I: acute crises

Phase I begins with the initial appearance of trypanosomes in peripheral circulation. The parasitaemia in this case is usually high, fluctuating and evident in most days (Maxie & Losos, 1979; Anosa and Isoun, 1980; Anosa, 1988; Abenga & Anosa, 2006; Mbaya *et al.*, 2009a, b, c; 2010; Mbaya and Ibrahim, 2011; Mbaya *et al.,* 2011). During this phase the anaemia is

morphologically classified as macrocytic and normochromic (Maxie & Losos, 1979; Anosa & Isoun, 1980). At this stage death commonly occurs due to severe pancytopoenia and other pathologies (Anosa, 1988). Sub-acute cases have been produced experimentally in rodents infected with *T. congolense* (Isoun and Esuroso, 1972) and with *T. brucei* (Mbaya *et al.*, 2007, 2010, 2011).

2.7.2.2 Phase II: chronic

Phase II follows the acute crises phase and is characterized by low levels of parasitaemia. The low to moderate erythrocyte value at this point persists with minor fluctuations. This period ranges from several weeks to months. With the *T. brucei* group, which mostly invade tissues, this is the aparasitaemic phase when the parasites becomes establish extravascularly and are less numerous in peripheral circulations (Rabo, 1995) or absent (Mbaya *et al.*, 2007, 2009a, d). In this chronic phase, the morphological classification of the anaemia is normochromic and normocytic (Maxie and Losos, 1979).

2.7.2.3 Phase III: recovery

The third phase is characterized by the low, infrequent or absence of parasitaemia. At this point, declined erythrocyte values begin to return towards pre-infection values and other pathological changes undergo resolution (Anosa, 1988) leading to self-recovery as commonly encountered in trypanotolerant wildlife (Mbaya *et al.*, 2009a).

2.7.3 The mechanism of anaemia in trypanosomosis

The interplay of several factors acting either individually or synergistically contributes to the development of haemolytic anaemia in human and animal trypanosomosis. Most common among these factors are erythrocyte injury caused by lashing action of trypanosome flagella, undulating pyrexia, platelet aggregation, toxins and metabolites from trypanosomes, lipid peroxidation and malnutrition (Murray and Morrison, 1978; Morrison et al., 1981; Saror, 1982; Igbokwe, 1994). Meanwhile, idiopathic (unknown) serum and tumuor necrosing factors are responsible for dyserythropoieses (Mabbot and Sternberg, 1995; Lieu and Turner, 1999; Maclean *et al.*, 2001).

2.7.3.1 Anaemia through mechanical injury to erythrocytes

Anaemia caused by mechanical injury to erythrocyte occurs by the lashing action of the powerful locomotory flagella and microtubule reinforced bodies of the millions of the organisms during parasitaemia (Vickerman and Tetley, 1978; Mbaya *et al*., 2012). Erythrocyte membrane damage has also been associated with adhesion of erythrocytes, platelets and reticulocytes to trypanosome surfaces via sialic acid receptors leading to damages to erythrocyte cell membranes (Bungener and Muller, 1976; Banks, 1980; Anosa and Kaneko, 1983; Shehu *et al*., 2006). As such, several areas of discontinuity occur along the surface of erythrocyte membranes where they adhere to the trypanosomes. Mechanical damage to vascular endothelium has been reported when tissue-invading trypanosomes such as the *T. brucei* group penetrate tissues via the interstices (Anosa and Kaneko, 1983; Mbaya *et al*., 2012).

2.7.3.2 Anaemia caused by trypanosome toxins and metabolites

Living and dead trypanosomes produce various forms of active chemical substances, which can cause erythrocyte injury (Tizzard and Holmes, 1976; 1977; Tizzard *et al*, 1977; 1978a, b, c; Zwart and Veenendal, 1978; Naessens *et al.,* 2005). Common among these chemical substances are proteases, neuraminidase, phospholipase, free fatty acids, pyruvates and aromatic by-products. Neuraminidase has been generated *in vitro* by *T. vivax* during periods of parasitaemia, making erythrocytes prone to phagocytosis (Esievo, 1979; 1982; Mbaya *et al*., 2012). One of the factors that make erythrocytes prone to phagocytosis by the expanded mononuclear phagocytic system (MPS) during trypanosomosis is associated with the activity of neuraminidase. This enzyme cleaves off sialic acids on the surface of erythrocytes and thereby disabling them (Verma and Gautam, 1978; Igbokwe, 1994; Adamu *et al.,* 2009) and by damaging erythropoietin (Igbokwe *et al.,* 1989). Trypanosomes are capable of releasing proteolytic lysosomal enzymes (proteases) from pockets on their flagella and from damaged or dead trypanosomes (Vickermon and Tetley, 1978; Rautenberg *et al*., 1982; Londsdale-Eccles and Grab, 1986; Igbokwe, 1994; Mbaya *et al*., 2012). The enzyme, when released into the general circulation is capable of damaging erythrocytes and vascular endothelium by cleaving sialic acid fractions from the cell membrane in the form of glycopeptides (Cook *et al.,* 1966; Mbaya *et al*., 2012). It was also reported that aromatic amino acids could be metabolized by trypanosome to produce toxic by–products, which acts directly on the erythrocyte cell membrane to cause osmotic fragility and lyses (Igbokwe, 1994). Similarly, phenylalanine could be catabolized to phenylpyruvate, which is proteolytic in nature and inhibitory to mitochondrial gluconeogenesis (Igbokwe, 1994; Mbaya *et al*., 2012). Tryptophan can also be broken down during trypanosomosis to indole-ethanol, which damages erythrocyte cell membranes (Igbokwe, 1994).

2.7.3.3 Anaemia due to lipid peroxidation

The mechanism of aneamia in trypanosomosis is greatly associated with the generation of free radicals and super oxides following lipid peroxidation. These oxidative products generally attack the cellular integrity of erythrocytes during trypanosomosis (Anosa and Kaneko, 1983; Igbokwe, 1994; Umar *et al*., 2007). They also particularly attack erythrocyte membrane polyunsaturated fatty acids and proteins (Slater, 1984; Mbaya *et al*., 2012) or red blood cells directly leading to oxidative haemolysis (Ameh, 1984; Igbokwe, *et al*., 1989; Umar *et al*., 2007). Sialic acids consist of about four derivatives of nine-carbon sugar neuraminic acids (Schauer and Kamerling, 1997; Mbaya *et al*., 2012). It was therefore concluded that anaemia in trypanosomosis might occur due to erythrophagocytosis (Holmes and Jennings, 1976) and may be associated with the formation of antigen-antibody complexes with sialic acids (Audu *et al.,* 1999).

It was also pointed out that trypanosomosis may cause a deficit in the systematic antioxidant capacity of the infected host (Esievo *et al.,* 1982) This has lsoabeen demonstrated in acute *T. b. gambiense* infection in rats (Ameh, 1984; Mbaya *et al*., 2012), *T. evansi* (Wolkmer *et al.,* 2009) and in *T. brucei* infected mice (Igbokwe *et al.,* 1989), where erythrocytes were susceptible to free radical-damage following hydrogen peroxidation. This process in mice led to enhanced oxidative haemolysis.

Peroxidation caused the erythrocytes to produce large quantities of lipid peroxidation byproducts. This is suggestive therefore that erythrocytes of the infected animals possessed decreased antioxidant ability, leading to its inability to withstand oxidative stress (Igbokwe, 1994). *Trypanosoma vivax* produced neuraminidase enzyme, which had a direct relationship with

parasitaemia. It was therefore concluded that neuraminidase produced by trypanosomes *in-vivo*, cleaved off erythrocyte surface sialic acid, making the red cells prone to phagocytosis. Similarly, Nok and Balogun (2003), showed a progressive increase in the level of serum sialic acid corresponding with anaemia and parasitaemia in *T. congolense* infected mice. *Trypanosoma vivax* was observed to be highly erythrogenic in mice, which was probably associated with depressed erythropoietin activity following the cleaving of sialic acid fragments (Igbokwe *et al*., 1989).

2.8 Trypanosomes and Immune Cells

The trypanosomes remain in the bloodstream as extracellular parasites and therefore constantly exposed to the host's immune system. Innate immune cells, especially macrophages are important host defence mechanism against trypanosomes (Oladiran and Beloosevic, 2012). Trypanosomes evades host immune cell recognition, activation and regulation by antigenic variation, over activation of complement system leading to hypocomplementaemia, poly clonal B cell activation and immunosuppression (Paulnock *et al.,* 2010; Magez *et al.,* 2008; Pan *et al.,* 2006). During development of trypanosomes in their invertebrate and vertebrate hosts, the parasite passes through several morphological distinct changes (Vickerman *et al.,* 1988). In case of *Trypanosoma brucei brucei*, the infection is initiated by a haematophagous arthropods, the tsetse fly (*Glossina* species), depositing metacyclic trypanosomes in mammalian host's dermal connective tissue, leading to local inflammatory reaction called 'chancre' characterized by accumulation of macrophages, granulocytes and lymphocytes (Barry and Emergy, 1984). These inflammatory cells are collectively called leukocytes. The parasites rapidly reach lymph and blood circulation and may traverse the wall of blood and lymph capillaries into connective

tissues, always remain extracellular. At a later stage, the parasites may cross the choroid plexus to reach the cerebro-spinal fluid (Turner *et al.*, 1995).

2.9 Control of Trypanosomosis

There is no effective vaccine against trypanosomes because of the phenomenon of antigenic variation exhibited by the parasites (Ezeh *et al.*, 2016). The available means of control involves tsetse control, chemoprophylaxis, chemotherapy and use of trypanotolerant livestock. At present, control of trypanosomosis is chiefly done by chemotherapy and chemoprophylaxis using the salts of three compounds: diminazine, homidium, and isometa-midium (Leach and Roberts, 1981; ILRAD Reports, 1990; Geerts and Holmes, 1998). However, complete dependence on drugs in many situations of trypanosomosis has been hampered in many areas by their toxic effects, high cost and frequent development of resistance to these drugs by the parasites (Ezeh *et al.*, 2016). This is considered a very serious problem in trypanosomosis control in Africa. This results from the fact that the drugs effectively eliminate the parasites from the blood stream and the animal appears recovered, and then undergoes relapse infection which may be characterized by severe neurological infection leading to the death of the affected animal.

In Nigeria, the occurrence of drug resistance to available trypanocides has been attributed to the presence of fake drugs, abuse of the existing drugs and inadequate dosing of the drugs in trypanosomosis therapy (Ezeokonkwo *et al*., 2010).

2.9.1 Mode of actions and side effects of diminazene aceturate

The trypanocidal actions of diminazene aceturate is thought to be by binding to kinetoplast DNA (Mamman *et al.,* 1993) thereby inducing complete and irreversible loss of kDNA in certain

strains of trypanosomes (Ezeh *et al.*, 2016). Due to its molecular structure, Berenil has particular affinity for A-T base pairs in circular DNA and kinetoplast DNA (Ezeokonkwo et al., 2010). Berenil is not licensed for use in humans because of serious side-effects observed in animals, which include tremors, itching, sweating, convulsions, dyspnea, recumbency and vomiting in camels and decreased blood pressure and diarrhea in dogs (Ezeh *et al.*, 2016). The toxic effect of the drug on certain body organs have been reported by several authors, Brander and Pugh (1982), have reported its hypotensive effect, Williamson (1970), also reported its nephro and hepatotoxic effects of diminazene. Similarly, Homeida *et al*, (1981), reported the nephrotoxic effect of the drug in camels.

2.10 History of Antioxidants

As part of the adaptation from marine life, terrestrial plants began producing non-marine antioxidants such as ascorbic acid (vitamin C), polyphenols and tocopherols. The evolution of angiosperm plants between 50 and 200 million years ago resulted in the development of many antioxidant pigments – particularly during the Jurassic period – as chemical defences against reactive oxygen species that are byproducts of photosynthesis (Benzie, 2003). Originally, the term antioxidant specifically referred to a chemical that prevented the consumption of oxygen. In the late 19^{th} and early 20^{th} centuries, extensive study concentrated on the use of antioxidants in important industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fueling of internal combustion engines. (Mattill, 1947).

Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity (German, 1999). Antioxidant

activity could be measured simply by placing the fat in a closed container with oxygen and measuring the rate of oxygen consumption. However, it was the identification of vitamins A, C, and E as antioxidants that revolutionized the field and led to the realization of the importance of antioxidants in the biochemistry of living organisms (Jacob, 1996; Knight, 1998). The possible mechanisms of action of antioxidants were first explored when it was recognized that a substance with anti-oxidative activity is likely to be one that is itself readily oxidized (Moure and Dufraisse, 1922). Research into how vitamin E prevents the process of lipid peroxidation led to the identification of antioxidants as reducing agents that prevent oxidative reactions, often by scavenging reactive oxygen species before they can damage cells (Wolf, 2005).

2.11 Flavonoids

Flavonoids are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection (Dixon *et al.,* 1983). Flavonoids are the most abundant polyphenols in plants, like other phytochemicals, they are the products of secondary metabolism of plants (de la Rosa *et al*., 2010; Han *et al*., 2007; Manach *et al*., 2004). Their activities are structure-dependent, the chemical nature of flavonoids depends on their structural class, degree of hydroxylation and degree of polymerization (Kelly *et al*., 2002).

Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions (Kumar *et al*., 2013; Kumar *et al.,* 2013). The chelation of metals is crucial as it prevent free radical generation which damage target biomolecules (Leopoldini *et al.,* 2006). The antioxidant activity of flavonoids depends upon the arrangement of functional groups about the molecular structure (Pandey *et al.,* 2012). The configuration, substitution, and total number of hydroxyl groups substantially influence several mechanisms of

antioxidant activity such as radical scavenging and metal ion chelation ability (Pandey *et al.*, 2012). The benzene ring hydroxyl configuration is the most significant determinant of scavenging of reactive oxygen species because it donates hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoids radical (Cao *et al.*, 1997).

Chemically, flavonoid have the general structure of 15-carbon skeleton, which consists of two phenyl rings (A and B) and heterocyclic ring (C) (Cappello *et al.*, 2015).

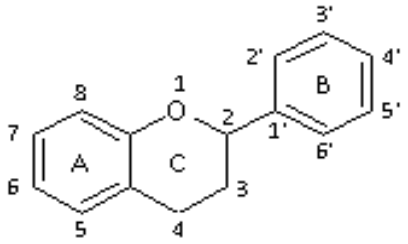


Figure 2.1: General Chemical structure of flavonoids. Adopted from (Calderon-montano *et al.*, 2011).

2.12 Antioxidants

Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals (Kobo *et al.*, 2014). Antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals might otherwise cause (Shetab-Boushehri, 2012). Free radical damage may lead to cancer. Examples of antioxidants include beta-carotene, lycopene, vitamins C, E, A and other substances (Sies, 1997; Eghianruwa, 2012). An antioxidant is also defined as a molecule capable of slowing or preventing the oxidation of other molecules (Abdollahi and Shetab-Boushehri, 2012).

Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which starts chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (Sies, 1997; Abdollahi and Shetab-Boushehri 2012).

Although oxidation reactions are crucial for life, they can also be damaging; hence plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases (Hamid *et al.,* 2010). Low levels of antioxidants, or inhibition of the antioxidant enzymes, causes oxidative stress and may damage or kill cells. As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases (Desoti, *et al.,* 2012).

2.13 Origin of Antioxidants

Antioxidants are abundant in fruits and vegetables, as well as in other foods including nuts and grains (Vijikumar *et al.,* 2011). Kaempferol (flavonoid), a yellow compound with low molecular weight is commonly found in plant-derived foods and in plants used in traditional medicines. Numerous preclinical studies have shown that kaempferol has a wide range oxidant activities (López-Lázar, 2009). Beta-carotene is found in many foods that are orange in colour, including sweet potatoes, carrots, cantaloupe, squash, apricots, pumpkin and mangoes. Some green, leafy vegetables including collard greens, spinach and kale, are also rich in betacarotene (Borek, 1991,

Hamid *et al.*, 2010). Lutein, best known for its association with healthy eyes, is abundant in green, leafy vegetables such as collard greens, spinach, and kale. Lycopene is a potent antioxidant found in tomatoes, watermelon, guava, papaya, apricots, pink grapefruit, blood oranges and other foods. Estimates suggest 85% of American dietary intake of lycopene comes from tomatoes and tomato products (Rodriguez-Amaya, 2003; Xianquan *et al.*, 2005).

Selenium is a mineral, not an antioxidant nutrient. However, it is a component of antioxidant enzymes (Hamid *et al.*, 2010; Pisoschi and Pop 2015). Plant foods like rice and wheat are the major dietary sources of selenium in most countries. The amount of selenium in soil, which varies by region, determines the amount of selenium in the foods grown in that soil. Animals that eat grains or plants grown in selenium-rich soil have higher levels of selenium in their muscle. In the United States, meats and bread are common sources of dietary selenium. Brazil nuts also contain large quantities of selenium (Hamid *et al.*, 2010).

Vitamin A is found in three main forms: retinol (Vitamin A1), 3, 4-didehydroretinol (Vitamin A2), and 3-hydroxyretinol (Vitamin A3). Foods rich in vitamin A include liver, sweet potatoes, carrots, milk, egg yolks and mozzarella cheese (Baublis *et al.*, 2000). Vitamin C is also called ascorbic acid and can be found in high abundance in many fruits and vegetables and is also found in cereals, beef, poultry, and fish (Jain *et al.*, 2013).

Vitamin E, also known as alpha-tocopherol, is found in almonds, in many oils including wheat germ, safflower, corn and soybean oils, and is also found in mangoes, nuts, broccoli, and other foods (Herrera and Barbas, 2001; Elnakish *et al.*, 2013).

2.14 Enzymatic and non-enzymatic antioxidants

Enzymatic antioxidants are mainly endogenous as they exist in our body and are very powerful antioxidants (Majeed *et al.,* 2016). Despite number of enzymes in our body belonging to this group, three super active enzymes are superoxide dismutase, catalase and glutathione peroxidase (Hassanuzaman *et al*., 2012). They catalyze the decomposition of superoxide, hydrogen peroxide and organic peroxides and detoxify oxy radicals and convert them finally into oxygen, water and alcohols. Metal binding proteins such as albumin, transferrin, ferritin, lactoferrin are present in our body and protect us from severe damage by trapping free radicals (Apak *et al.,* 2016).

Non-enzymatic antioxidants are mostly exogenous as our body hardly produce them and need to be outsourced (Majeed *et al.,* 2016), which includes: nutritional antioxidants (such as vitamin C, vitamin E and carotenoids), and minerals (e.g selenium) (Hassanuzaman et al., 2012).

2.15 Plants as Potential Sources of Antioxidants

Synthetic and natural food antioxidants are used routinely in foods and medicine especially those containing oils and fats to protect the food against oxidation (Sindhi *et al.,*2013). There are a number of synthetic phenolic antioxidants which have been widely used in the food, cosmetics, and pharmaceutical industries (Papas, 1999; Rajan *et al.,* 2011). In view of the increasing risk factors of various deadly diseases, there has been a global trend toward the use of natural substance present in medicinal plants and dietary plants as therapeutic antioxidants (Sindhi *et al.,* 2013). Various herbs and spices have been reported to exhibit antioxidant activity (Sindhi *et al.,* 2013).

Many antioxidant compounds, naturally occurring in plants sources have been identified as free radical or active oxygen scavengers (Brown and Rice-Evan, 1998; Jayachitra and Krithiga, 2012). Attempts have been made to study the antioxidant potential of a wide variety of vegetables like potato, spinach, tomatoes, legumes and fruits (Wang *et al.,* 1996; Furuta *et al.*, 1997; Aggarwal *et al.,* 2013). Strong antioxidant activities have been found in berries, cherries, citrus, prunes and olives. Green and black teas have been extensively studied in the recent past for antioxidant properties since they contain up to 30% of the dry weight as phenolic compounds such as flavonols, flavandiols, flavonoids and phenolic acids. In addition to it, there are other phenolic acids (gallic acids) and characteristic amino acids (theanine) in tea (Lin *et al*., 1998; Stoilova *et al.,* 2007). The antioxidant activity of these plants is due to the presence of antioxidants such as flavones, isoflavones, flavonoids, anthocyanin, coumarin lignans, catechins and isocatechins (Nair, *et al.*, 1998; Aqil *et al*., 2006).

2.16 Effects of Antioxidants

Free radicals are highly reactive species having unpaired electrons in their outermost shell (Hamid *et al.,* 2010). Free radicals react rapidly with the membranes eventually causing cellular degeneration and finally death. To overcome this problem, the living system produces many antioxidants or takes the supplement through diet (Samaranayaka and Li-Chan, 2011). Free radicals occur in blood by combining with different chemicals found in polluted air and water etc. They react with the polyunsaturated lipids (lipid peroxidation) of cell membranes along with the normal RNA and DNA, the genetic material of the cell (Warner *et al.,* 2004). An effective antioxidant complex has various types of radical catching antioxidant sites that seek and destroy free radicals at many cellular sites (Imosemi, 2013). A number of scientific studies are going

about addressing the varied health benefits of antioxidant supplementation in processes like stress, ageing, pathogen infestation, apoptosis and neurological diseases (Bonnefont-Rousselot and Collin, 2010), by reducing the cell damaging effects of free radicals. The brain is uniquely vulnerable to oxidative injury, due to its high metabolic rate and elevated levels of polyunsaturated lipids, and hence antioxidants are used as medications to treat various forms of brain injury (Imosemi, 2013). Antioxidants are also being investigated as possible treatments for neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis and as a way to prevent noise induced hearing loss (Warner *et al.,* 2004). People take antioxidant supplements directly from fresh fruits and vegetables containing large amount of flavonoids and antioxidant supplements that contribute to protection against different types of cancers and cardiovascular diseases (Hamid *et al.,* 2010).

2.16.1 Antioxidants as anti-cancer agents

Studies have confirmed the role of antioxidants (e.g. lanthanides, selenium, flavonoids, lycopene and glutathione) as anti-cancerous compounds in bio-coordination chemistry (Sindhi *et al*., 2013). Recent developments in medicinal chemistry have become crucial for improving the design of the compounds, reducing toxic side effects and understanding their mechanism of action. Numerous metal based drugs are widely used in the treatment of cancer.

