COMPARATIVE THERAPEUTIC EVALUATION OF ASTAXANTHIN AND DIMINAZENE ACETURATE IN *Trypanosomabruceibrucei*-INFECTED WISTAR RATS

BY

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AUGUST, 2021

DECLARATION

I solemnly declare that the work in the thesis titled "**Comparative therapeutic evaluation of astaxanthin and diminazeneaceturate in *Trypanosomabruceibrucei*-infected Wistar rats**"has been performed by me in the Department of Veterinary Pharmacology and Toxicology, under the supervision of Professors M. M. Suleiman, J. O. Ayo, and I. A. Lawal. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any University.

Ishaku Patricia KOBO

_____	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This thesis titled: COMPARATIVE THERAPEUTIC EVALUATION OF ASTAXANTHIN AND DIMINAZENE ACETURATE IN *Trypanosomabruceibrucei*-INFECTED WISTAR RATS; by Patricia Ishaku Kobo meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this work to God Almighty, the omnipotent, omniscient and omnipresent God who has been the source of my strength, inspiration, wisdom, knowledge and understanding throughout this work. He kept close watch on my paths, to him be the glory and honour forever, amen. I also dedicate this work to my beloved husband, Rev. Ishaku Kobo and lovely children, Hyeinmen, Cynthia, Emmanuella and Israel, for their moral, spiritual and emotional support.

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ABSTRACT

The experiment was conducted with the aim of evaluating the effects of administration of astaxanthin singly or in combination with diminazene aceturate on *Trypanosoma brucei brucei*-infection in Wistar rats. Eighty (80) adult male Wistar rats were randomly allotted into eight groups (I, II, III, IV, V, VI, VII and VIII) of 10 rats each. Group I (DW) and II (S/OIL) served as negative controls and they were only administered *per os* with distilled water (2 mL/kg) and soya oil (2 mL/kg), respectively. Group III (T) rats served as positive controls and were inoculated with *Trypanosoma brucei brucei* only. Rats in groups IV (PreA + T) and V (PreA +T + DZ) were first pre-treated with astaxanthin orally, at 100 mg/kg body weight once daily for three weeks, after which they were inoculated with *T. brucei brucei*. Treatment with astaxanthin continued after inoculation to the end of the experiment. In addition, group V rats were administered diminazene aceturate, once at 3.5 mg/kg body weight intraperitoneally, when infection was established (at day 4 post-innoculation). Groups VI (T + A) and VII (T + A + DZ) rats were first inoculated with *T. brucei brucei*, followed by treatment with astaxanthin once daily to the end of the experiment. In addition, group VII rats were administered intraperitoneally with diminazene aceturate, once at 3.5 mg/kg. Rats in group VIII (T + DZ) were inoculated with *T. brucei brucei* and administered diminazene aceturate. All the inoculated rats were given 10^6 trypanosomes/mL of blood intraperitoneally. Infection of Wistar rats with *T. brucei brucei* resulted in classical symptoms of animal trypanosomosis. At the end of the experiment, blood and serum samples were collected for evaluation of haematological and biochemical parameters. Tissue samples of the heart, lungs, kidneys and liver were harvested and assessed for activities of antioxidant enzymes and 8-isoprostane concentration. The level of parasitaemia in T, PreA + T and T + A groups progressively increased till the termination of the experiment; but was

significantly ($P < 0.05$) lower in PreA + T group, when compared to T and T + A groups at days 6 and 8 post-infection. There was resurgence of parasitaemia in 10% of the rats in T + A + DZ group at day 7 post-treatment. There was a significant ($P < 0.05$) decrease in erythrocyte (RBC) count, packed cell volume (PCV) and haemoglobin (Hb) concentration in T and T + A groups, when compared to other treatment groups. The PCV and Hb concentration in PreA + T + DZ were significantly ($P < 0.05$) higher compared to values obtained in T + A + DZ rats. The T and T + A groups had a significant ($P < 0.05$) increase in neutrophil and total leucocyte counts, compared to the PreA + T and PreA + T + DZ. At 0.9%, 0.7% and 0.5% NaCl concentrations, the percentage haemolysis of rats in T and T + A groups were significantly ($P < 0.05$) higher when compared to all other treatment groups. The percentage haemolysis of rats in PreA + T + DZ was lower ($P < 0.05$) in comparison to PreA + T and T + A + DZ. The concentrations of 8-isoprostane in the liver, kidneys and heart of rats in T and T + A groups were significantly ($P < 0.05$) higher, when compared to other treatment groups. The level of immunoglobulin G was significantly ($P < 0.05$) higher in PreA + T and PreA + T + DZ groups than in other treatment groups. The concentration of tumour necrosis factor-alpha (TNF-α), interleukin-1 alpha (IL-1α) and interleukin-6 (IL-6) were significantly ($P < 0.05$) higher in T and T + A compared to other treatment groups. The concentrations of 8-isoprostane decreased significantly ($P < 0.05$) in the liver and kidneys of rats in PreA + T + DZ group when compared to those of T + A, and T + A + DZ groups. The kidneys of rats in PreA +T + DZ group had lower ($P < 0.05$) concentration of 8-isoprostane than those of PreA + T. The activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in the heart, lungs and liver of rats in the T and T + A groups were significantly ($P < 0.05$) higher, when compared to PreA + T and PreA + T + DZ groups. The liver of rats in PreA + T + DZ and T + A + DZ groups had lower ($P < 0.05$)