Lanthanides are antioxidants with known pharmacological activity in radioimmuno and photodynamic therapy and are of specific interest due to their therapeutic radioisotopes nature (Kostova, 2005). It has been reported that lanthanides are coordination compounds with improved pharmacological properties and a broader range of antitumour activity (Blot *et al.,* 1993).

Flavonoids are considered to have potential anticarcinogenic effects (Jain *et al*., 2013) because they are good scavengers of free radicals in the body. Lycopene is a flavonoid and it has been found that lycopene can significantly reduce the risk of prostate cancer in men (Sindhi *et al.,* 2013). Similarly, it is found to prevent cancer of the pancreas, colon, rectum, oesophagus, oral cavity, large bowel, ovaries, cervix and mouth. Lycopenes have a specific role in preventing heart disease and protect the skin against sun damage (Sharoni *et al.,* 2000).

Glutathione is an intracellular antioxidant in the body. It is a tripeptide thiol compound and has been reported to be effective against hepatocellular carcinoma. In another study, it was found that oral administration of glutathione caused regression of liver tumours and increased the survival of tumour bearing in rats (Novi, 1981).

2.16.2 Antioxidants as hepatoprotective agents

Reports have shown that antioxidants have been used in the treatment of various types of liver diseases (Sindhi *et al.,* 2013). Several clinical trials have confirmed the effectiveness of antioxidants (Vitamin C, E, and selenium) in the treatment of hepatocellular carcinoma (Singal *et al.,* 2011).

2.16.3 Antioxidant and nervous system

The importance of the cerebellum is well known in controlling various motor activities in the body and the developing brain is susceptible to the detrimental effects of ROS. It has been reported that antioxidants prevent oxidative damage in cerebellar development and play an important role in the general well-being as well as maintenance of health (Imosemi, 2013). Few

antioxidants have also been reported as therapeutic agents for acute central nervous injury (Singal *et al.,* 2011).

2.16.4 Antioxidants and red blood cells

Their main function of erythrocytes is O_2 and CO_2 transports and repeatedly circulate through the lungs and capillaries during their 120-day life span. As these RBCs are continuously exposed to intracellular ROS derived from antioxidation of oxyhaemoglobin, there is damage to these RBCs (Singal *et al.,* 2011). Studies have confirmed that superoxide dismutase and catalase get accumulated at RBC membrane as first line defence against oxidative stress. It was speculated that glutathione peroxidase act in concert with catalase to protect the whole RBCs (membrane and cytoplasm) from ROS induced damage (Bing, 2009).

2.17 Free Radicals

Free radicals are reactive chemical entities that are short lived species containing one or more unpaired electrons. They also served as signaling agents in normal process of differentiation and migration. Free radicals induce damage to cells by passing the unpaired electron resulting in oxidation of cell components and molecules (Ayepola *et al.,* 2014). They are generally very unstable and reactive.

Oxygen is however essential for survival, reduction of molecular oxygen sequentially produces oxy radicals such as hydroxyl radicals, hydrogen peroxide, superoxide anion radicals often called as free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) like nitric oxide radical (Majeed *et al.,* 2016). Some amount of these free radicals are essentially involved in normal body function, therefore, excess of unsuppressed ROS and RNS could be deleterious

to health (Urquiza-Martínez and Navarro, 2016), even though the body has its own self-defense mechanism to neutralize oxy radicals through the use of endogenous antioxidants. However, oxidative stress, environmental effect, feeding habits as well as aging process, reduce the body defence system (Majeed *et al.,* 2016). Once these free radicals overpower innate anti-oxidant capacity (‘state of oxidative stress’), they attack healthy cells and other organs and ultimately damage healthy cellular DNA causing the progression of several life-threatening diseases like cancer (Singh *et al*., 2015)., diabetes mellitus (Watson, 2014), rheumatoid arthritis (*Kumar et al.*, 2012), ulcers (Demir *et al.*, 2003), stroke (Flamm *et al.*, 1978) and cardiovascular diseases (Maxwell and Lip, 1997; Fiedor and Burda, 2014). Uncontrolled free radicals are responsible for other health disorders such as Parkinson’s and eye diseases such as cataracts (Fletcher, 2010), noise induced hearing loss (Thiagarajan and Manikandan, 2013), aging related disorder (Watson, 2014), and disorient humans being from leading healthy life-style.

2.18 Oxidative Stress

It is universally accepted that oxygen is the major factor that has made the life finite and is one of the important components of aerobic life. However in some circumstances, oxygen may be killer of cells when it generates reactive species that causes necrosis and ultimately the cell death. RNS and ROS also cause oxidation by the generation of certain mechanism that interferes with the normal physiological processes inside the cell (Weseler and Bast, 2010). ‘‘Oxidative stress’’ can be defined as any disturbance in the balance of antioxidants and pro-oxidants in favour of the later due to different factors such as aging, drug actions and toxicity, inflammation and/or addiction (Somogyi *et al.*, 2007), which results in excess formation or/and insufficient removal of highly reactive molecules such as reactive nitrogen species (RNS) and reactive

oxygen species (ROS) (Johansen *et al.,* 2005). Oxygen is highly reactive specie that has the ability to become part of potentially harmful and damaging molecules (Free Radicals). Oxidative stress causes healthy cells of the body to lose their function and structure by attacking them. Until now, the pathogenesis of more than 50 diseases has been implicated to free radicals. It is when the antioxidant level is limited that this damage can become debilitating and cumulative (Kobo *et al.,* 2014). Damage to DNA, proteins, and other macromolecules due to oxidation has been implicated in the pathogenesis of a wide variety of diseases, most notably cancer and heart disease (Halliwell, 1994).

2.19 Biomarkers of Oxidative Stress

2.19.1 Lipids

Studies have shown that polyunsaturated fatty acids in cell membrane are extremely prone to attack by free radicals due to the presence of multiple bond (Butterfiel *et al*., 1998). Lipid hyperperoxides (LHP) through intermediate radical reactions produce such fatty acids that generate highly reactive and toxic lipid radicals that form new LHP (Matough *et al.,* 2012). A critical biomarker of oxidative stress is lipid peroxidation which is the most explored area of research when it comes to ROS (Hatice *et al.,* 2004). Malondialdehyde (MDA) is formed as a result of lipid peroxidation that can be used to measure lipid peroxides after reacting it with thiobarbituric acid (Esterbauer *et al*., 1991).

2.19.2 Vitamins

Vitamins are very important part of biological system as they play important role in different biochemical processes. Among such vitamins are the A, C and E which act as antioxidants by

detoxifying the free radicals. Any alteration in their levels is significant biomarkers of oxidative stress. These vitamins also promote toxicity by acting as pro-oxidants in certain conditions (Maritim *et al.,* 2003).

2.19.3 Glutathione

Disease conditions induce alterations in activity of the enzymes glutathione peroxidase and glutathione reductase. These enzymes are found in cells that metabolize peroxide to water and converting glutathione disulfide back into glutathione (Maritim *et al.,* 2003). Any alteration in their levels will make the cells prone to oxidative stress and hence cell injury.

2.19.4 Catalase (CAT)

Catalase is a regulator of hydrogen peroxide metabolism that can, in excess, cause serious damage to lipids, RNA and DNA. Catlase converts H_2O_2 catalytically into water and oxygen and thus neutralizes it. In the case of catalase deficiency, the cells of the body that contain large amount of mitochondria, undergoes oxidative stress by producing excess ROS that results in dysfunction of the cell involved (Dana, 1991; Patel *et al.,* 2013).

2.19.5 Superoxide dismutase (SOD)

Superoxide dismutase provides first line defense against ROS mediated cell injury by catalyzing the proportion of superoxide into molecular oxygen and peroxide. We can say that superoxide is dismutated to other compounds that are less toxic by SODs (Tiwari *et al.,* 2013).

2.20 Kaempferol

Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a yellow compound, commonly found in plant derived foods (Calderon-Montano *et al.,* 2011). It is a natural flavonol, a type of flavonoid found in a variety of plants and plant-derived foods. Kaempferol acts as an antioxidant by reducing oxidative stress. Many studies suggest that consuming kaempferol may reduce the risk of many cancers, and it is currently under consideration as a possible cancer treatment (Leopoldini *et al.,* 2006). Kaempferol is a yellow crystalline solid with a melting point of 276–278 °C (529–532 °F) (Liu, 2013). It is slightly soluble in water and highly soluble in hot ethanol, ethers, and DMSO. Kaempferol acts as an antioxidant by reducing oxidative stress (Liu, 2013).

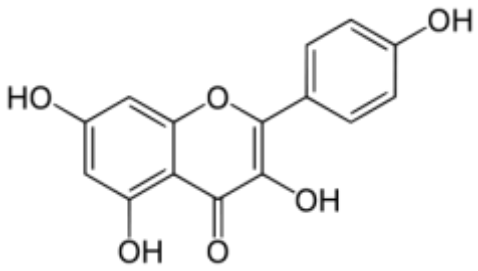


Figure 2.2: Chemical structure of kaempferol adopted from (Calderon-Montano *et al.,* 2011)

2.20.1 Biosynthesis of kaempferol

The biosynthesis of kaempferol occurs in four major steps: (Calderon-Montano *et al.,* 2011).

- Kaemferol is synthesized through the phenylpropanoid pathway, transforming phenylalanine into 4-coumaroyl-CoA.

- 4-coumaroyl-CoA combines with three molecules of malonyl-CoA to form naringenin chalcone (tetrahydroxychalcone) through the action of the enzyme chalcone synthase
- Naringenin chalcone is converted to naringenin and then a hydroxyl group is added to form dihydrokaempferol
- Dihydrokaempferol has a double bond introduced into it to form kaempferol

2.20.2 Pharmacokinetics of kaempferol

Kaempferol when given orally is absorbed in the small intestine, usually by passive diffusion due to kaempferol's lipophilicity, and metabolized in various areas of the body. In the small intestine, kaempferol is metabolized to glucuronides and sulfoconjugates by intestinal enzymes (*Liu*, 2013). It can also be metabolized by colon microflora which can hydrolyze the glycosides to aglycones or form simple phenolic compounds. These compounds can be absorbed or excreted. Kaempferol is also extensively metabolized in the liver to form glucurono-conjugated and sulfo-conjugated forms. These forms of kaempferol, and kaempferol itself, can then be excreted in urine. About 2.5% of ingested kaempferol is excreted in urine. Much of the rest of ingested kaempferol is present in the plasma and tissues in nanomolar concentrations (Calderon-Montano *et al.,* 2011).

2.20.3 Pharmacological activities of kaempferol

Kaempferol has been shown to have an array of antioxidant effects *in vitro* and *in vivo*. At low concentrations, it acts as a super-oxide scavenger, specifically against the highly reactive hydroxyl radical and peroxynitrite species (Kim and Choi, 2013). At high concentrations it increases the activity or expression of antioxidant enzymes such as superoxide dismutase,

catalase, and heme oxygenase-1. Kaempferol can prevent the oxidation of low-density lipid proteins indicating a potential protective role in atherosclerosis (Calderon-montano *et al.,* 2011). Numerous preclinical studies have shown kaempferol and some glycosides of kaempferol to have a wide range of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, antiosteoporotic, estrogenic/antiestrogenic, anxiolytic, analgesic, and antiallergic activities (Calderon- montano *et al.,* 2011).

Epidemiological studies suggest an inverse association between flavonoids intake and risks for certain disease such as cardiovascular and neurodegenerative disease and certain human cancers (Spencer, 2009; Yang *et al.,* 2009; Perez-Vizcaino and Duarte, 2010; Chen *et al.,* 2013a,b). These beneficial roles are closely associated with their antioxidative activities, cardiovascular protection, neuroprotection, anti-inflammation and/or antitumor effects (Chen *et al.,* 2014).

Kaempferol has also been found to protect rats from doxorubicin-induced cardiotoxicity both *in vivo* and *in vitro* Xiao *et al.,* (2012), and has been shown to improve chronic hyperglycemia due to impaired pancreatic beta-cell viability and insulin secretory function (Zhang and Liu, 2011). Li and Pu, (2011), showed the neuroprotective effect of kaempferol in mice with experimental Parkinson's disease. Studies have also showed the antioxidant (Choi, 2011), antiapoptotic (Siegelin *et al.,* 2008), free radical scavenging and anti-inflammatory effects of kaempferol (Singh *et al*., 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Location of the Research

This work was conducted in the Departments of Veterinary Pharmacology and Toxicology and Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University (A.B.U), Zaria, Kaduna State, Nigeria.

3.2 Experimental Animals

Thirty six adult swiss albino mice of either sex weighing between 18 and 22 grams were used in this study. Thee mice were reared in the animal house, Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria. These animals were housed in locally fabricated mice cages at room temperature, 25 ^{o}C. Wood shavings were used as beddings and changed once every week. The experimental mice were allowed free access to rat chow and water *ad-libitum*.

All animal experiments were carried out according to international guidelines as approved by the postgraduate (ethical) committee of the Faculty of Veterinary Medicine, Ahmadu Bello University Zaria.

3.3 The Parasite

Trypanosoma brucei brucei was obtained from the National Veterinary Research Institute (N.V.R.I) Vom, Jos, Plateau State, Nigeria. The parasites was maintained by continuous passage in a donor mouse. Parasitaemia was monitored by use of wet mount viewed under × 400 magnifications (Herbert and Lumsden, 1976).

3.4 Drugs, Sources and Preparation

Kaempferol was sourced from whitehead scientific (pty) limited, South Africa, it came along with the following details; CAS number (520-18-3), Catalog number (3603), EC number (208-287-6) and batch number (3). Diminazene aceturate was purchased from pharmacy unit of the Veterinary Teaching Hospital (VTH).

The drugs (kaempferol and diminazene aceturate) were dissolved in distilled water and administered to each mouse according to the body weight. The concentrations of kaempferol and diminazene aceturate used were 0.5 mg/ml and 3 mg/12.5 ml, respectively.

3.5 Experimental Infection of the Mice

Trypanosomes infected blood was obtained from the tail of the infected donor mice at peak of parasitaemia (10^9) and used to maintain parasite suspension in phosphate buffer saline glucose solution, which was inoculated into peritoneal cavity of uninfected mice. The suspension contained 3 or 4 trypanosomes per microscopic field at × 100 magnification (approximately 10^6 trypanosomes per ml) as described by Ekanem and Yusuf (2008). The Thirty six adult mice were randomly divided into six groups of six mice each and were treated as follows:

Group I- The mice in this group were neither infected with the parasites nor treated with any substance and therefore served as neutral control group.

Group II- Each of the mice in this group was pre-treated individually with kaempferol (1 mg/kg *per os*) for 14 days. Thereafter, each mouse was infected with *Trypanosoma brucei brucei* (10^6 trypanosomes/ml of blood i.p).

Group III- Each of the mice in this group was infected with *Trypanosoma brucei brucei* (10^6 trypanosomes/ml of blood i.p). After infection was established, each mouse was treated once with diminazene aceturate (3.5 mg/kg i.p) intraperitoneally. Animal in this group served as treated controls.

Group IV- Each of the mice in this group was infected with *Trypanosoma brucei brucei*(10^6 trypanosomes/ml of blood). After the establishment of infection, each mouse was treated with diminazene aceturate (3.5 mg/kg i.p) intraperitoneally, and then treated with kaempferol (1 mg/kg *per os*) for 9 consecutive days.

Group V- Each of the mice in this group was infected with *Trypanosoma brucei brucei* (10^6 trypanosomes/ml of blood i.p) and then treated with with kaempferol (1 mg/kg *per os*) for 9 consecutive days.

Group VI- Each of the mice in this group was infected with *Trypanosoma brucei brucei* (10^6 trypanosomes/ml of blood i.p) and then administered normal saline at (5 ml/kg *per os*) for 9 consecutive days. Animals in this group served as untreated controls.

. The parasitaemia in the infected and treated groups was monitored daily using the rapid matching counting method (Herbert and Lumsden, 1976).

3.6 Determination of Parasitaemia in Mice

Parasitaemia was monitored in mice from blood obtained from the tail vein. The number of parasite was determined microscopically at × 400 magnification using rapid matching method (Herbert and Lumsden, 1976).

3.7. Haematological Analyses

At the end of experiment, mice from each group were euthanized by severing the jugular vein. About 1 ml of blood was collected from each mouse in a 10 ml vial containing EDTA as an anti-coagulant and was used to determine the haematological parameters. The packed cell volume (PCV), total erythrocyte count, haemoglobin concentration, and a total leucocyte counts were determined using methods described by Coles (1986). The method descrbed by Schalm *et al.* (1975) was also used to calculate erythrocytic indices from PCV, haemoglobin concentration and erythrocyte count.

3.7.1 Evaluation of packed cell volume (PCV*)*

Packed cell volume was determined using the microhaematocrit method as decribed by Coles (1986).

3.7.2 Evaluation of haemoglobin (Hb) concentration

Cyanmethaemoglobin method described by Coles (1986) was used to determine haemoglobin concentration.

3.7.3 Evaluation of total erythrocyte count

Haemocytometer method described by Coles (1986) was used to determine total erythrocyte count.

3.7.4 Evaluation of erythrocyte indices

The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean morpuscular haemoglobin concentration (MCHC) was calculated from the values of PCV, Hemoglobin concentration and erythrocyte count as described by Schalm *et al.* (1975).

3.7.5 Evaluation of total white blood cell count

The total leucocytes count was determined using haemocytometer method as described by Coles (1986).

3.7.6 Determination of differential leucocyte count

The method described by Decie and Lewis (1991) was used to determine the differential leucocyte ount.

3.7.7 Determination of erythrocyte osmotic fragility (EOF)

Erythrocyte osmotic fragilty of blood obtained from all the mice was determined using method described by Faulker and King (1970) and modified by Oyewale (1991).

3.8. Serum Analyses

Briefly, 2.0 ml of blood was collected from each mouse and allowed to clot. The clotted blood was centrifuged at 1000 × *g* for 10 minutes. The supernant (serum) from each tube was decanted into clear test tube and was used to determine serum level of liver enzymes and biomarkers of oxidatve stress.

3.8.1 Evaluation of enzymes in the serum

Serum level of alanine amino transferase (ALT), aspartate amino trasferase (AST), and alkaline phosphatase (AP) were determined using chemical auto-analyzer (Bayer® Clinical Chemistry Analyzer, Germany).

3.8.2 Determination of serum malondialdehyde concentration

Fifty microlitres of serum from each animal were used to determine the level of serum malondialdehyde by double heating method as described by Draper and Hadley (1990) and modified by Altuntas (2002).

3.8.3 Determination of serum superoxide dismutase (SOD) specific activity

The specific activity of SOD was measured using the method described by Misra and Fridovich (1972) as modified by Rabideau (2001).

3.8.4 Determination of serum catalase (CAT) activity

The specific activity of catalase in the serum collected from each mouse was determined using method descrbed by Beers and Sizer (1952).

3.8.5 Determination of serum glutathine peroxidase (GPx) specific activity

Modified method of Paglia and Valentine (1967) was used to determine glutathione peroxidase (GPx) activity spectrophotometrically.

3.9 Histopathology

Sample of the brain, heart, liver, spleen, lungs and kidneys were obtained from each group, and preserved in 10% buffered formalin for histopathological examination as described by Luna, (1960).

3.10 Statistical Analysis

Data obtained were expressed as means ± standard errors of mean (S.E.M), subjected to one-way analysis of variance (ANOVA) and compared with Tukey post-hoc test. The level of significance was set at $p< 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Clinical Signs and Mean Survival rate in Mice Infected with *Trypanosoma brucei brucei*

Four days post-infection, mice in all infected groups showed anorexia, weakness, pale ocular mucus membrane, loss of weight, rough hair coat and difficulty in breathing as clinical signs of infection with *T. brucei brucei*. These signs were mild in mice treated with diminazene aceturate only (groups III), keampferol and diminazene aceturate (group IV), and keampferol only (group V), but severe with mortality recorded in mice pretreated with keampferol (group II) and those that were administered normal saline (group VI).

Figure 4.1 shows the mean survival rate in mice infected with *T. brucei brucei* and treated with kaempferol, diminazene aceturate and combination of kaempferol and diminazene aceturate. There was no significant difference in the mean survival rate of mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V) when compared to untreated uninfected mice (group I). Similarly, there were significant decreases ($P < 0.001$) in the mean survival rate of mice pretreated with kaempferol (group II) and those that were administered normal saline (group VI) when compared to mice treated with diminazene aceturate only (groups 3), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V).

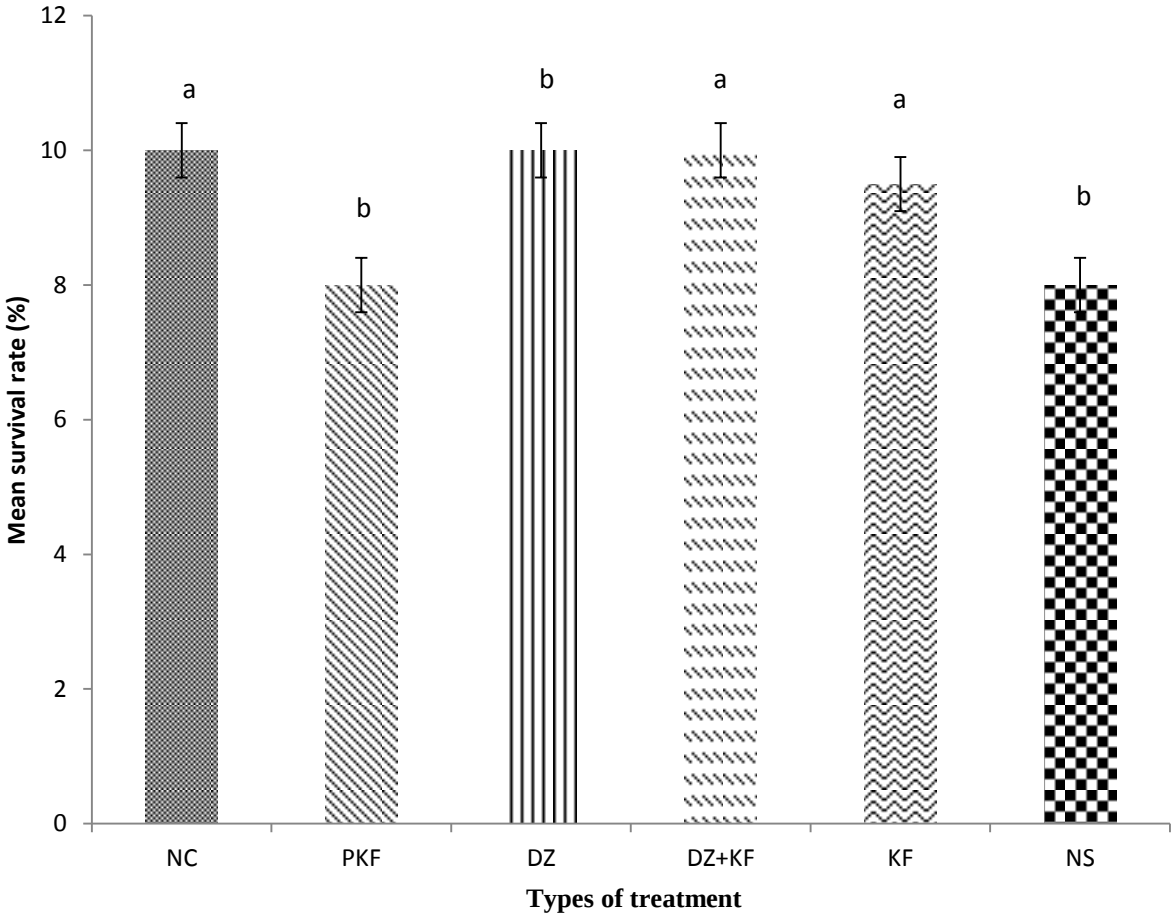


Figure 4.1: Effects of treatments with kaempferol and/or diminazene aceturate on mean survival rate in *Trypanosoma brucei brucei* experimentally infected mice

Means with different letters differ significantly ($P < 0.05$). Values are express as mean ± SEM (6 animals per group).

Key: NC= Neutral control, DZ= Diminazene aceturate, PKf= pre-treated with kaempferol, DZ+Kf= Diminazene aceturate and kaempferol, Kf= kaempferol and NS= Normal saline

4.2 Effects of Treatments with Kaempferol and/or Diminazene Aceturate on the Level of Parasitaemia in Infected Mice

The effects of treatments with kaempferol and/or diminazene aceturate on the level of parasitaemia in all infected mice are shown in Figure 4.2. The level of parasitaemia increased progressively in mice pre-treated with kaempferol (group II) and those that were treated with kaempferol only (group V) and normal saline (group VI) up to day nine post-infection when all animals were sacrificed. The parasites were cleared completely from the blood stream of mice treated with diminazene aceturate only (group III) and those that were treated with diminazene aceturate and kaempferol (group IV) on day six post-infection. Although the onset of parasitaemia was not different from that in other groups, there was a significant ($P < 0.05$) reduction in the level of parasitaemia in mice treated with kaempferol only (group IV) when compared to mice pre-treated with kaempferol (group II) and those that were administered normal saline (group VI).

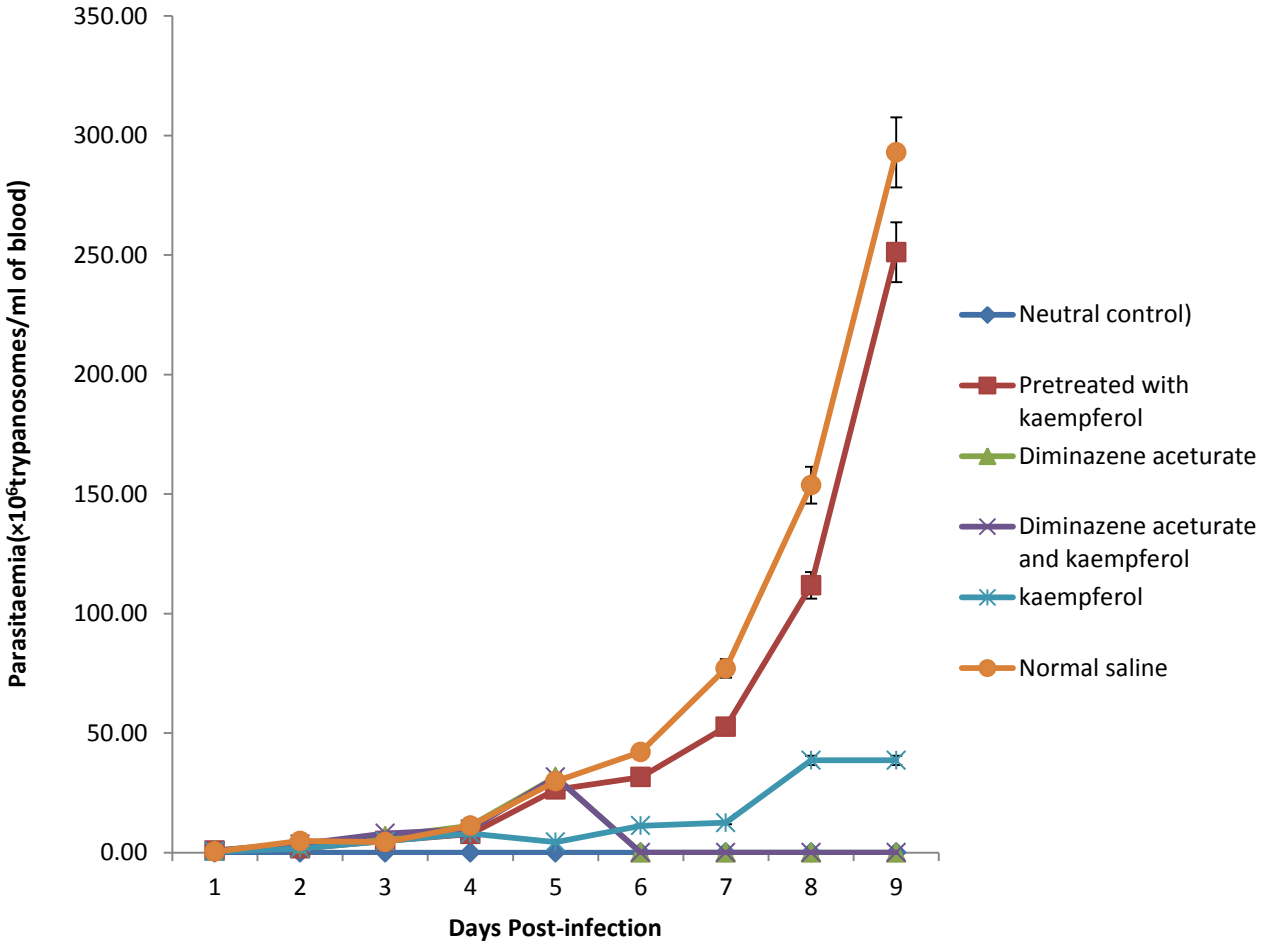


Figure 4.2: Effects of treatments with kaempferol and/or diminazene aceturate on the level of parasitaemia in *Trypanosoma brucei brucei* experimentally infected mice

4.3 Effects of Treatments with kaempferol and/or Diminazene Aceturate on Haematological Parameters

4.3.1 Effects of treatments with kaempferol and/or diminazene aceturate on packed cell volume

Figure 4.3 shows the effects of treatments with kaempferol and/or diminazene aceturate on packed cell volume in mice infected with *T. brucei brucei*. The mean packed cell volume significantly ($P < 0.05$) decreased in mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V) and normal saline (group VI) when compared to untreated uninfected mice (group I). Similarly, there were significant ($P < 0.05$) increased mean packed cell volume in mice treated with treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V) when compared to mice pre-treated with kaempferol (group II) and those that were administered normal saline (group VI).

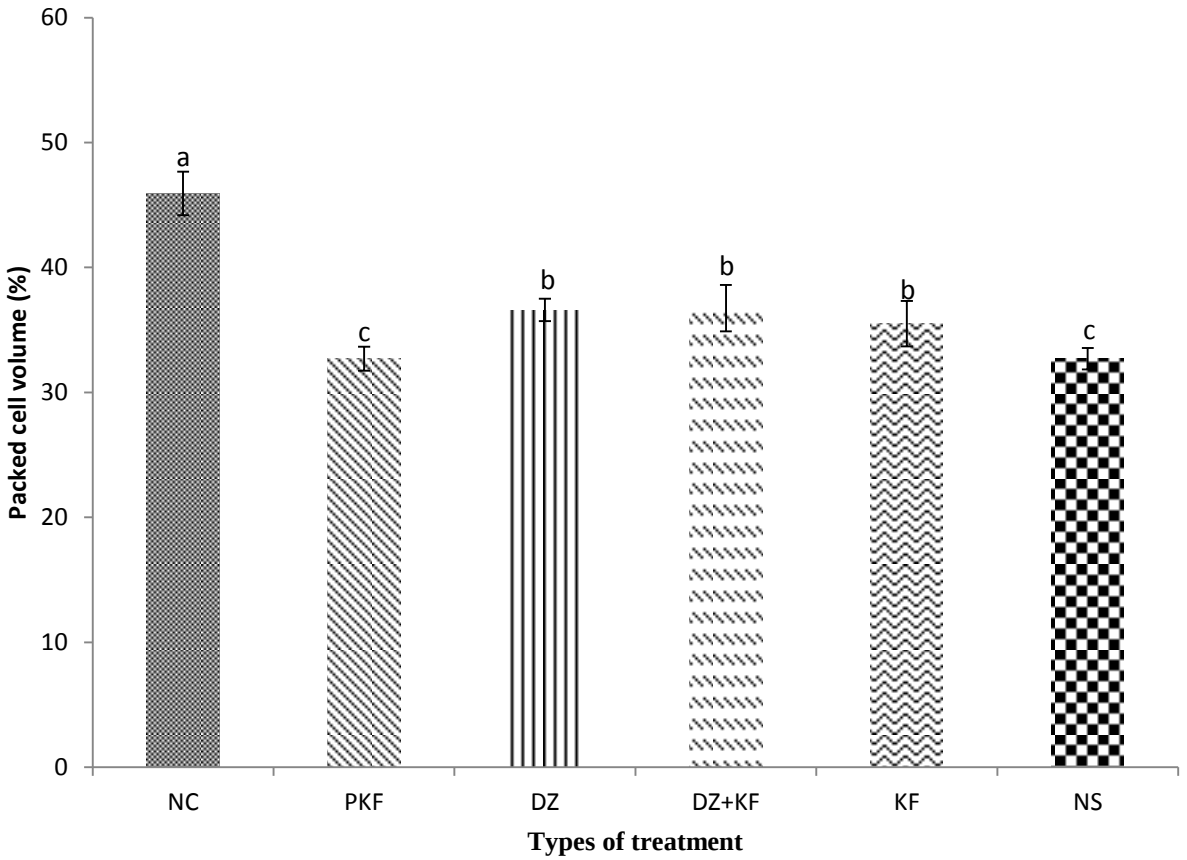


Figure 4.3: Effects of treatments with kaempferol and/or diminazene aceturate on packed cell volume in *Trypanosoma brucei brucei* experimentally infected mice

Means with different letters differ significantly ($P < 0.05$). Values are express as mean ± SEM (6 animals per group).

Key: NC= Neutral control, DZ= Diminazene aceturate, PKf= pre-treated with kaempferol, DZ+Kf= Diminazene aceturate and kaempferol, Kf= kaempferol, NS= Normal saline

4.3.2 Effects of treatments with kaempferol and/or diminazene aceturate on haemoglobin concentration

Figure 4.4 shows the effects of treatments with kaempferol and/or diminazene aceturate on haemoglobin concentrations of mice infected with *T. brucei brucei*. There were significant ($P < 0.05$) increase in the mean heamoglobin concentrations in untreated uninfected mice (group I) when compared to mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV), kaempferol only (group V) and normal saline (group VI). Similarly, there were significant ($P < 0.05$) decrease in the mean haemoglobin concentration in mice pre-treated with keampferol (group II) and those administered normal saline (group VI) when compared to mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V).

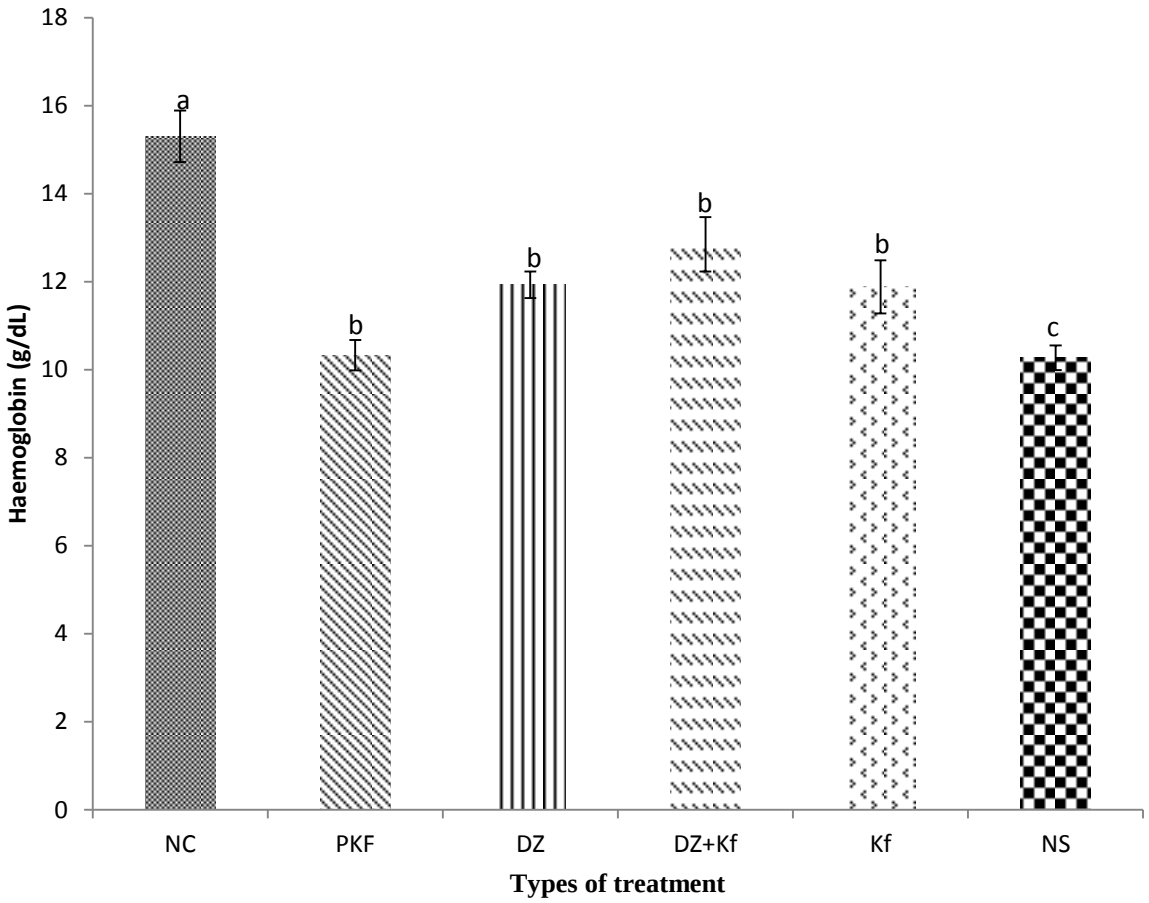


Figure 4.3: Effects of treatments with kaempferol and/or diminazene aceturate on haemoglobin concentration in *Trypanosoma brucei brucei* experimentally infected mice

Means with different letters differ significantly ($P < 0.05$). Values are express as mean ± SEM (6 animals per group).

Key: NC= Neutral control, DZ= Diminazene aceturate, PKf= pre-treated with kaempferol DZ+Kf= Diminazene aceturate and kaempferol, Kf= kaempferol NS= Normal saline

4.3.3 Effects of treatments with kaempferol and/or diminazene aceturate on red blood cell count

Figure 4.5 shows the effects of treatments with kaempferol and/or diminazene aceturate on red blood cell counts of mice infected with *T. brucei bruce.* Mean red blood cell count decreased significantly ($P < 0.05$) in mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV), kaempferol only (group V) and normal saline (group VI) when compared untreated uninfected mice (group 1). Similarly, significant ($P < 0.05$) increase in the mean values of red blood cell counts were recorded in mice treated with diminazene aceturate only (groups 3), kaempferol and diminazene aceturate (group IV), kaempferol only (group V) when compared to mice pretreated with kaempferol (group II) and those that were administered normal saline (group VI).

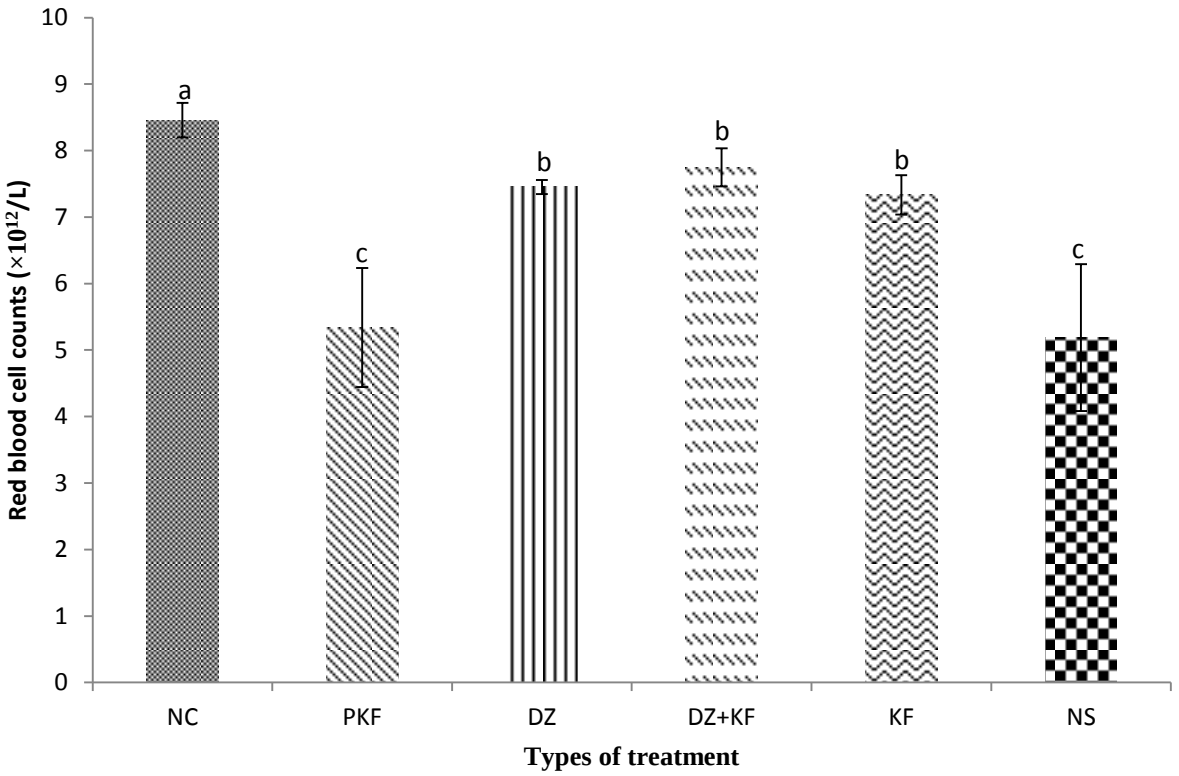


Figure 4.5: Effects of treatments with kaempferol and/ or diminazene aceturate on red blood cell count in *Trypanosoma brucei brucei* experimentally infected mice

Means with different letters differ significantly ($P < 0.05$). Values are express as mean ± SEM (6 animals per group).

Key: NC= Neutral control, DZ= Diminazene aceturate, PKf= pre-treated with kaempferol, DZ+Kf= Diminazene aceturate and kaempferol, Kf= kaempferol and NS= Normal saline

4.3.4 Effects of treatments with kaempferol and/or diminazene aceturate on erythrocytic indices

4.3.4.1 Effects of treatments with kaempferol and/or diminazene aceturate on mean corpuscular volume (MCV)

Figure 4.6 shows the effects of treatments with kaempferol and/or diminazene aceturate on MCV of mice infected with *T. brucei brucei*. There were significant ($P < 0.05$) increase in the mean corpuscular volume (MCV) in untreated uninfected mice (group I) when compared to mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV), kaempferol only (group V) and normal saline (group VI). Similarly, MCV decreased significantly ($P < 0.05$) in mice pre-treated with kaempferol (group II) and those that were administered normal saline (group VI) when compared to mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V).