GPxactivity, when compared to PreA + T and T + DZ groups, respectively. There was a decrease ($P < 0.05$) in catalase activity in the liver of rats in PreA + T + DZ in comparison to PreA + T and T + A + DZ.

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LIST OF ABBREVIATIONS AND SYMBOLS

ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ApoA1	Apolipoprotein A1
ApoLI	Apolipoprotein LI
AST	Aspartate aminotransferase
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CSF	Cerebrospinal fluid
Cu	Copper
DNA	Deoxyribonucleic acid
DW	Distilled water
DZ	Diminazeneaceturate
EDTA	Ethylenediaminetetra acetic acid
Fe^{2+}	Ferrous iron
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Oxidised glutathione
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HAT	Human African trypanosomosis
Hb	Haemoglobin
HDL	High density lipoprotein
HNE	4 –hydroxy-2-nonenal

$HO^{\cdot}$	Hydroxyl radical
HOCI	Hypochlorous acid
Hpr	Haptoglobin related protein
HRP	Horseradish peroxidase
IFN-γ	Interferon – gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1α	Interleukin-1 alpha
IL-6	Interleukin-6
LOOH	Lipid hydroperoxide
mAb	Monoclonal antibody
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MDA	Malondialdehyde
Mn	Manganese
NaCI	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NWLSS^{TM}	Northwest Life Science Specialties
$^{1}O_2$	Singlet oxygen
O_2	Diatomic oxygen
O_2^{-}	Superoxide anion
$O_2^{\cdot}$	Superoxide radical
O_3	Ozone
ONOO	Peroxynitrite
PBS	Phosphate buffered saline

PCV	Packed cell volume
RBC	Red blood cell
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S/OIL	Soya oil
Se	Selenium
Se-OH	Selenole
SOD	Superoxide dismutase
T	Trypanosome
TBARS	Thiobarbituric acid reactive substance
TLF	Trypanosome lytic factor
TLR	Toll-like receptor
TMB	3,3′5,5′tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
UVA	Ultraviolet – A
VSG	Variant specific surface glycoprotein
WK	Week
Zn	Zinc

CHAPTER ONE

1.0 INTRODUCTION

Trypanosomosis is a complex debilitating and often fatal disease of humans and domestic animals (Umar *et al*., 2014;Kivali *et al*., 2020). It is caused by infection with one or more of the pathogenic tsetse-transmitted haemoflagellate protozoan parasite of the genus *Trypanosoma* (Yakubu *et al*., 2014). The causative agents of African animal trypanosomosis are *Trypanosoma brucei brucei*, *Trypanosoma vivax*, and*Trypanosoma congolense* (Ngure *et al*., 2009; Feyera *et al*., 2014; Igbo *et al*., 2015). Anaemia is a consistent feature of trypanosome infections associated with oxidative stress; inflicting damage to the erythrocyte membrane, resulting in haemolysis (Ngure *et al*., 2009; Abdullahi *et al*., 2018). Oxidative stress, involved in the pathogenesis of the disease (Omer *et al*., 2007; Umar *et al*., 2007, 2010; Saleh *et al*., 2009),is theincreased production of reactive oxygen species (ROS), including superoxides and hydrogen peroxide (H_2O_2) by macrophages of the mononuclear phagocytic system (Askonas, 1985), *T. brucei* (Meshnick *et al*., 1977), and depletion of tissue antioxidant reserves during trypanosome infections. Depletion of tissue glutathione (GSH) has been reported in *T. gambiense* (Ameh, 1984), *T. brucei*-infected rats (Igbokwe *et al*., 1998; Umar *et al*., 2000, 2001), *T. brucei*-infected mice (Igbokwe *et al*., 1994) and *T. vivax*-infected cattle (Igbokwe *et al*., 1996). The levels of plasma and tissue ascorbic acid are depleted in *T. brucei*-infected rats (Umar *et al*., 2000, 2001). Similarly, levels of retinol and carotenoids are depleted by the infection (Ihedioha and Anwa, 2002). Thus, oxidative stress causes damages to plasma membranes, thereby contributing to the cellular degenerative changes observed in trypanosomosis. Oxidative stress has been alleviated in experimental infections with various species of trypanosomes by administration of exogenous antioxidants, including flavonoids, ascorbic acid and vitamin E, to infected rats and rabbits

(Umar *et al*., 2007, 2010; Kobo *et al*., 2014). Antioxidant administration considerably reduces the degree and rate of degeneration of the tissues and organs; and may reduce the parasitaemia and anaemia intrypanosome-infected animals (Umar *et al*., 2007, 2010; Kobo *et al*., 2014).