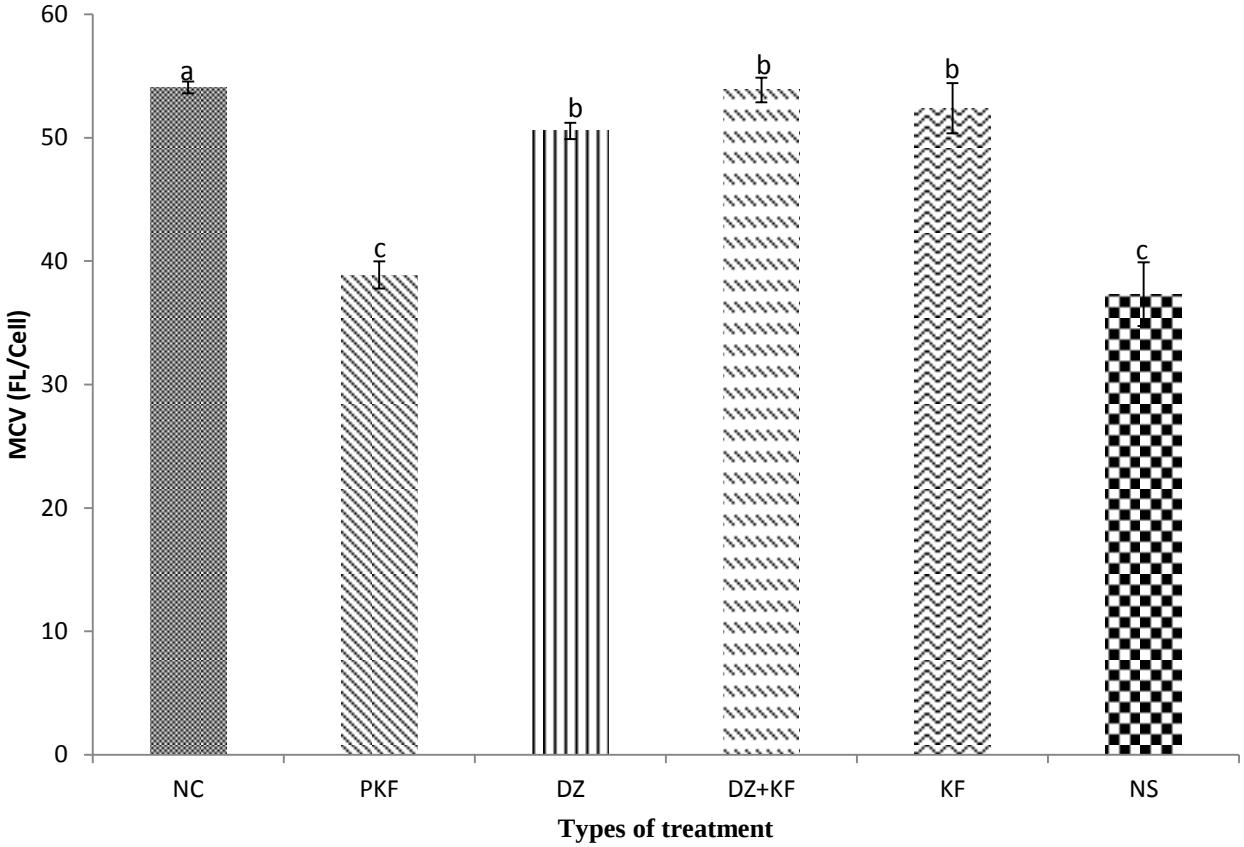


Figure 4.6: Effects of treatments with kaempferol and/or diminazene aceturate on MCV in *Trypanosoma brucei brucei* experimentally infected mice

Means with different letters differ significantly ($P < 0.05$). Values are express as mean ± SEM (6 animals per group).

Key: NC= Neutral control, DZ= Diminazene aceturate, PKf= pre-treated with kaempferol, DZ+Kf= Diminazene aceturate and kaempferol, Kf= kaempferol and NS= Normal saline

4.3.4.2 Effects of treatments with kaempferol and/or diminazene aceturate on mean corpuscular haemoglobin concentration (MCHC)

Figure 4.7 shows the effects of treatments with kaempferol and/or diminazene aceturate on MCHC of mice infected with *T. brucei brucei.* There were significant ($P < 0.05$) decrease in the mean corpuscular haemoglobin concentration (MCHC) in mice pre-treated with kaempferol (group II) and those that were administered normal saline (group VI) when compared to mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V). Similarly, MCHC decreased significantly ($P < 0.05$) in mice pre-treated with kaempferol (group II) when compared to mice administered normal saline (group VI)

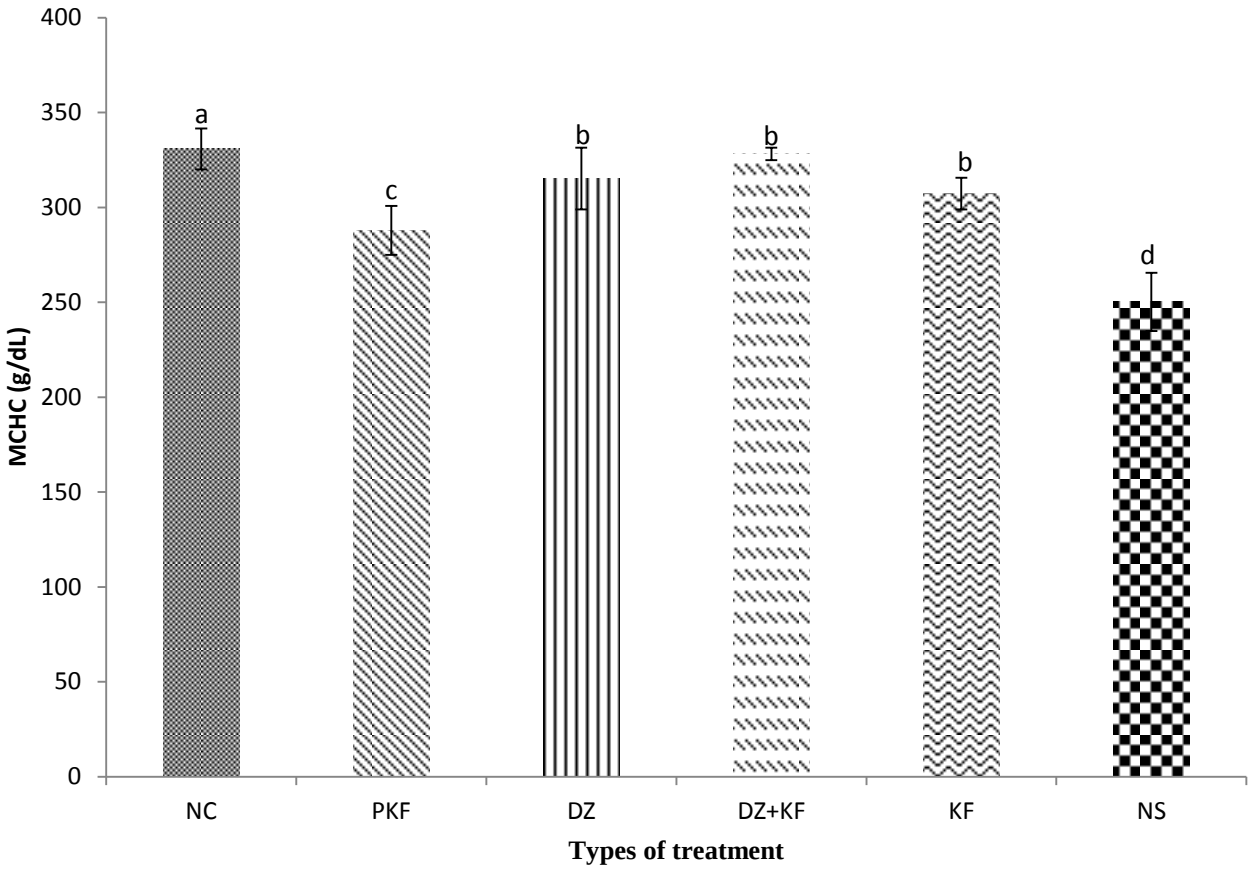


Figure 4.7: Effects of treatments with kaempferol and/or diminazene aceturate on MCHC in *Trypanosoma brucei brucei* experimentally infected mice

Means with different letters differ significantly ($P < 0.05$). Values are express as mean ± SEM (6 animals per group).

Key: NC= Neutral control, DZ= Diminazene aceturate, PKf= pre-treated with kaempferol, DZ+Kf= Diminazene aceturate and kaempferol, Kf= kaempferol and NS= Normal saline

4.3.4.3 Effects of Treatments with kaempferol and/or diminazene aceturate on mean corpuscular haemoglobin (MCH)

Figure 4.8 shows the effects of the treatments with kaempferol and/or diminazene aceturate on MCH of mice infected with *T. brucei brucei*. There were no significant ($P < 0.05$) difference in mean corpuscular haemoglobin (MCH) in mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V) when compared to untreated uninfected mice (group I). Similarly, MCH decreased significantly ($P < 0.05$) in mice pre-treated with kaempferol (group II) and those that were administered normal saline (group VI) were recorded when compared to mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V). So also, a significant ($P < 0.05$) decrease in the mean MCH values of mice pre-treated with kaempferol (group II) was recorded when compared to mice that were administered normal saline (group VI)

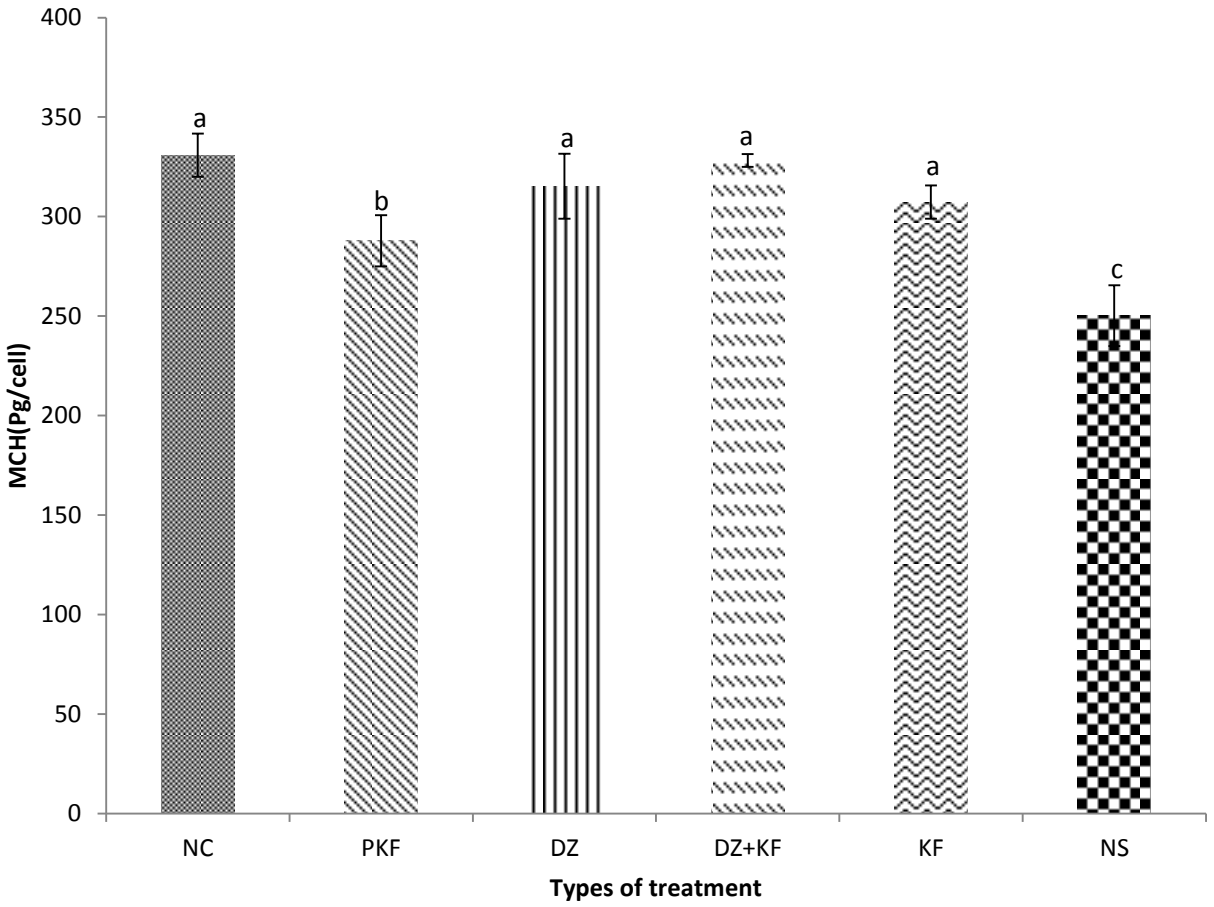


Figure 4.8: Effect of treatment with kaempferol and/ or diminazene aceturate on MCH in *Trypanosoma brucei brucei* experimentally infected mice

Means with different letters differ significantly ($P < 0.05$). Values are express as mean ± SEM (6 animals per group).

Key**:** NC= Neutral control, DZ= Diminazene aceturate, PKf= pre-treated with kaempferol, DZ+Kf= Diminazene aceturate and kaempferol, Kf= kaempferol and NS= Normal saline

4.3.5 Effects of treatments with kaempferol and/or diminazene aceturate on erythroyte osmotic fragility (EOF)

Figure 4.9 shows the effects of the treatments with kaempferol and/or diminazene aceturate on erythrocyte osmotic fragility of mice infected with *T. brucei brucei*. At 0.0 % and 0.1 % NaCl concentration, no significant ($P > 0.05$) difference in the mean percentage of red blood cell haemolysis were observed in mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV), kaempferol only (group V) and normal saline (group VI) when compared to untreated uninfected mice (group 1).

At 0.3 % NaCl concentration, the mean percentage of red blood cell haemolysis of untreated uninfected mice (group I) significantly ($P < 0.05$) decreased when compared to mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups 3), kaempferol and diminazene aceturate (group IV), kaempferol only (group V) and normal saline (group VI). Similarly, significant ($P < 0.05$) increase in the mean percentage of red blood cell haemolysis were observed in mice administered normal saline (group VI) when compared to mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V).

At 0.5 % NaCl concentration, there were significant ($P < 0.05$) decrease in the mean percentage of red blood cell haemolysis of mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group III) and kaempferol only (group V) when compared to mice pre-treated with kaempferol (group II) and those that were administered normal saline (group VI) .

At 0.7 % NaCl concentration, mean percentage of red blood cell haemolysis significantly ($P < 0.05$) decreased in untreated uninfected mice (group I) when compared to mice pretreated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV), kaempferol only (group V) and normal saline (group VI). Similarly, there were significant ($P < 0.05$) decrease in the mean percentage of red blood cell haemolysis of mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V) when compared to mice pre-treated with kaempferol (group 2) and those that that were administered normal saline (group VI).

At 0.9 % NaCl concentration, no significant ($P > 0.05$) difference in the mean percentage of red blood cell haemolysis were observed in mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V) when compared to untreated uninfected mice (group I). Similarly, a significant ($P < 0.05$) decrease in the mean percentage of haemolysis was recorded in mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V) when compared to mice pre-treated with kaempferol (group II) and those that that were administered normal saline (group VI).

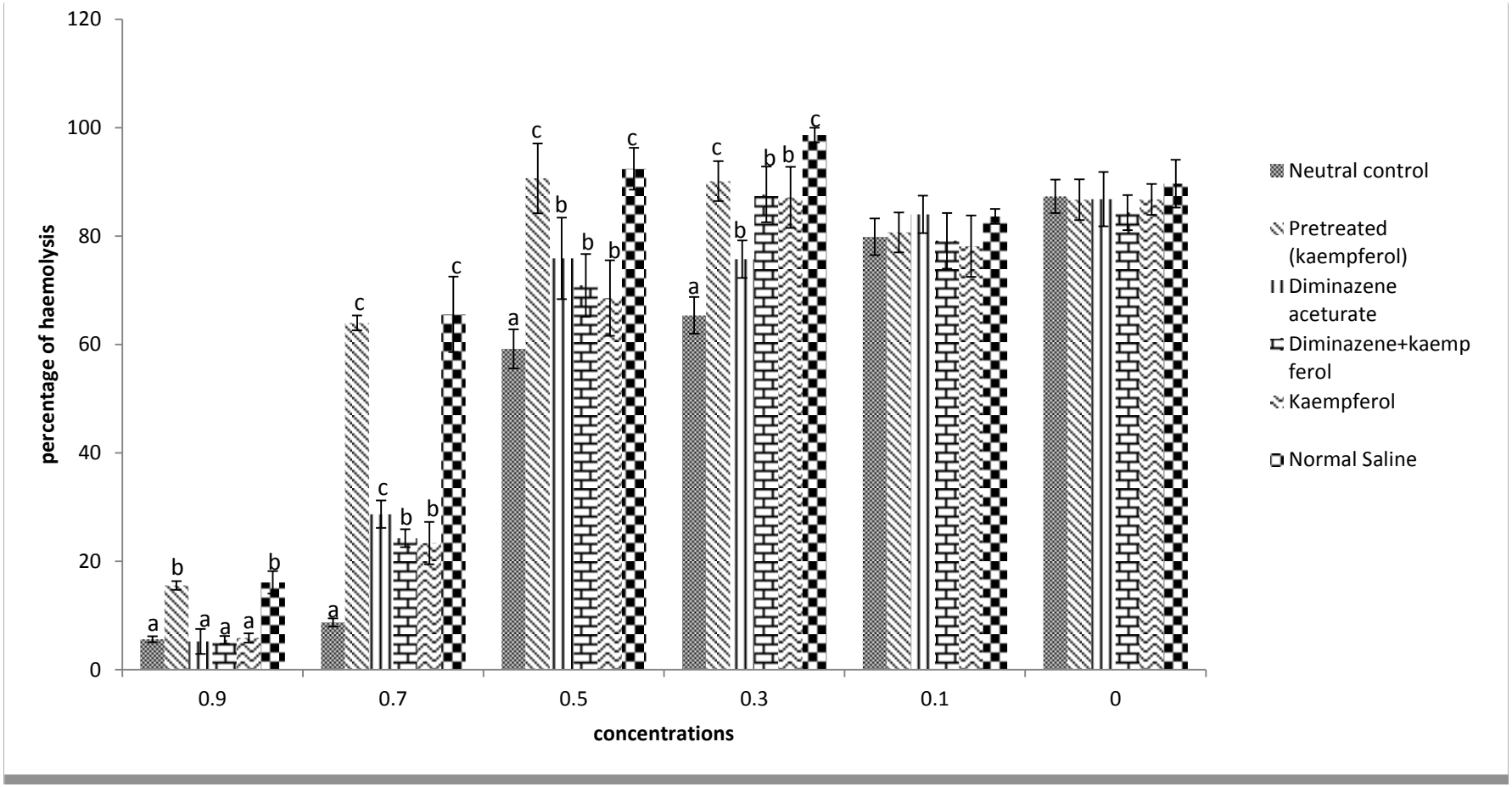


Figure 4.9: Effects of treatments with kaempferol and/or diminazene aceturate on erythrocyte osmotic fragility in *Trypanosoma brucei brucei* experimentally infected mice

Means with different letters differ significantly ($P < 0.05$). Values are express as mean ± SEM (6 animals per group

4.3.6 Effects of the treaments with kaempferol and/or diminazene aceturate on total white blood cell count

Figure 4.10 shows the effects of the treaments with kaempferol and/or diminazene aceturate on total white blood cell count of mice infected with *T. brucei brucei*. There were no significant ($P > 0.05$) difference recorded in the means total leucocyte count of mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V) when compared to untreated uninfected mice (group I). Similarly, the mean total leucocyte count significantly ($P < 0.05$) decreased in mice pre-treated with kaempferol (group II) and those that were administered normal saline (group VI) when compared to mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V).

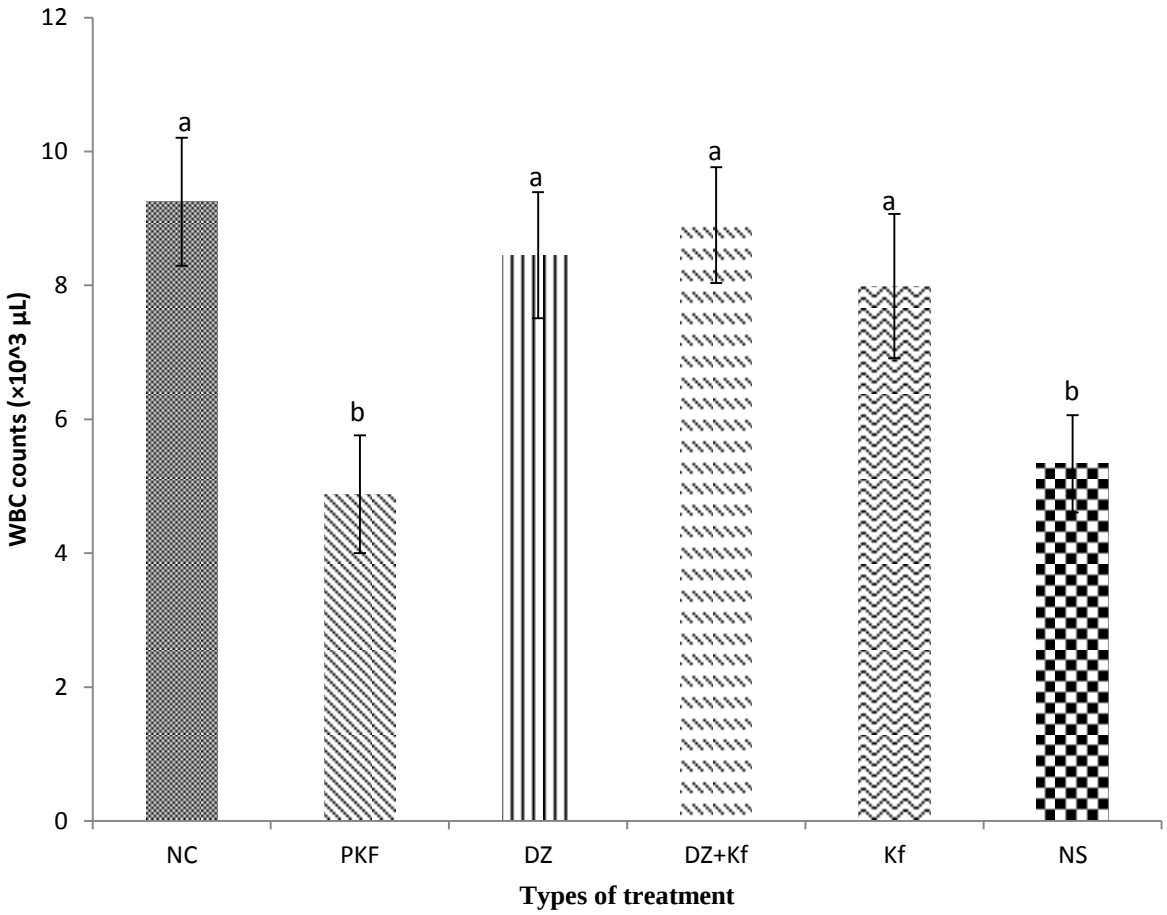


Figure 4.10: Effects of treatments with kaempferol and/or diminazene aceturate on absolute white blood cell count in *Trypanosoma brucei brucei* experimentally infected mice

Means with different letters differ significantly ($P < 0.05$). Values are express as mean ± SEM (6 animals per group).

Key: NC= Neutral control, DZ= Diminazene aceturate, PKf= pre-treated with kaempferol, DZ+Kf= Diminazene aceturate and kaempferol, Kf= kaempferol and NS= Normal saline

4.3.6 Effects of treatments with kaempferol and/or diminazene aceturate on differential leucocyte count

Figure 4.11 shows the effects of treatments with kaempferol and/or diminazene aceturate on lymphocyte and neutrophil counts of mice infected with *T. brucei brucei.* There were significant ($P < 0.05$) increase in the mean lymphocytes count in untreated uninfected mice (group I) when compared to mice mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV), kaempferol only (group V) and normal saline (group VI). Similarly, there were significant ($P > 0.05$) decrease in the mean values of lymphocytes in mice pre-treated with kaempferol (group II) and those that were administered normal saline (group VI) when compared to mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III).

Significant ($P < 0.05$) increase in the mean values of neutrophils were observed in untreated uninfected mice (group I) when compared mice to mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV), kaempferol only (group V) and normal saline (group VI). Similarly, there were significant ($P > 0.05$) decrease in the mean lymphocytes in mice pre-treated with kaempferol (group II) and those that were administered normal saline (group VI) when compared to mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V).

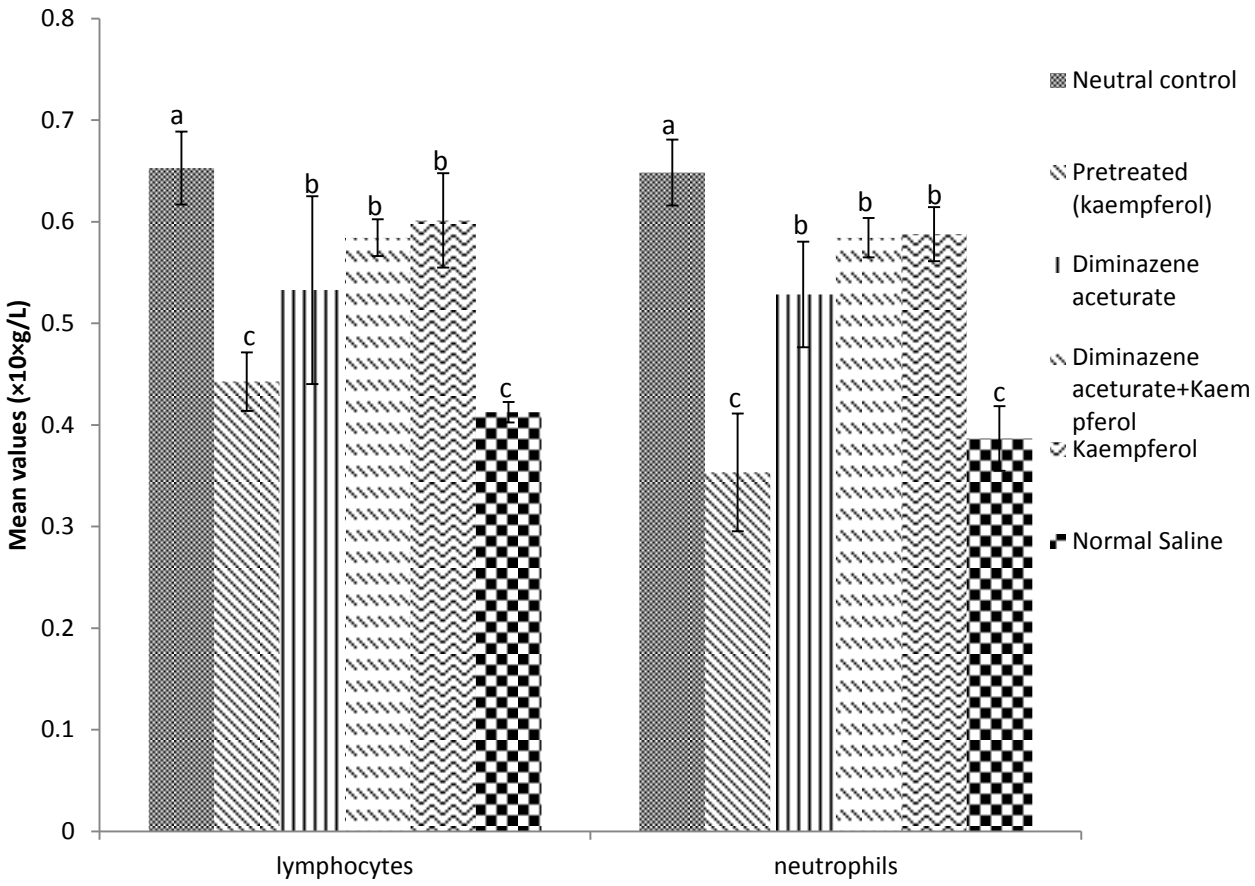


Figure 4.11: Effects of treatments with kaempferol and/ or diminazene aceturate on mean lymphocytes and neutrophils count in *Trypanosoma brucei brucei* experimentally infected mice

Means with different letters differ significantly ($P < 0.05$). Values are express as mean ± SEM (6 animals per group).

4.4. Effects of the Treatments with Kaempferol and/or Diminazene Aceturate on the Activities of Liver Enzymes

Figure 4.12 shows the effects of treatments with kaempferol and/or diminazene aceturate on the activities of liver enzymes of mice infected with *T. brucei brucei*. There were significant ($P < 0.05$) decrease in the mean of ALT in untreated uninfected mice (group I) when compared to mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV), kaempferol only (group V) and normal saline (group VI). Similarly, significant ($P < 0.05$) increase in the mean ALT were recorded in mice pre-treated with kaempferol (group II) and those that were administered normal saline (group VI) when compared to mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V).

No significant ($P > 0.05$) difference were observed in the mean AST in mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V) when compared to untreated uninfected mice (group I). Significant ($P < 0.05$) decrease in the mean AST were observed in mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V) when compared to mice pre-treated with kaempferol (group II) and those that were treated with normal saline (group VI).

No significant ($P > 0.05$) difference were recorded in the mean ALP in mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V) when compared to untreated uninfected mice (group I). Similarly, significant ($P < 0.05$) decrease in the mean ALP were observed in mice treated with diminazene

aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V) when compared to mice pre-treated with kaempferol (group II) and those that were administered normal saline (group VI).

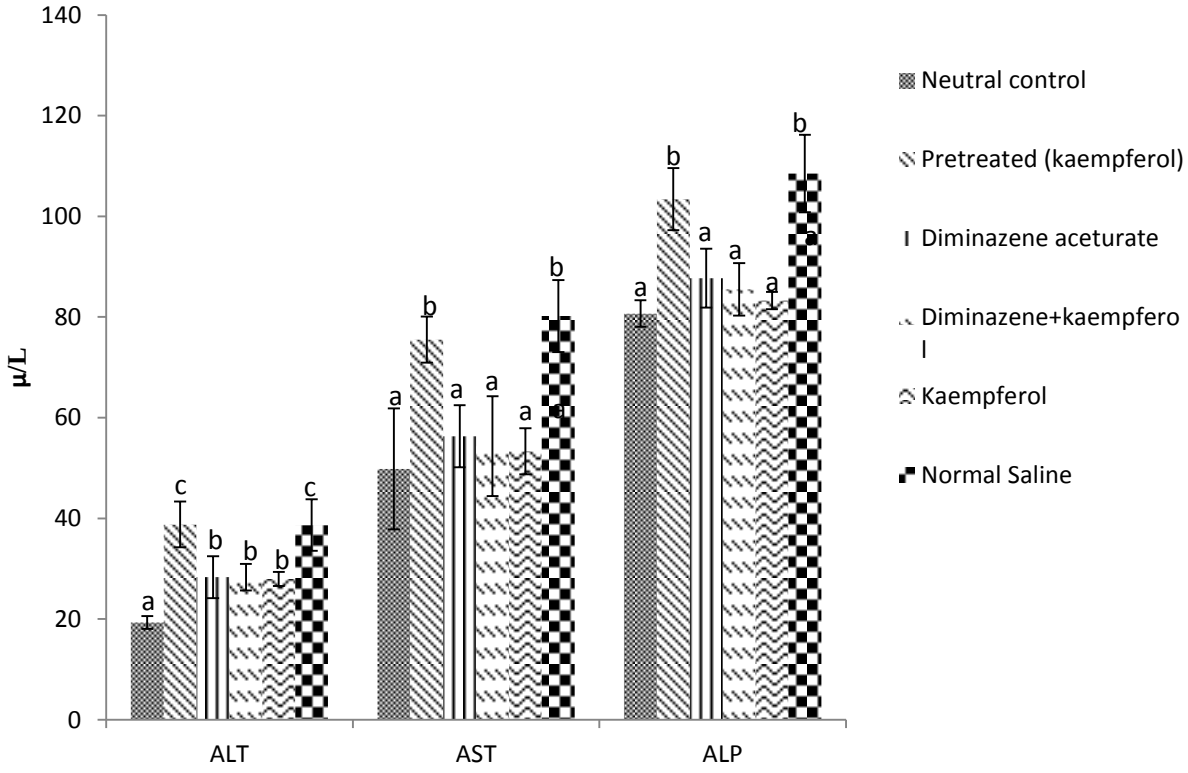


Figure 4.12: Effects of treatments with kaempferol and/ or diminazene aceturate on the activities of liver enzymes (ALT, AST and ALP) in *Trypanosoma brucei brucei* experimentally infected mice

Means with different letters differ significantly ($P < 0.05$). Values are express as mean ± SEM (6 animals per group).

4.5 Effects of the Treatments with Kaempferol and/or Diminazene Aceturate on the Activities of Antioxidant Enzymes

Figure 4.13 shows the effects of the treatments with kaempferol and/or diminazene aceturate on antioxidant enzymes of mice infected with *T. brucei brucei*. No significant ($P > 0.05$) difference were recorded in the mean values of SOD, CAT and GPx in mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V) when compared to untreated uninfected mice (group I). There were significant ($P < 0.05$) increase in the mean of SOD, CAT and GPx in mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol l only (group V) when compared to mice pre-treated with kaempferol (group II) and those that were treated with normal saline (group VI). No significant ($P > 0.05$) difference was recorded in the mean values of SOD, CAT and GPx in mice pre-treated with kaempferol (group II) when compared to mice treated with normal saline (group VI).

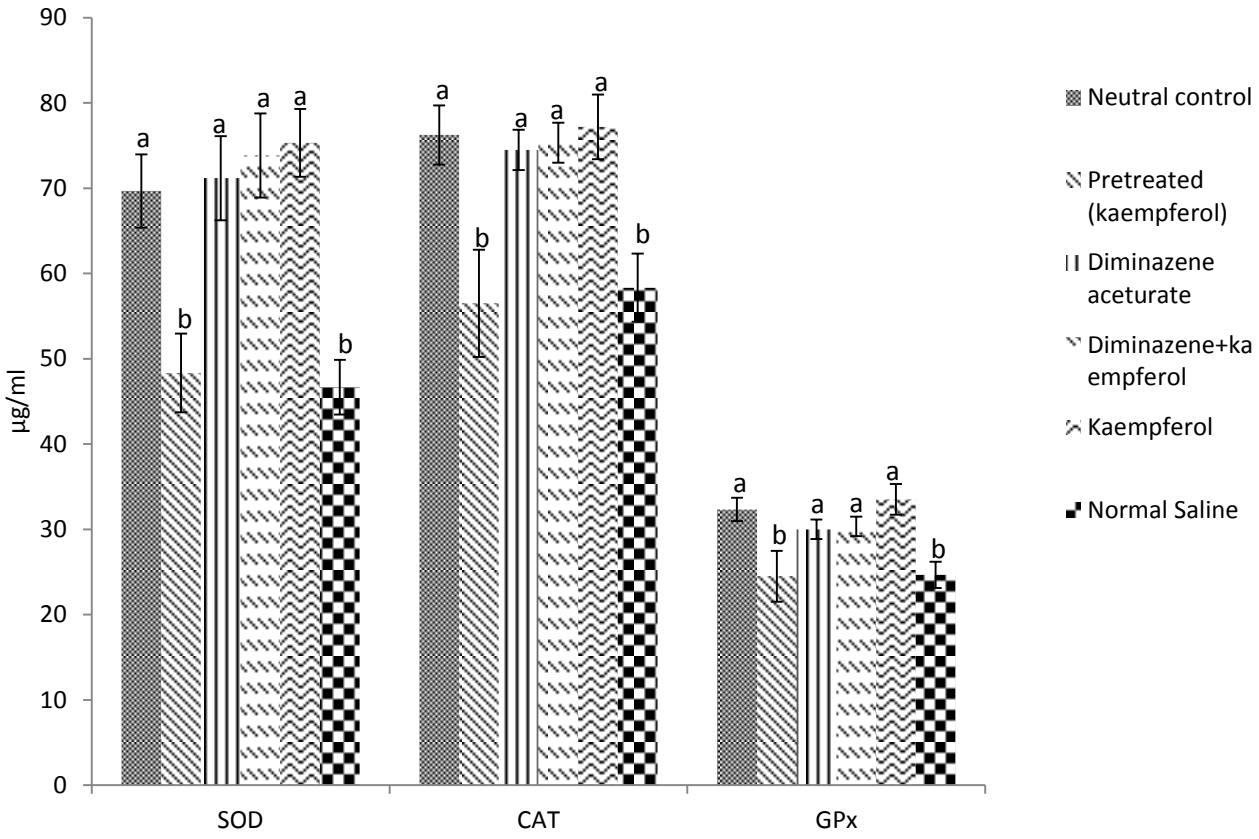


Figure4.13: Effects of treatments with kaempferol and/ or diminazene aceturate on antioxidant enzymes (SOD, CAT and ALP) in *Trypanosoma brucei brucei* experimentally infected mice

Means with different letters differ significantly ($P < 0.05$). Values are express as mean ± SEM (6 animals per group).

4.6 Effects of Treatments with Kaempferol and/ or Diminazene Aceturate on the Serum Level of Malondialdehyde (MDA)

Figure 4.14 shows the effects of the treatments with kaempferol and/ or diminazene aceturate on the serum level of malondialdehyde of mice infected with *T. brucei brucei.* There were significant ($P < 0.05$) increase in the mean values of serum MDA concentration of mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV), kaempferol only (group V) and normal saline (group VI) when compared to untreated uninfected mice (group I). Similarly, significant ($P < 0.05$) increase in the mean values of serum MDA concentration were recorded in mice pre-treated with kaempferol (group II) and those treated with normal saline (group VI) when compared to mice diminazene aceturate only (group III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V).

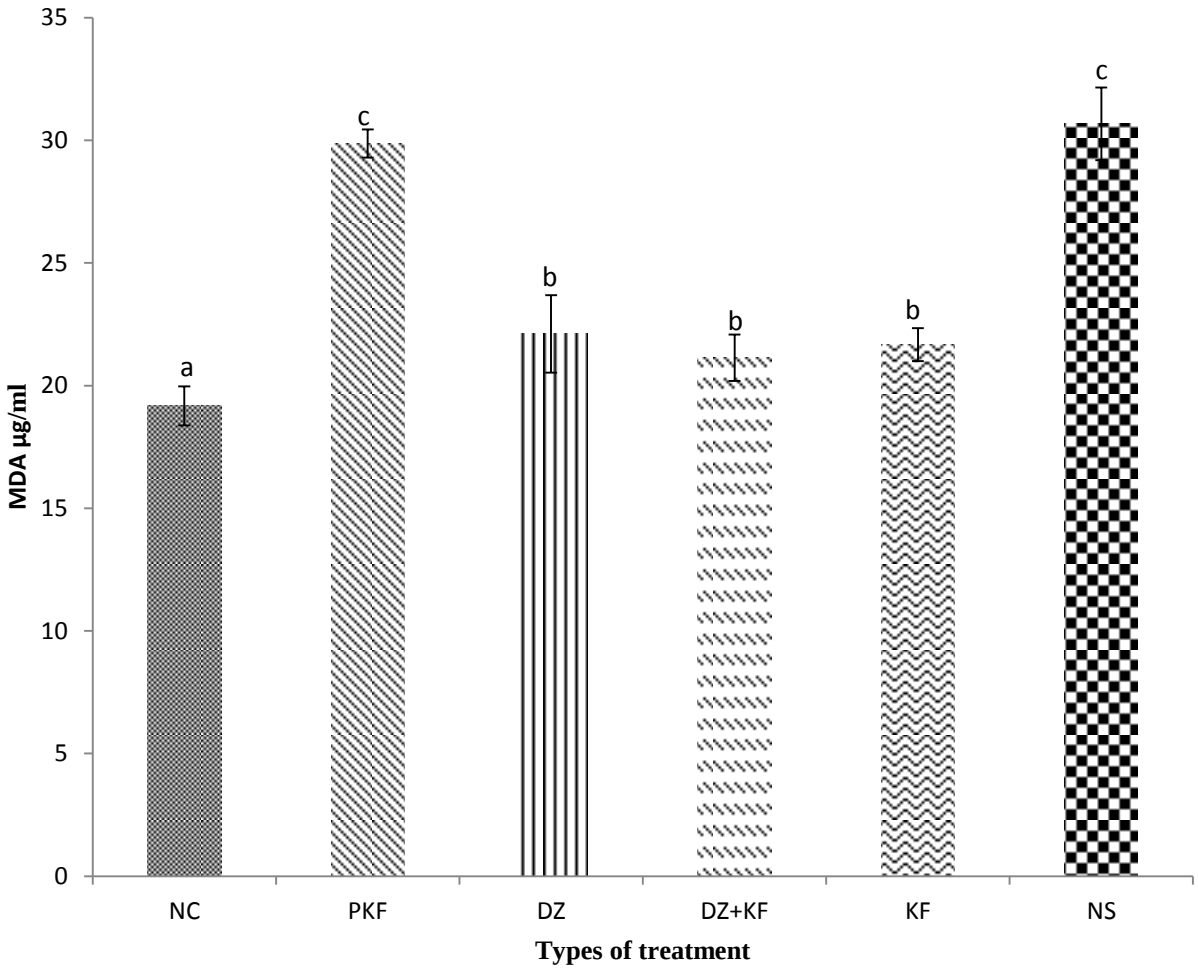


Figure 4.14: Effects of treatments with kaempferol and/ or diminazene aceturate on the serum level MDA concentration in *Trypanosoma brucei brucei* experimentally infected mice

Means with different letters differ significantly ($P < 0.05$). Values are express as mean ± SEM (6 animals per group).

Key**:** NC= Neutral control, DZ= Diminazene aceturate, PKf= pre-treated with kaempferol, DZ+Kf= Diminazene aceturate and kaempferol, Kf= kaempferol and NS= Normal saline

4.7 Effect of the Treatment with Kaempferol and/or Diminazene Aceturate on Histopathology

The histopalogical changes observed in the infected and treated mice were slight necroses in the brain of mice infected and that were administered normal saline (group VI) (Plate I B). However, no histopathology was seen in the brain of infected mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V).

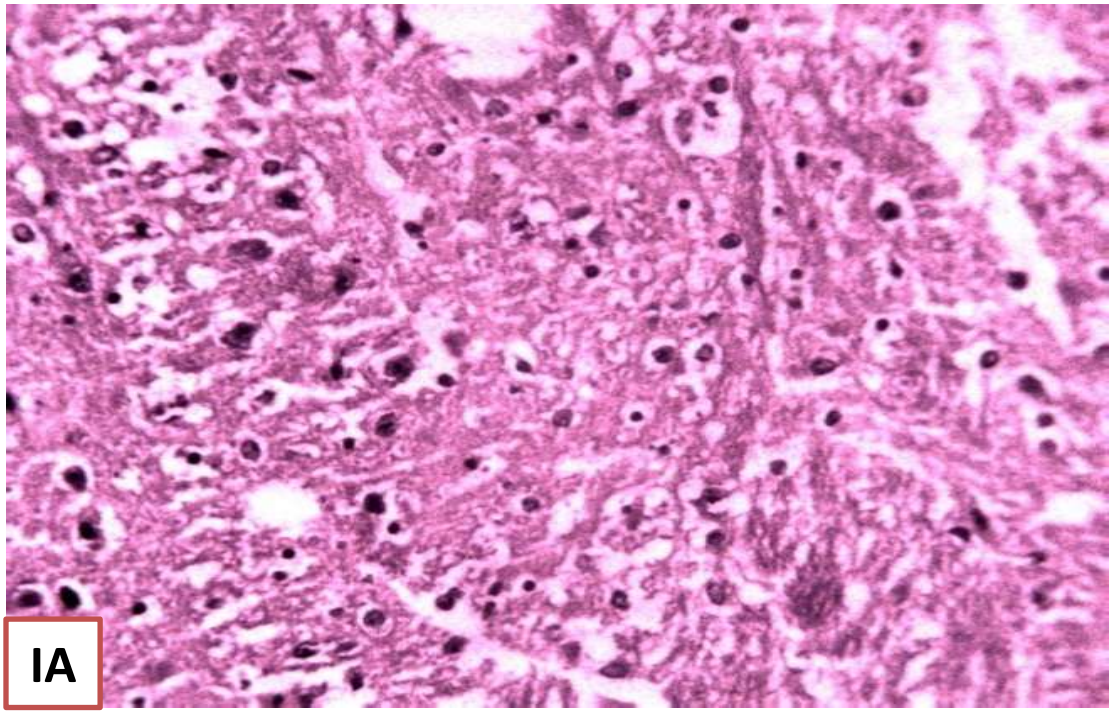
Slight muscular necroses were observed in the heart section of mice pre-treated with kaempferol (group II) and those that were administered normal saline (group VI) (Plate II).

Lymphocyte hyperplasia and slight tubular necroses were observed in the kidney section of mice pre-treated with kaempferol (group II) and those that were treated with kaempferol only (group V) and normal saline (group VI) (Plate III). However, only lymphocyte hyperplasia were seen in the heart section of infected mice treated with diminazene aceturate only (groups III) and kaempferol and diminazene aceturate (group IV)

Lymphocyte hyperplasia were seen in the liver section of infected mice pre-treated with kaempferol (group II) and those that were treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V), while both lymphocyte hyperplasia and hepatic necroses were observed in the liver section of mice infected and those that were administered normal saline (group VI) (Plate IV).

Peritubular lymphocyte infiltrations and alveolar congestion were observed in the lung section of mice pre-treated with kaempferol (group II) and those that were treated with kaempferol only

(group V) and normal saline (group VI) (Plate V), while no pathology was observed in the lung section of infected mice treated with diminazene aceturate only (groups III) and kaempferol and diminazene aceturate (group IV).



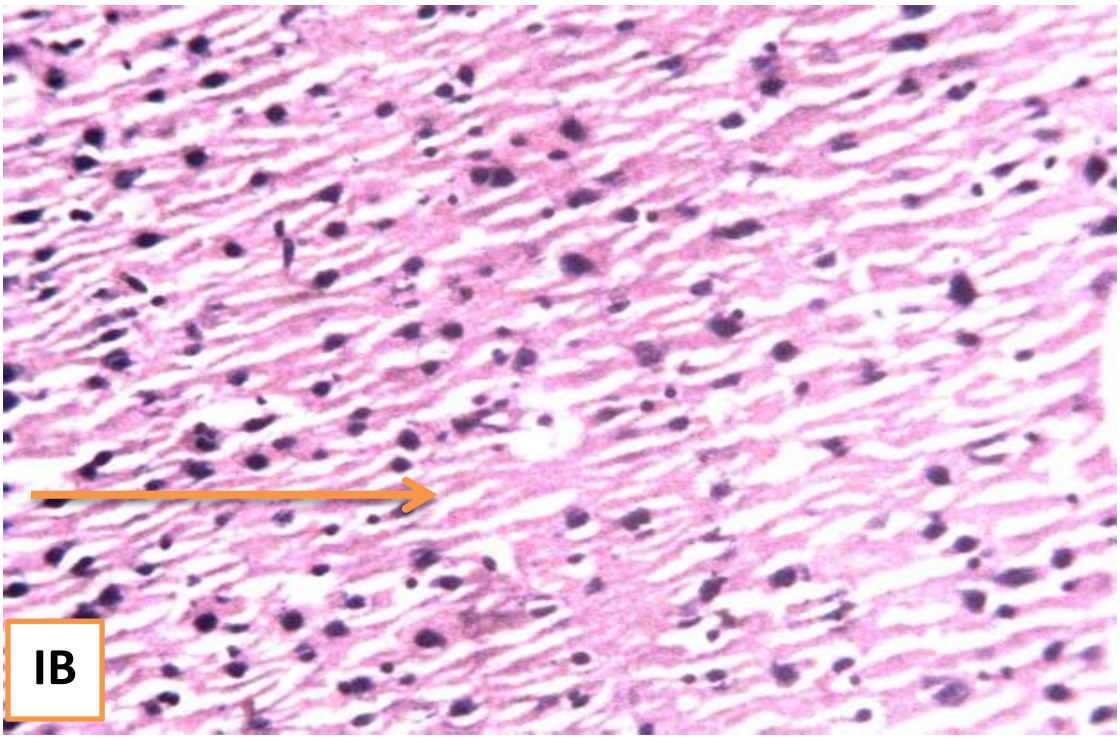
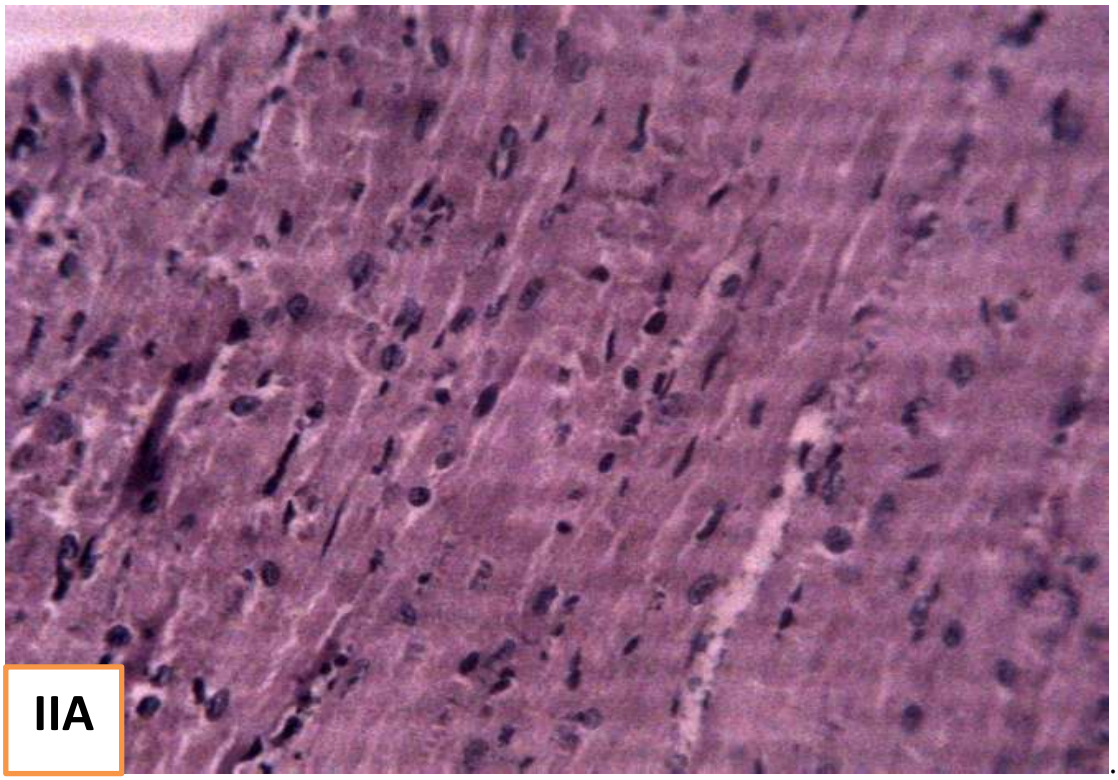


Plate I: Photomicrograph of the section of the brain of a mouse showing normal tissue (A) and slight necrosis (B) in the brain of a mouse infected with *Trypanosoma brucei brucei* (untreated control) (H and E) × 200



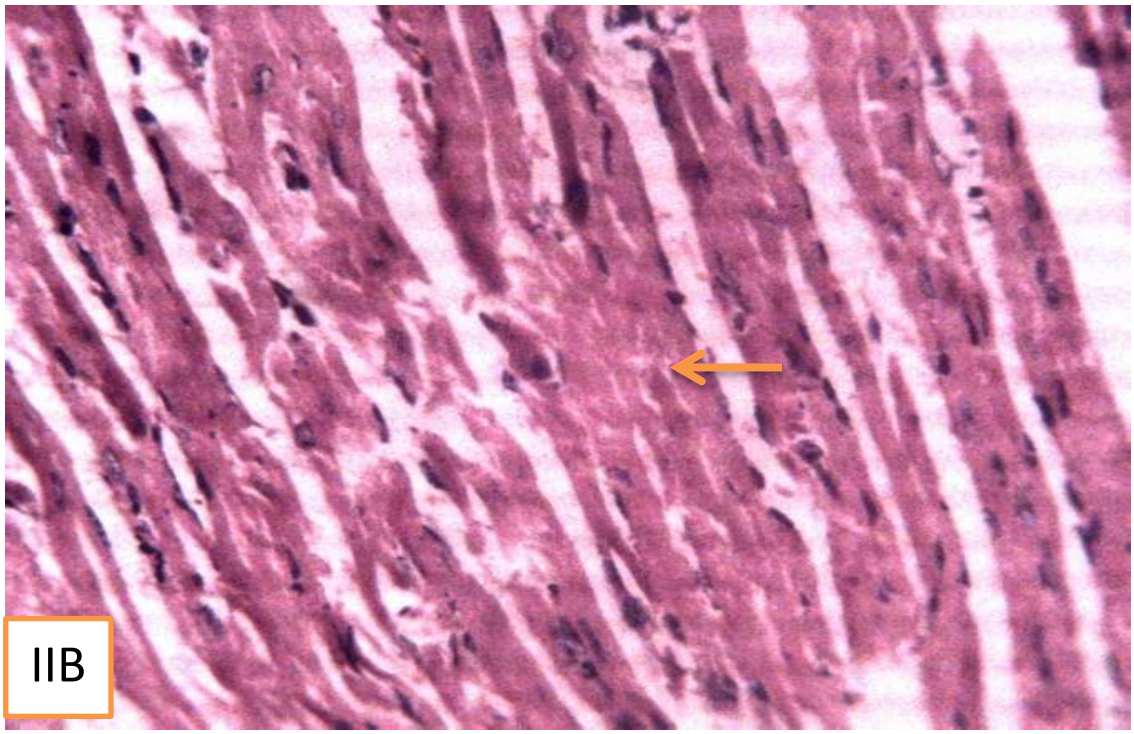
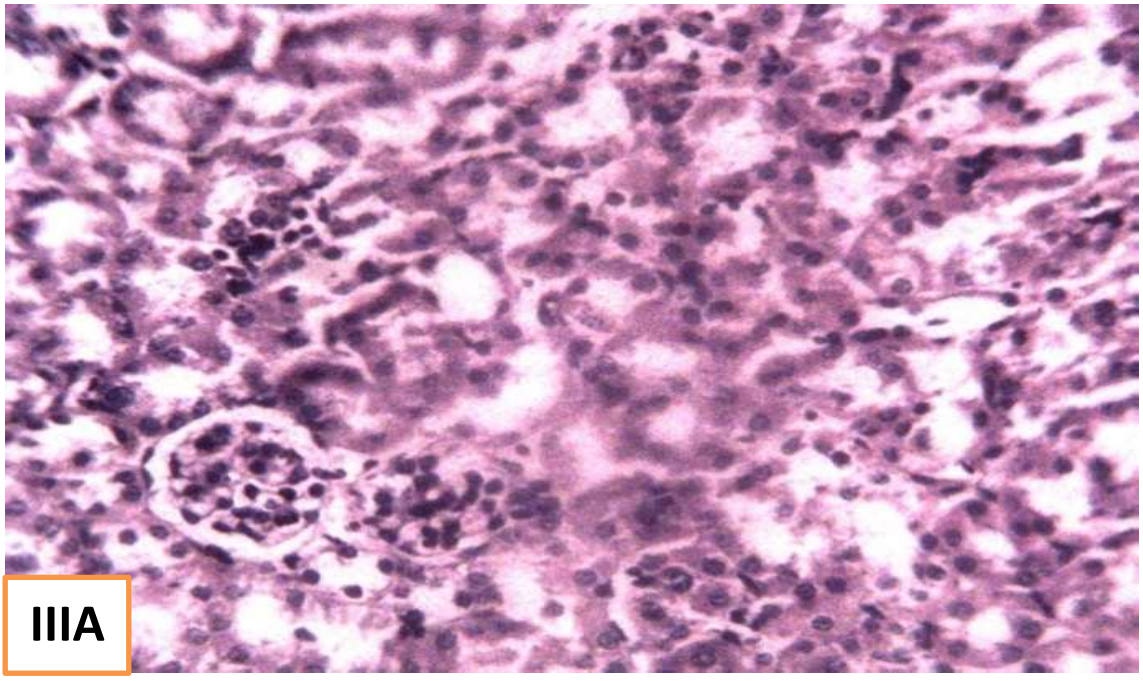


Plate II: Photomicrograph of the section of the heart of a mouse showing normal tissue (A) and myocardiac necrosis (B) in the heart of a mouse infected with *Trypanosoma brucei brucei* (untreated control) (H and E) × 200



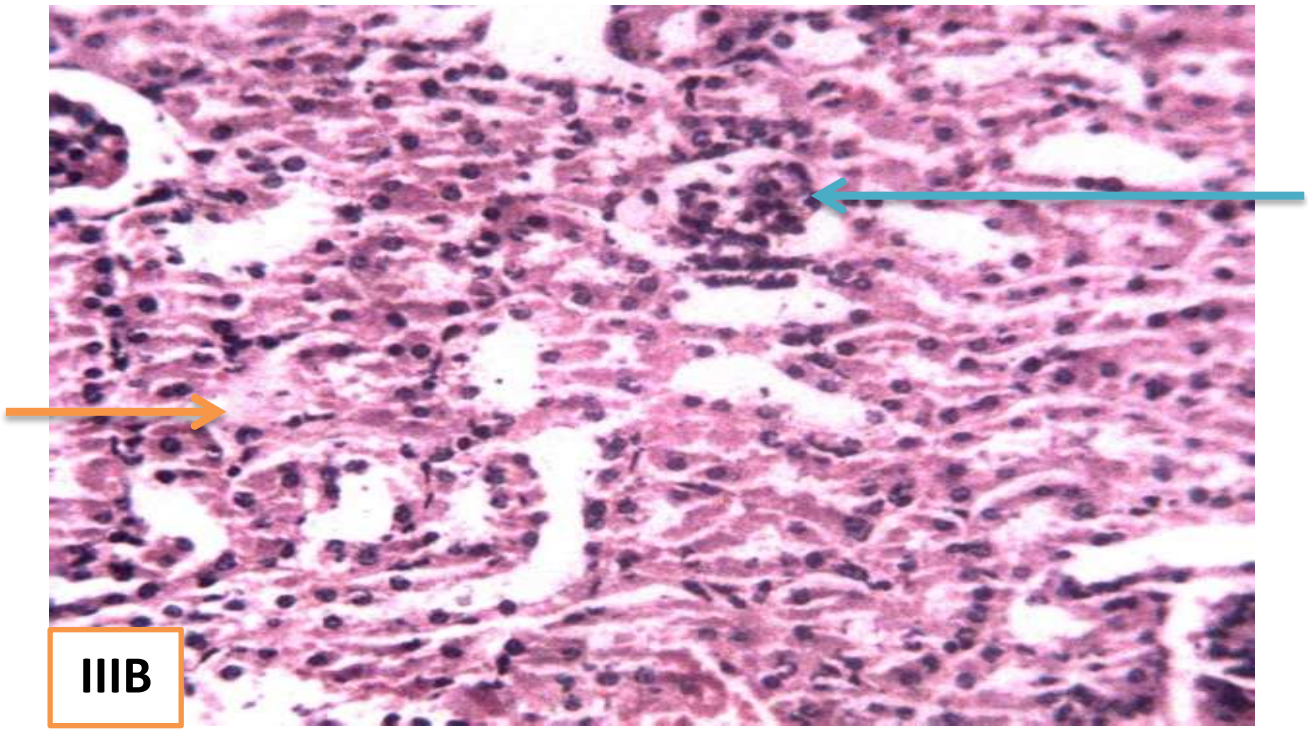
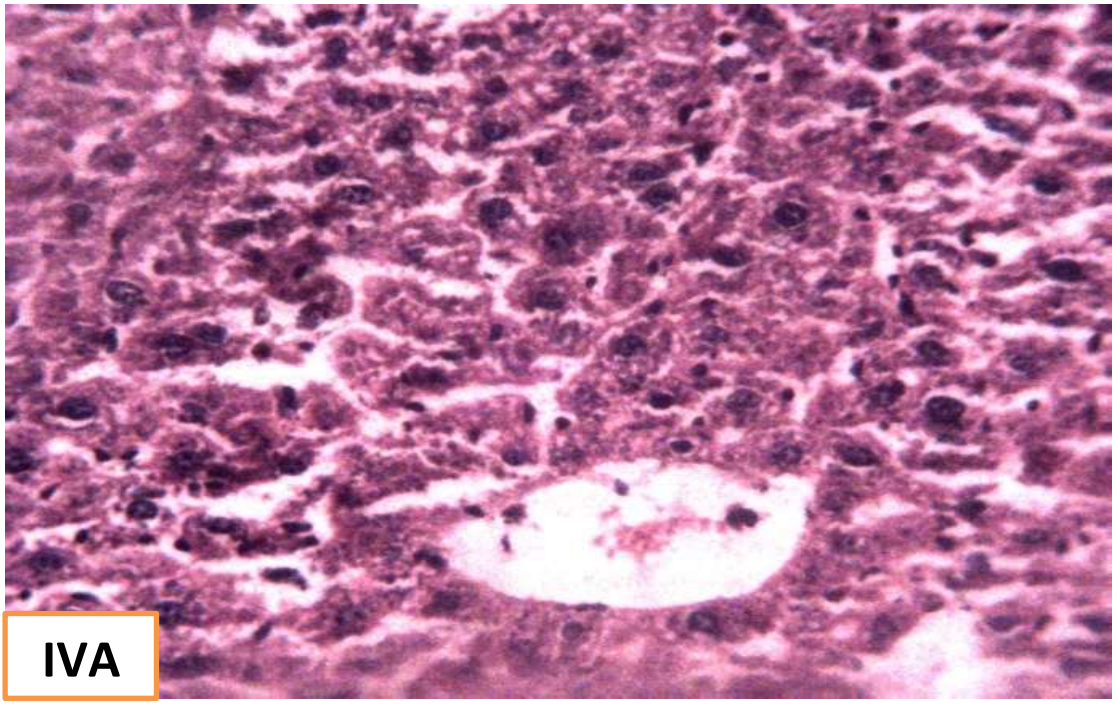


Plate III: Photomicrograph of the section of the kidney shwing normal tissue (A) and lymphocyte hyperplasia (blue arrow) and tubular necrosis (red arrows) in the kidney of a mouse infected with *Trypanosoma brucei brucei* (untreated control) (B) (H and E) × 200



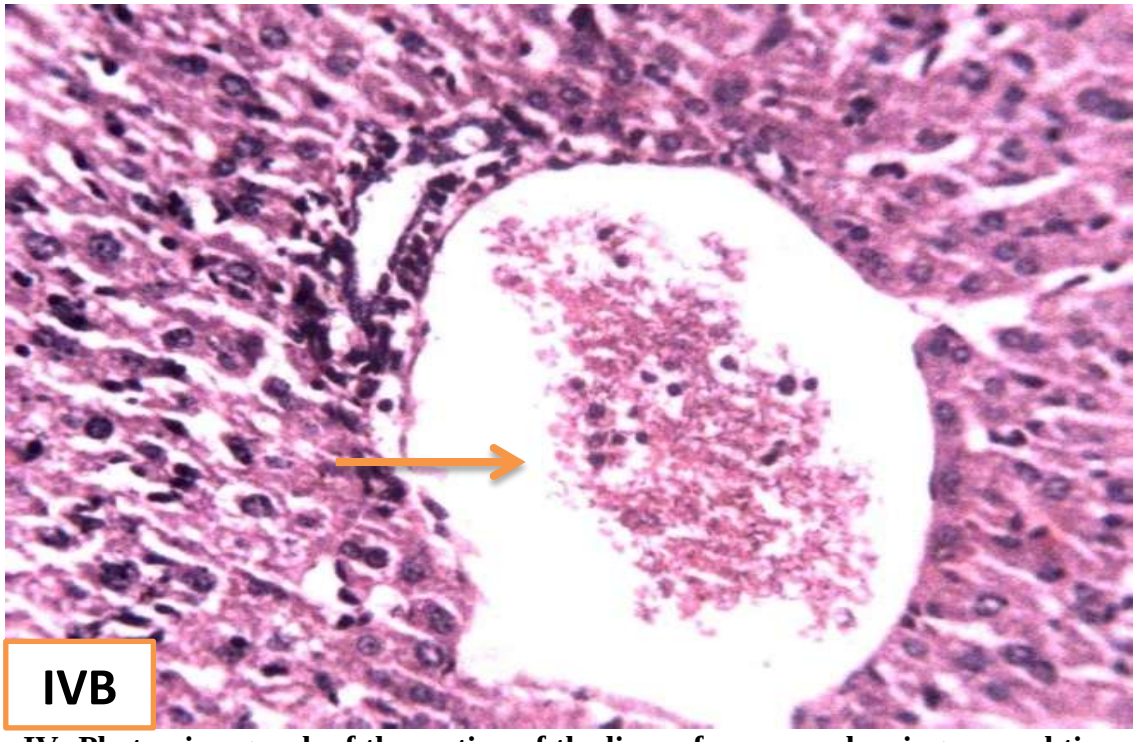


Plate IV: Photomicrograph of the section of the liver of a mouse showing normal tissue (A) and vascular congestion in the liver of a mouse infected with *Trypanosoma brucei brucei* (untreated control) (B) (H and E) × 200.

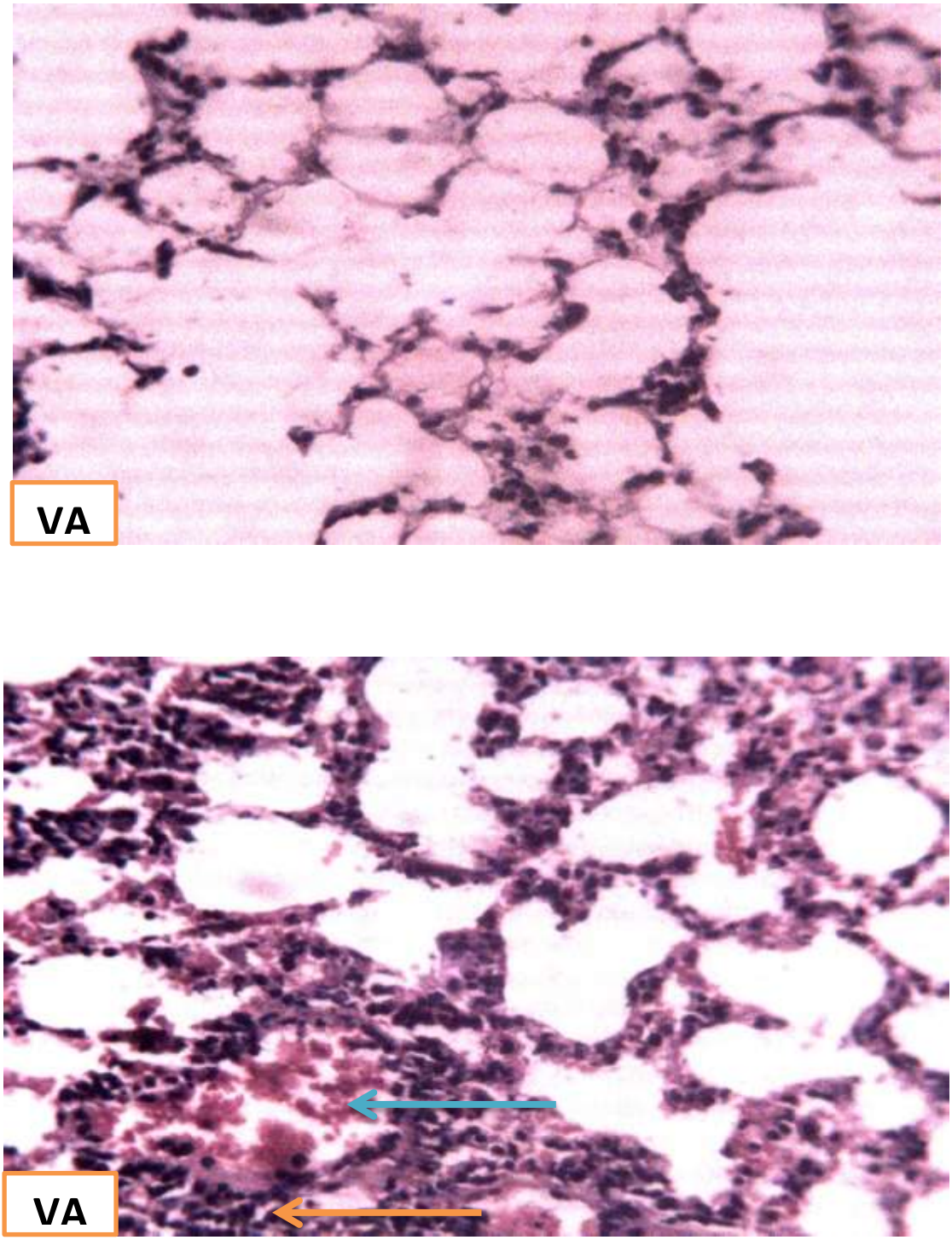


Plate V: Photomicrograph of the section of the lung of a mouse showing normal tissue (A) and perivascular lymphocyte infiltration (red arrow) and alveolar congestion (blue arrow) in the lung of a mouse infected with *Trypanosoma brucei brucei* (untreated control) (B) (H and E) × 200

CHAPTER FIVE

5.0 DISCUSSION

The clinical signs observed in mice infected with *T. brucei brucei* include; anorexia, loss of body weight, pale ocular mucous membrane and weakness, these signs were in agreement with the findings of Ibrahim *et al* (2016) in experimental *T. brucei brucei* infection in mice, rats and rabbits. Variable disorders occur sequel to trypanosome infection in animals (Adamu *et al.,* 2009), depending on the virulence of the infecting trypanosome, the infective dose and the immune status of the host. The symptoms usually associated with trypanosomosis includes; pallor of the mucous membranes, enlargement of lymph nodes, anoraexia and emaciation (Shimelis *et al.,* 2015).

Reactive oxygen species (ROS) are incriminated in the pathogenesis of African trypanosomosis which are known to cause degenerative changes in cells, tissues and organs of infected animals (Umar *et al.,* 2007). The ROS attack both the membrane polyunsaturated fatty acids and proteins of RBCs, leading to haemolysis and, consequently, anaemia. There is also depletion of endogenous antioxidant reserves in the blood and other tissues of trypanosome-infected animals (Umar *et al.,* 2007). The signs observed in this study were not severe in mice treated with kaempferol only and mice treated with kaempferol and diminazene aceturate this may be due to ability of kaempferol to scavenge free radicals generated during the course of the infection and this agrees with the findings of Kobo *et al* (2014), which showed that the Daflon 500® (mixture of two flavonoids) enhanced the effect of diminazene aceturate in rats infected with *T. brucei brucei*.

Four days following inoculation of the parasites (*T. brucei brucei*) all infected mice showed the presence of parasites. So far, little has been achieved in terms of understanding the variations prepatent period and course of infection in various laboratory animal species especially as relates to *T. b. brucei* (Ibrahim *et al*., 2016). The prepatent period of four days in this study is consistent with that reported by Kobo *et al* (2014) in rats and by Ibrahim *et al* (2016) in mice and rats and experimentally infected with *Trypanosoma brucei brucei*. However, in rabbits the prepatent period was observed to be eight days (Ibrahim *et al*., 2016). Trypanosomes rapidly divide in the blood stream of mammalian host by binary fission, resulting in the formation of a large population of the parasites within 2-4 days (Merck, 2015). There was a significant ($P < 0.05$) decrease in the parasite count in mice treated with kaempferol only when compared to mice pretreated with kaempferol and those treated with normal saline. Similarly, there was a progressive increase in parasitaemia in mice pre-treated with kaempferol and those treated with kaempferol and normal saline up to nine days post-infection. This result is consistent with that reported by Kobo *et al* (2014) where mixture of two different flavonoids; hesperidin and Daflon® reduced parasitaemia in rats infected with *Trypanosoma brucei brucei* by preventing the rate of the parasite prolifiration.

Kaempferol is extensively metabolized in the liver to form glucurono-conjugated and sulfo-conjugated forms. These forms of kaempferol, and kaempferol itself, can then be excreted in urine. About 2.5% of kaempferol ingested is excreted in the urine. Ingested kaempferol is found in the plasma and tissues in nanomolar concentrations (Calderon-Montano *et al.,* 2011). In addition, some flavonoids (e.g kaempferol) may regenerate other antioxidants with known immune-enhancing activity, such as vitamin E (Zhu *et al.,* 2000) and carotenoids (Pietta and

Simonetti, 1998). This may explain why kaempferol was effective at reducing the level of parasiataemia in this study. Production of ROS and mitochondrial dysfunction caused by quercetin (a flavonoid) in rats was reported by Fonseca-Silva *et al* (2011). Similarly, Lapidot *et al* (2002) reported the prooxidant effect of quercetin where it induced production of superoxide anion, hydrogen peroxide and other ROS in mice with leishmaniosis. Maritim *et al* (2003) reported that prolonged administration of vitamin A and E in laboratory animals promote the development of toxic effect by acting as prooxidants. In this study, mice that were pretreated with kaempferol for 14 consecutive days showed high parasitaemia; perhaps this could be due to over production of ROS in the animals leading to oxidative stress and immunosuppression which eventually resulted in high parasitaemia. There was an absolute clearance of the parasites from the blood stream of mice treated with diminazene aceturate only and those treated with diminazene aceturate and kaempferol a day after treatments. Diminazene aceturate is an antitrypanosomal drug with good activity against *T. brucei brucei* (Saba *et al.*, 2007).

The mean PCV, Hb concentration and RBC count reduced significantly ($P<0.05$) in mice pre-treated with kaempferol and those that were administered normal saline when compared to untreated uninfected mice, indicating anaemia since their values falls below normal range, this agrees with the work of Ukpai and Nwabuko (2014) who reported the effects of *Trypanosoma brucei brucei* on haematological parameters and pathology of internal organs in albino rats. Anaemia which is regarded as the most consistent pathology in trypanososmosis of man and domesticated animal has been reported in *T. vivax* infected cattle and goats (Saror, 1980), *T. congolense* infected sheep (Mohammed *et al.*, 2007), *T. congolense* infected dogs (Gow *et al.*, 2007) and *T. brucei bruce* infected goats, sheep and rabbits (Taiwo *et al.*, 2003; Seed, 1969). The pathophysiology of anaemia in *Trypanosomiasis* is complex and multi factorial in origin

(Naessens *et al.,* 2005). Trypanosomes initiate a cascade of events in the animal host leading to anaemia and cardiovascular collapse (Anosa, 1988). This anaemia might be due to haemolysis induced by the trypanosomes (Soulsby, 1982). The anaemia is caused by mechanical injury to erythrocyte and occurs by the lashing action of the powerful locomotory flagella and microtubule reinforced bodies of the millions of the organisms during parasitaemia (Vickerman *et al.,* 1988). Erythrocyte membrane damage has also been associated with adhesion of erythrocytes, platelets and reticulocytes to trypanosome surfaces via sialic acid receptors leading to damages to erythrocyte cell membranes (Shehu *et al.,* 2006). Kaempferol used in this study showed significant level of protection of mice against anaemia caused by the trypanosomes. This may be due to its antioxidant effect and ability to scavenge free radicals generated by the parasite during the course of infection, thereby reducing the free radical loads hence prevent erythrocyte membrane from oxidative damage (Procházková *et al.,* 2011). Umar *et al* (2007; 2001) have reported protective effect of of vitamin E and C in trypanosomes induced anaemia. Murray and Dexter (1988) showed that the severity of anaemia during trypanosome infection could be related to differences in virulence among trypanosome strains.

Erythrocytic indices were determined so as to classify anaemia morphologically. There was a significant reduction in the mean values of MCV and MCHC in infected untreated mice, resulting in microcytic hypochromic anaemia, therefore the results obtained in this study agrees with that reported by Kobo *et al.,* (2014) where microcytic hypochromic anaemia was observed only in untreated rats infected with *T. brucei brucei* and disagrees with the finding of Shimelis *et al.,* (2015) who reported macrocytic normochromic anaemia in young Zebu cattle experimentally infected with *Trypanosoma vivax.*These findings also disagree with that of Rwakishaya *et al.* (1992) who reported that ovines infected with *Trypanosoma congolense*

developed macrocytic normochromic anemia. Igbokwe *et al*. (1989) reported that dyserythropoiesis is associated with animal trypanosomosis. Microcytic hypochromic anaemia is a blood disorder characterized by small red blood cells (erythrocytes) which have insufficient haemoglobin and hence have a reduced ability to carry oxygen through the body (Ford, 2013). The red blood cells are small due to a failure of haemoglobin synthesis or insufficient quantities of haemoglobin available. The blood disorder is usually caused by conditions such as iron deficiency (Ford, 2013). Jenkins and Face (1985), proposed several causes of anaemia in acute and chronic African trypanosomosis such as: increased red cell destruction, extravascular and intravascular haemolysis by immune system (trypanosome antigen/antibody complex and antierythrocyte antibodies), haemolysins, non-specific reticulo endothelial system activation and direct traumatic effect on trypanosomes. Microcytic hypochromic anaemia (iron deficiency) also occurs due to decreased iron intake and decreased absorption and increased requirement. Small laboratory animals (e.g rats and mice) have high demand of iron because of their high metabolic activities (Kagira *et al.,* 2006). Hepcidin is a peptide hormone produced primarily by the liver and secreted into the circulation. Hepcidin synthesis increases in response to iron and inflammation and decreases in response to erythropoiesis (Enrico, 2005). In mice, hepcidin has been found to have anti-inflammatory properties. This is a negative feedback: it reduces the inflammation which caused the elevated hepcidin level (De Domenico *et al.,* 2010). Ganz (2003) reported that the rate of iron efflux into the plasma depends primarily on the plasma level of hepcidin; when iron levels are high the synthesis of hepcidin increases and the release of iron from enterocytes and macrophages are diminished. It could be possible in this study there was increased production of hepsidin by the liver as a result of inflammation caused by the trypanosomes in mice treated with normal saline hence low level of iron in circulation.

Since decrease MCV and MCHC are associated with decreased PCV and Hb concentration (Kagira *et al.,* 2006), there is also a significant increase in the MCV and MCHC in kaempferol treated mice when compared to mice treated with normal saline, this may be due to the ability of kaempferol to protect red blood cells from oxidative damage by scavenging free radicals that are detrimental to biomolecules. No parasite was seen in the blood of mice treated with diminazene aceturate and those that were treated with diminazene aceturate and kaempferol. Studies have shown the effect of flavonoids in potentiating the activity of diminazene aceturate in ameliorating anaemia induced by *T. brucei brucei* (Kobo *et al.,* 2014) and *T. evansi* (Aron *et al.,* 2017).

The significant ($P < 0.05$) decrease in WBC, lymphocytes and neutrophils observed in mice pretreated with kaempferol and and those that were treated with normal saline is not in aggreement with the work of Adenike and Stephen (2010) who reported a significant ($P < 0.05$) increase in WBC, lymphocytes and neutrophils in rats infected with *Trypanosoma brucei brucei* and agrees with that of Shimelis *et al* (2015) in young Zebu cattle experimentally infected with *Trypanosoma vivax and* Ekanem and Yusuf (2008). It also agrees with the the work of Takeet and Fagbemi (2009) in rabbits experimentally infected with *trypanosoma congolense*. The decrease could be attributed to the immunosuppressive actions of trypanosomes in the body.

Leucopenia after an initial period of leucocytosis is a common finding in *T. b. brucei* infection in rats and rabbits and other aniamals (Ibrahim *et al.,* 2016). Leucopaenia in animals with trypanosomosis has been reported to be largely due to ineffective or depressed granulopoiesis in the bone marrow (Anosa *et al.,* 1997a). The immunosuppression may result in ROS accumulation; therefore, in *T. brucei brucei* infection with increased ROS production, the immune cells are vulnerable to oxidative damage, which may be responsible, among other

factors, for the leucopenia observed in the infected untreated and pretreated groups. The increase in the mean values of WBC, lymphocytes and neutrophils count in mice treated with kaempferol combined with diminazene aceturate and those that were treated with kampferol only, showed the protective action of the treatments against trypanosome induced leucopenia, probably due to kaempferol antioxidant effect. The antioxidants could reduce this oxidative damage of a tissue indirectly by enhancing natural defences of cell and/or directly by scavenging the free radical species (Ukachi *et al.,* 2015). Antioxidants can also elicit a number of *in vivo* effects such as promotion of increased synthesis of endogenous antioxidant defences or they act directly to scavenge free radicals (Ukachi *et al.,* 2015). The increase in WBC, lymphocytes and neutrophils count was also observed in mice treated with diminazene aceturate only that also agrees with the work of Kobo *et al* (2014). The effect was attributed to the increase in cell mediated immunity induced by the drug.

Studies have also shown direct association between trypanosomes infection and erythrocyte osmotic fragility. Several mechanisms have been suggested to explain the effect of trypanosomes on RBCs. These effects includes: mechanical damage produced by the interaction of trypanosomes with RBC (Mbaya *et al.* 2012), adhesion and membrane fusion mediated filopodia (Anosa and Kaneko, 1983), immune complexes adsorbed to erythrocytes (Anosa,1988), and biologically active substances released by live or dead trypanosomes which produce erythrocyte injury (Huson, *et al.*, 2009). The high level of RBC haemolysis observed in infected untreated mice is in agreement with that reported by Ogunsanmi and Taiwo (2004), they showed that in grey duiker (*Sylvicapra grimmia*), sheep and goats that were experimentally infected with *Trypanosoma congolense,* have high percentage of haemolysis in goat relative to sheep and

duiker. The significant ($P < 0.05$) reduction in the mean percentage of RBC haemolysis in mice treated with kaempferol and diminazene and those treated with kaempferol only when compared to mice treated with normal saline agrees with the work of Kobo *et al* (2014), where mixture of two different flavonoids; hesperidin and Daflon ameliorated the effects trypanosomes on erythrocyte osmotic fragility in rats. The effect was attributed to ability of flavonoids to enhance the antioxidant defence mechanism. It may be possible that kaempferol enhanced the antioxidant defence mechanism thereby reducing the intensity of oxidant stress and suppressed the oxidative stress induced by *T.b.brucei*. The reduction in red blood cell haemolysis observed in mice treated with diminazene only may be due to the abilty of the drug to eliminate the parasites from the blood of mice thereby preventing the RBCs destruction by the parasites, hence decrease osmotic fragility.

Furthermore, it has been shown that oxidative stress plays an important role in the pathogenesis of African trypanosomosis (Ogunsanmi and Taiwo, 2004). The erythrocytic membrane is rich in polyunsaturated fatty acids, a highly susceptible and primary target for free radical lipoperoxidation, which results in loss of membrane fluidity and cellular lysis (Wahab *et al*., 2010). By-products of lipid peroxidation have been shown to cause profound alterations in the structural organisation and functions of the cell membrane, including decreased membrane fluidity, increased membrane permeability, inactivation of membrane-bound enzymes and loss of essential fatty acids (Maikai *et al.,* 2013). These changes in the cell surface of erythrocytes lead to increase in erythrocyte osmotic fragility (Umar *et al*., 2007). It has been established that infections caused by *T. brucei* group of parasites alter the antioxidant defence of the host (Igbokwe *et al*., 1994; Umar *et al*., 2007) and, thus, increase the susceptibility of the erythrocytes to oxidative haemolysis (Igbokwe *et al*., 1994). Increased production of hydrogen peroxide and

free reactive oxygen species (ROS) have been reported in parasitized erythrocytes (Maikai *et al.*, 2013). Antioxidants are compounds that are involved in the scavenging of free radicals and in suppressing the actions of ROS generated during infections.

There was a significant increase in the levels of ALT, AST and ALP in mice pretreated with kaempferol and those treated with normal saline. The elevated serum levels of ALT and AST in mice in this study is in agreement with earlier reports of Eghianruwa *et al.*, (2012), Srivastava *et al.*, (2012), Taiwo *et al.*, (2003), in various trypanosome-infected animals. The elevated values of ALT and AST enzymes may be due to tissue death (necrosis) and inflammation in the host, particularly of the liver, heart, muscle and kidney (Taiwo *et al.*, 2003; Takeet and Fagbemi 2009). Another possibility of increased levels of ALT and AST is the lyses of RBCs by trypanosomes (Taiwo *et al*. 2003; Takeet and Fagbemi 2009) at different stages of the infection. Serum levels of ALT and AST are two of the most useful indications of liver cell injury, although the AST is less liver specific than is ALT (Murali, 2014). Elevations of the AST level may also be seen in acute injury to cardiac or skeletal muscle (Murali, 2014). The AST, ALT, and alkaline phosphatase tests are most useful to make the distinction between hepatocellular and other disease conditions (Murali, 2014). *Trypanosoma brucei* may induce stress, thereby weakening the immune system and eventually alter the serum biochemical parameters. Awobode (2006), reported that many enzymes were found in the serum and the level of their activity can be determined, and that depression or elevation of particular enzyme indicate the presence of disease or damage to specific tissue or organ. The elevated serum level of AST may be attributed to cellular damage as reported by Kobo, (2012), and that of ALT may be attributed tissue destruction by the parasites as previously reported by Enwezor and Sakey (2005).

The elevated ALP in mice pretreated with kaempferol and those that were treated with normal saline disagrees with the work of Nwoha *et al*., (2013), who reported that dogs infected with *Trypanosoma brucei* and *Ancylostoma caninum* (hook worm) had low levels of ALP. Kwem *et al* (2000) reported progressive abnormal decrease in ALP in association with liver fibrosis in cattle infected with *Trypanosoma vivax*, but agrees with the work of Kobo (2012) in rats infected with *T brucei brucei*. Oyewale and Malomo (2009) attributed the increase to inflammatory condition involving liver and gatro-intestinal tract. But Ekanem and Yusuf (2008) attributed the increase in ALP to infiltration and inflammation of vital organ especially liver and kidney.

A significant ($P < 0.05$) increase in the serum level of specific antioxidants enzymes were recorded in mice treated with diminazene aceturate and kaempferol and kaempferol only when compared to mice treated with normal saline. This finding agrees with the report of Mandal *et al* (2007) in rats chemically induced hepatocarcinogenesis. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are enzymatic antioxidants that mop-up free radicals, SOD are the first line of defense against oxidative stress. It dismutates superoxide anion to H_2O_2 and water. The H_2O_2 generated from the activity of SOD will be detoxified to water and oxygen by CAT and GPx. Hence, CAT and GPx are positioned in the second line of defence to scavenge free radical and ROS. Trypanosomes were reported to induce oxidative stress and deplete the body antioxidants. Exogenous antioxidants such as Vitamin C and dimethyl sulphoxide gave a better clinical improvement and survival in *T. brucei brucei* infection in rats (Eghianruwa, 2012). Reactive oxygen species (ROS) are produced during normal cellular function. ROS include hydroxyl radicals, superoxide anion, hydrogen peroxide and nitric oxide Lapidot *et al*., (2002). They are transient species due to their high chemical reactivity that leads to lipid peroxidation (Fonseca-Silva *et al.,* 2011). Under normal conditions, antioxidant systems of the cell minimize

the perturbations caused by ROS. When ROS generation is increased to an extent that overcomes the cellular antioxidants, the result is oxidative stress (Fonseca-Silva *et al.,* 2011). Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. The various antioxidants exert their effect by scavenging superoxide, or by activating of a battery of detoxifying/defensive proteins (Cao *et al.,* 1997). The prevention of oxidation is an essential process in all the aerobic organisms, as decreased antioxidant protection may lead to cytotoxicity (Miura, *et al.,* 1998). Although, the precise molecular mechanism of action of flavonoid have not yet been demonstrated (Fonseca-Silva *et al.,* 2011), but there are various ways in which ROS can be produced; flavonoids (e.g quercetin) can induce the production of superoxide anion, hydrogen peroxide, and other reactive oxygen species (ROS) (Bischoff, 2008). ROS are generated in cells infected by pathogens to combat infection (Fonseca-Silva *et al.,* 2011). ROS can also be generated in response to some drugs, and the same principle works for certain antiprotozoan drugs in killing parasites in an infected cell (Mamani-Matsuda *et al.,* 2004). These could be the reason for the significant ($P < 0.05$) reduction in serum level of some specific antioxidants enzymes in mice pretreated with kaempferol for 14 consecutive days.

The significant reduction in the serum level of MDA in mice treated with diminazene aceturate only, diminazene aceturate and kaempferol and kaempferol only when compared to those that were treated with normal saline agrees with the work reported by Kobo *et al* (2014) who reported the ameliorative effect of Daflon® (a mixture of two different flavonoids) on erythrocyte osmotic fragility and malondialdehyde concentration induced by *Trypanosoma brucei brucei*-infection in Wistar rats. They attributed the in increase MDA concentration to the action of *T. brucei brucei* on the erythrocyte membranes. Since malondialdehyde is one of the end-products of lipid peroxidation, and its extent is measured by concentration of MDA in tissues (Eze *et al.,* 2008).

These changes on the surface of erythrocyte membranes, caused by *T. brucei brucei* infection couple with increased level of free radical production by the parasites could be responsible for the increased MDA concentration obtained in infected untreated mice. Infection by *T. brucei brucei* may alter the host's antioxidant defence system against free radicals, which results in an imbalance between radical-generating and radical-scavenging activities and, hence, the results in oxidative stress.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

In summary, the present study showed that experimental infection of mice with *Trypanosoma brucei brucei* causes decreased mean survival rate, increased erythrocyte osmotic fragility, increased serum level of MDA concentration and some level of damage in the brain, heart, liver, kidney and lungs. The mice infected with *Trypanosoma brucei brucei* developed parasitaemia four days following intraperitoneal inoculation with the parasite.

Treatment with kaempferol in mice experimentally infected with *Trypanosoma brucei bruce* causes increased survival rate, reduced level of parasitaemia, erythrocyte osmotic fragility, serum level of MDA concentration and damage to tissues and organs.

6.2 Conclusion

In conclusion:

Pre-treatment with kaempferol did not significantly suppressed parasitaemia and did also not improve PCV, Hb concentration and RBC count in relation to infected untreated mice.

Treatment with Kaempferol in mice infected with *Trypanosoma brucei brucei* significantly suppressed parasitaemia and improved PCV, Hb concentration, RBC count and erythrocyte osmotic fragility in relation to infected untreated mice.

Treatment with kaempferol significantly reduced serum level of MDA and liver enzymes in mice infected with *Trypanosoma brucei brucei* in relation to infected untreated mice.

Treatment kaempferol significantly improved antioxidant statue in mice infected with *Trypanosoma brucei brucei* in relation to infected untreated mice.

Treatment with the combination of diminazene aceturate and kaempferol did not revealed superior effect over diminazene aceturate alone in mice infected *Trypanosoma brucei brucei.*

6.3 Recommendations

Based on the results of this study, it is therefore recommended that:

Further study should be carried out to evaluate the effect of kaempferol using different routes and time of administration.

Further determine the mechanism by which kaempferol enhances some haematological parameters and suppression of parasitaemia.

Need to determine the effect of combination of kaempferol with the sub-therapeutic dose of diminazene aceturate in mice with experimental *Trypanosoma brucei brucei* infection.

There is also need to study the mechanism of interaction between kaempferol and diminazene aceturate in mice with experimental *Trypanosoma brucei brucei* infection.

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Appendix 1: Mean ± SEM level of survival rate in mice infected with *Trypanosoma brucei brucei* and treated with Kaempferol and/ or diminazene aceturate

SEM

NC	PKF	DZ	DZ+KF	KF	NS
10 ± 0.0	8.7 ± 0.54	10 ± 0.0	10 ± 0.0	9.9 ± 0.1	8.4 ± 0.6

Key: NC= Neutral Control, PKF= Pretreated with kaempferol, DZ=Diminazene aceturate, DZ+KF= Diminazene aceturate and kaempferol Kf= Kaempferol and NS=Normal Saline

Appendix 2: Mean level of parasitaemia in mice infected with *Trypanosoma brucei brucei* and treated with kaempferol and/ or diminazene aceturate

Days	NC(×10^6)	PKF(×10^6)	DZ(×10^6)	DZ+KF(×10^6)	Kf(×10^6)	NS(×10^6)
1	0.00	0.87	0.88	0.87	0.44	0.44
2	0.00	1.77	3.98	3.53	1.77	4.90
3	0.00	4.86	6.83	8.00	4.79	4.42
4	0.00	7.79	11.22	9.76	7.94	11.22
5	0.00	26.36	31.62	31.62	4.42	29.86
6	0.00	31.62	0.00	0.00	11.22	42.11
7	0.00	52.59	0.00	0.00	12.55	77.06
8	0.00	111.80	0.00	0.00	38.62	153.70
9	0.00	251.20	0.00	0.00	38.62	292.90

Key: NC= Neutral Control, PKF= Pretreated with kaempferol, DZ=Diminazene aceturate, DZ+KF= Diminazene aceturate and kaempferol Kf= Kaempferol and NS=Normal Saline

Appendix 3: Mean ± SEM of haematological parameters in mice infected with *Trypanosoma brucei brucei* and treated with kaempferol and/ or diminazene aceturate

	Treatments					
Test	NC	PKF	DZ	DZ+KF	KF	NS
PCV	45.93±1.746	29.07±0.8977	36.60±0.9706	37.88±1.860	38.65±1.814	28.70±0.868
Hb	15.30±0.5854	9.69±0.3040	11.93±0.3459	12.88±0.6174	12.85±0.6030	9.57±0.284
RBC	8.46±0.2587	5.34 ±0.1063	6.89±0.8953	7.75±0.2852	7.27±0.2973	5.11±1.105

Key: NC= Neutral Control, PKF= Pretreated with kaempferol, DZ=Diminazene aceturate, DZ+KF= Diminazene aceturate and kaempferol Kf= Kaempferol, NS=Normal Saline, PCV= packed cell volume, Hb= haemoglobin concentration, RBC= red blood cell count

Appendix 4: Effect of treatments with kaempferol and/ or diminazene aceturate on erythrocytic indices; MCV (mean corpuscular volume); MCH (mean corpuscular haemoglobin); MCHC (mean corpuscular haemoglobin concentration) of mice experimentally infected with *Trypanosoma brucei brucei*

Treatment	NC	SEM PKF	DZ	DZ+KF	KF	NS
MCV (FL/Cell)	54.07±0.4688[a]	40.87±0.6627[b]	48.55±1.104[a]	47.87±1.005[a]	53.38±2.035[a]	37.33±2.582[b]
MCH (Pg/cell)	15.3±0.1673[a]	14.77±0.3293[a]	16.00±0.2266[a]	15.63±0.2418[a]	16.82±0.1759[a]	9.947±0.3420[b]
MCHC (g/dl)	33.58±1.833[a]	26.98±1.631[b]	32.32±1.890[a]	32.72±1.670[a]	30.03±1.325[a]	21.02±1.530[b]

Key: Key: NC= Neutral Control, PKF= Pretreated with kaempferol, DZ=Diminazene aceturate, DZ+KF= Diminazene aceturate and kaempferol Kf= Kaempferol, NS=Normal Saline, MCV= Mean corpuscular volume, MCH= Mean corpuscular haemoglobin AND MCHC= Mean corpuscular haemoglobin concentration

Appendix 5: Mean ± SEM of erythrocyte osmotic fragility in mice infected with *Trypanosoma brucei brucei* and treated with kaempferol and/ or diminazene aceturate

%haemolysis

Conc	NC	PRF	DZ	DZ+KF	KF	NS
0.9	5.64±.0.5619	15.56±2.317	5.23±.0.7851	5.13±.0.917	5.89±0.8506	16.12±2.081
0.7	15.72±0.7492	69.98±2.524	22.69±1.363	22.27±1.665	23.38±3.902	68.57±6.950
0.5	69.20±3.615	93.65±7.503	75.88±6.432	72.90±5.783	68.57±6.943	92.43±3.810
0.3	65.37±3.387	90.12±3.456	85.73±3.684	87.68±5.127	87.13±5.647	98.63±1.370
0.1	71.86±6.164	76.68±5.131	73.98±6.544	76.13±5.214	73.14±4.125	75.62±3.315
0	87.33±3.097	86.70±4.999	86.81±3.750	84.33±2.233	86.77±2.853	86.66±4.424

Key: NC= Neutral Control, PKF= Pretreated with kaempferol, DZ=Diminazene aceturate, DZ+KF= Diminazene aceturate and kaempferol Kf= Kaempferol and NS=Normal Saline

Appendix 6: Mean ± SEM of total and differential white blood cell count in mice infected with *Trypanosoma brucei brucei* and treated with kaempferol and/or diminazene aceturate

	Treatments					
	NC	PKf	DZ	DZ+Kf	Kf	NS
WBC	9.250±0.9566	4.880±1.643	7.650±0.878	7.3±1.064	7.993±1.077	5.333±1.126
Lymphocytes	0.6528±0.046	0.4427±0.033	0.4903±0.039	0.58±0.031	0.6013±0.046	0.4127±0.030
Neutrophils	0.6483±0.032	0.3533±0.052	0.5283±0.016	0.5823±0.027	0.5879±0.026	0.3868±0.032

Key: NC= Neutral Control, PKF= Pretreated with kaempferol, DZ=Diminazene aceturate, DZ+KF= Diminazene aceturate and kaempferol Kf= Kaempferol and NS=Normal Saline

Appendix 7: Mean ± SEM of serum biochemical parameters in mice infected with *Trypanosoma brucei brucei* and treated with kaempferol and/or diminazene aceturate

	Treatments					
	NC	PKF	DZ	DZ+KF	KF	NS
ALT	19.33±1.256	38.79±3.177	28.33±2.551	28.33±2.642	27.95±1.408	38.67±3.136
AST	49.83±3.102	75.53±4.173	56.33±4.566	54.36±3.903	53.33±3.566	80.2±5.139
ALP	80.69±2.643	103.4±5.876	87.72±6.164	85±5.216	83.27±5.703	108.5±7.707

Key: NC= Neutral Control, PKF= Pretreated with kaempferol, DZ=Diminazene aceturate, DZ+KF= Diminazene aceturate and kaempferol Kf= Kaempferol, NS=Normal Saline, ALT= Alanine aminotransferase, AST= Aspartate aminotransferase and ALP= Alkaline phosphatate

Appendix 8: Mean ± SEM of serum biomarkers of oxidative stress parameters in mice infected with *Trypanosoma brucei brucei* and treated with Kaempferol and/ or diminazene aceturate

	Treatments					
Test	NC	PKF	DZ	KF+DZ	KF	NS
SOD	69.67±4.302	48.33±3.442	71.17±4.615	75.83±4.936	75.31±3.992	46.67±3.211
CAT	72.67±3.474	46.5±3.085	73.50.5±6.29	75.33±2.362	77.17±3.790	48.33±3.981
GPx	32.33±1.358	24.15±1.478	30±2.989	30.33±1.145	33.5±1.803	24.67±1.544
MDA	19.17±0.7923	29.87±1.579	22.11±0.577	21.13±0.9458	21.67±0.6667	30.67±1.476

Key: NC= Neutral Control, PKF= Pretreated with kaempferol, DZ=Diminazene aceturate, DZ+KF= Diminazene aceturate and kaempferol Kf= Kaempferol, NS=Normal Saline, SOD= Superoxide dismutase, CAT= Catalase, GPx= Glutathion peroxidase and MDA= Malondialdehyde

Appendix 9: Evaluation of packed cell volume (PCV*)*

PCV was determined using the microhaematocrit method as decribed by Coles (1986). Briefly, heparinized capillary tubes were filled with 75 µL of blood samples by capillary action. Piece of gause was carefully used to dry the outside of the tubes, while the opposite end of the tubes were sealed with flame. The tubes were then loaded onto a microhaematocrit centrifuge (Hawksley® England) and centrifuged at 3000 × *g* for ten minutes. The PCV values was measured using hematocrit reader (Hawksley® England).

Appendix 10: Evaluation of haemoglobin (Hb) concentration

Cyanmethaemoglobin method described by Coles (1986) was used to determine haemoglobin concentration. About 0.02 ml of blood was pipetted into a clean cuvette and diluted with 5 ml of cyanmethemoglobin reagent (Modified Drabkin Fluid). Following addition of blood, the cuvette was inverted 2-3 times and allowed to stand for 10 minutes for maximum conversion of haemoglobin to cyanmethaemoglobin. The absorbance of the resulting mixture was taken using a UV-spectrophotometer (Beckham Coulter, Model B U520, Austria) at 540 nm against blank. The percent transmission or optical density at 540 nm was recorded and compared with the reading obtained using standard solution of cyanmethaemoglobin.

Appendix 11: Evaluation of total erythrocyte count

Haemocytometer method described by Coles (1986) was used to determine total erythrocyte count. Blood sample was drawn carefully by aspiration to 0.5 mark of the pipette and transferred into a clean cuvette containing diluent(0.9% of normal saline). With another pipette the dilution fluid was drawn to 101 marks and mixed gently, the diluted blood was then discharged into haemocytometer counting chamber and allow to settle. The Total erythrocyte count was determined by viewing under microscope at 40 × magnification. The total number of cells in five squares in the centre of the counting chamber was calculated and multiplied by 10,000. The value obtained represented the total number of the erythrocyte per microliter (µL).

Appendix 12: Evaluation of erythrocyte indices

The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean morpuscular haemoglobin concentration (MCHC) was calculated from the values of PCV, Hemoglobin concentration and erythrocyte count as described by Schalm *et al.* (1975). The formula for calculating each parameter is shown below:

$$\text{MCV} = \frac{\text{PCV} \times 10}{\text{RBC Count}} \text{ (femtoliter per cell)}$$

$$\text{MCH} = \frac{\text{Hb} \times 10}{\text{RBC Count}} \text{ (picogram per cell)}$$

$$\text{MCHC} = \frac{\text{Hb} \times 100}{\text{PCV}} \text{ gram per deciliter}$$

Appendix 13: Evaluation of total white blood cell count

The total leucocytes count was determined using haemocytometer method as described by Coles (1986). A clean micropipette was used to draw blood by aspiration up to 0.5 mark using rubber mouth piece attached to the stem end of the micropipette above the bulb. Cotton wool was used to wipe off the excess blood from the stem of the pipette. Dilution fluid (2% glacial acetic acid in 100 ml of distilled water to which 1 ml of a 1% aqeous solution of gentian violet has been added) was drawn to the 11.0 mark. Blood and dilution fluid were then being mixed gently and properly

by inversion (up and down) for 2-3 minutes, six drops of of the mixture was removed by releasing the thumb immediately into haemocytometer counting chamber. The haemocytometer was filled with blood by capillary action and was examined under microscope at low power objectives × 40 magnification. The leucocytes in the 4 squares within the larger area of the corner of the haemocytometer were counted and the value obtained was multiplied by a factor 50 so as to obtain the total leucocyte counts.

Appendix 14: Determination of differential leucocyte count

The method described by Decie and Lewis (1991) was used to determine the Differential Leucocyte Count. A thin blood was made, air dried and fix in methanol for 4 minutes before staining with giemsa for 30 minutes. Thereafter, the smear was rinsed with distilled water and

dried with blotting paper. The thin blood smear slide was later viewed under oil immersion at × 100 magnification. Examination began at the thin layer of the smear where RBCs were well separated and the leucocytes were thinly spread. From thin end, systemic meandering system of slow, careful and detailed examination of leucocytes was made and differentiated WBC was finally counted.

Appendix 15: Determination of erythrocyte osmotic fragility (EOF)

Erythrocyte osmotic fragilty of blood obtain from all the mice was determined using method described by Faulker and King (1970) and modified by Oyewale (1991). Different concentrations of sodium chloride (NaCl) 0.0, 0.1, 0.3, 0.5, 0.7, 0.9 g/L (pH =7.4) were prepared in six different test tubes. About 1 ml of blood from each mouse was collected and transfered into sample bottles containing EDTA as an anticoagulant. Twenty microlitres of blood sample from each mouse was pipetted into test tubes containing varying concentrations of the prepared NaCl solution. Each tube were carefully mixed and incubated for 30 minutes at room temperature. All the test tubes were centrifuged at 2000 × g for 10 minutes. The supernatants were transfered into glass cuvette and the absorbance of each preparation was measured using UV spectrophotometer (Bausch and Lomb, USA) at 540 nm. The percentage haemolysis for each sample was calculated using the formula below:

$$\%\ \text{Haemolysis} = \frac{\text{Optical density of test solution}}{\text{Optical density of standard solution}} \times\ 100$$

Appendix 16: Evaluation of enzymes in the serum

Briefly, 1.0 ml of blood was collected from each mouse and allowed to clot. The clotted blood was centrifuged at 1000 × *g* for 10 minutes. The supernant (serum) from each tube was decanted into clear test tube and was used to analyse for the level of alanine amino transferase (ALT), aspartate amino trasferase (AST), and alkaline phosphatate (AP) using chemical auto-analyzer (Bayer® Clinical Chemistry Analyzer, Germany).

Appendix 17: Determination of serum malondialdehyde concentration

Fifty microlitres of serum from each animal were used to determine the level of serum malondialdehyde by double heating method as described by Draper and Hadley (1990) and modified by Altuntas (2002). The principle will be based on spectrophotometric measurement of the colour produced during the reaction of thiobarbituric acid (TBA) with the serum malondialdehyde. Two hundred and fifty microlitres of 100 *g*/L of trichloro acetic acid solution was added to fifty microliters of serum in a centrifuge tube and placed in a boiling water bath for 15 minutes. After cooling in tap water, the mixture was centrifuged at 1000 × *g* for 10 minutes and two hundred microlitres of the supernatant was added to one hundred microlitres 6.7 *g*/L of TBA in a test tube, then placed in a boiling water bath for 15 minutes. The solution was cooled in tap water and the absorbance was measured using UV-spectrophotometer (Jenway, 6405 Model, Japan) set at 532 nm.

Appendix 17: Determination of serum superoxide dismutase (SOD) specific activity

The specific activity of SOD was measured using the method described by Misra and Fridovich (1972) as modified by Rabideau (2001). The method is an indirect method of inhibiting auto-oxidation of adrenaline to its adrenochrome using SOD activity in the serum sample. By measuring the rate at which SOD decreases oxygen-dependent auto-oxidation reaction, the specific activity of SOD in each sample was calculated. One hundred a microlitres (0.1 ml) of serum was diluted with 0.9 ml of distilled water to make 1:10 dilution of microsome. Twenty microlitres of the diluted microsome were added to two hundred of and fifty microlitres of 0.05 M carbonate buffer. The reaction started by addition of thirty microlitres of 0.3 mM adrenaline. The reference mixture contain two hundred and fifty microlitres of 0.05 M of carbonate buffer, thirty microliters of 0.3mM adrenaline and twenty microlitres of distilled water. Thereafter, absorbance was measured for 30 seconds to 150 seconds at 480 nm. The percentatage inhibition was calculated as:

$$\% \text{ inhibition} = 100 - \left(\frac{Increase\ in\ absorbance\ \ for\ sample}{Increase\ in\ absorbance\ of\ blank} \times\ 100\right)$$

Appendix 18: Determination of serum catalase (CAT) activity

The specific activity of catalase in the serum collected from each mouse was determined using method descrbed by Beers and Sizer (1952). Catalase is an enzyme that scavenges H_2O_2 and converts it to water and molecular oxygen. By measuring the rate of breakdown of H_2O_2 spectrophotometrically, the specific activity of CAT in each sample was measured. Briefly, 167 µL of subst9rate(0.053 M H_2O_2) in 0.05 M potassium phosphate buffer (pH 7.0, 25 ºC) and 25 µL of the serum from each mouse was added to a 0.5 ml quartz cuvette and brought to final volume of 0.5 ml at 25 ºC, using 0.05 M potassium phosphate buffer,(pH 7.0). The rate of decrease in absorbance at 240 nm was measured with UV spectrophotometer. The catalase activity was calculated using the co-efficient of H_2O_2 43.6 M^{-1} Cm^{-1} as follow:

$$\text{Unit/mg protein} = \frac{\Delta A \text{ at } 240\ nm\ x\ cuvette\ volume(ml) x\ Dilution\ factor(1000)\ x\ 1cm\ light}{43.6\ \text{M}-1\ \text{Cm}-1\ \ x\ mg\ protein}$$

Appendix 19: Determination of serum glutathine peroxidase (GPx) specific activity

Modified method of Paglia and Valentine (1967) was used to determine glutathione peroxidase (GPx) activity spectrophotometrically by coupling the oxidation of glutathione and beta nicotinamide adenine dinucleotide phosphate (NADPH) using GP and glutathione reducctase (GR). Briefly, 1 mL of assay mixture containing an optimized concentrations of the following chemicals: 0,5 M K_2HPO_4 (pH 7.0), 2,5 mM EDTA, 0,18 U/mL GR, 100 mM glutathione and 10 mM reduced NADPH and plasma (0,5 mL) was added into the spectrophotometer cuvette along with 0,1 mL of 60 mM cumene hydroperoxide, a suitable substrate for GP. The mixture was placed into a 1 mL cuvette and read with Shimadzu UVPC 2100 spectrophotometer set at 366 nm at 37°C.

Glutathione Peroxidase

$GSH + H_2O_2$----------> $GSSG + 2\ H_2O$

Glutathione Reductase

GSSG + ß-NADPH----------> ß-NADP + 2 GSH

Abbreviations used:

GSH = Glutathione, Reduced Form

GSSG = Glutathione, Oxidized Form

ß-NADPH = ß-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form

ß-NADP = ß-Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form.

Appendix 20: Equipments and chemical/reagents used

S/N	Equipment	Drugs/chemical/reagents
1	Microscope	Normal saline
2	Micro haematocrit centrifuge	kaempferol
3	Coverslip	Diminazene aceturate
4	Glass slides	Glacial acetic acid
5	Scissors	Methanol
6	Cotton wool	EDTA
7	Surgical blade	Thiobarbituric aci
8	Spectrophotometer	Trichloroacetic acid solution
9	Fabricated mice cage	Adrenaline
10	Insulin syringe	Carbonate buffer
11	Capillary tube	Potassium phosphate buffer
12	Micro Pipette	10 % buffered formalin

13	Cuvette	Bouin solution
14	Haemocytometer	Haematoxylin
15	Water bath container	Eosin
16	Microtome	