African trypanosomes modulate the mammalian host immune responses (Beschin *et al*., 2014) by manipulating cells of the myeloid phagocytic system, including macrophages, monocytes, and granulocytes (neutrophils) (Stijlemans *et al*., 2015). Antioxidants, thus, increase the capacity of the host to control parasite growth by increasing resistance to infection. They limit tissue pathogenicity caused by immune responses, mounted for resistance to infection (trypanotolerance to infection). Infections caused by the *Trypanosoma brucei* group of parasites decrease the antioxidant defence mechanism of the host (Igbokwe *et al*., 1994, 1996; Omer *et al*., 2007; Umar *et al*., 2007, 2010), increase lipid peroxidation of the cell membranes(Saleh *et al*., 2009; Kobo *et al*., 2014) and, thus, increase the susceptibility of the erythrocytes to oxidative haemolysis (Igbokwe *et al*., 1994; Habila *et al*., 2012). The combined administration of antioxidant vitamins and flavonoids ameliorates the anaemia and organ damage, caused by *T. brucei* infection in rats (Umar *et al*., 2007; Kobo *et al*., 2014). Lipid peroxidation, the oxidation of polyunsaturated fatty acids, is a central feature of oxidant stress, a phenomenon increasingly implicated as causative in numerous pathological conditions (Yin *et al*., 2006), including trypanosomosis (Omer *et al*., 2007; Saleh *et al*., 2009; Kobo *et al*., 2014). Lipid peroxidative products are frequently used to quantify oxidative injury and may be assessed by measuring either primary or secondary peroxidative end-products. Primary end-products of lipid peroxidation include conjugated dienes and lipid hydroperoxides (Kenar *et al*., 1996), while secondary end-products include thiobarbituric acid reactive substances, gaseous alkanes and 8-

iso-prostaglandin $F_{2\alpha}$ (8-isoprostane) (Yin and Porter, 2003). The 8-isoprostane is a stable end product of oxidative stress and one of the most reliable biomarkers of lipid peroxidation in the body used to quantify oxidative injury (Mizuno and Kataoka, 2015). Products of lipid peroxidation formed in various biochemical reactions are scavenged by antioxidants. This informed the use of antioxidant agents, which scavenge ROS, and prevent the induced damage by ROS, in the treatment of trypanosomosis (Umar *et al*., 2007; Kobo *et al*., 2014). Oxidative stress induced by infection with trypanosomes results in the production of large amounts of reactive oxygen species (ROS), which act as cytotoxic agents (Gutteridge, 1995) damaging vital components of the cells, including proteins and lipids (Mishra *et al*., 2017). The evaluation of oxidative stress markers and haemato-biochemical indices is beneficial in the determination of the degree of damage to host tissues caused by the infection (Otesile *et al*., 1991; Akinseye *et al*., 2020), and the health status of the infected animals (Mishra *et al*., 2017). The major enzymatic antioxidants directly involved in ROS scavenging are:superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (Halliwel, 2007; Pacher *et al*., 2007; Pham-Huy *et al*., 2008; Kurutas, 2016).Catalase is used by cells to metabolise the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water (Chelikani *et al*., 2004). It is an important enzyme that protects the cells from ROS-induced damage. Glutathione peroxidase is a cytosolic enzyme that maintains redox equilibrium and protects the cells from oxidative damage. The biological function is to reduce free hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water (Muthukumar *et al*., 2010). Superoxide dismutase is one of the most potent intracellular enzymatic antioxidants. It catalyses the conversion of superoxide anions to dioxygen and hydrogen peroxide. Immune response to trypanosomes infection is mediated by various cytokines (Bakari *et al*., 2017).

Cytokines orchestrate a Type I and/or Type II immune responses that play a role in African trypanosomosis outcomes (Isaac *et al*., 2016). Trypanosome-derived products activate the generation of pro-inflammatory mediators;interleukin-1alpha (IL-1α) and tumournecrosis factor-alpha (TNF-α) (Tachado and Schofield, 1994).They have been proposed to be part of the immune process, leading to the pathological conditions of human African trypanosomosis (HAT) (Okomo-Assoumou *et al*., 1995; Rhind *et al*., 1997; Bakari *et al*., 2017). Similarly, interleukin-7 (IL-7), a Type I cytokine-triggered the production of IL-1 α and TNF-α reportedly influence the disease outcome (Weitzmann *et al*., 2000). The TNF-α has been implicated in the dysfunction of the blood-brain-barrier, enabling entry of trypanosomes into the central nervous system (CNS), thus, initiating late stage of infection (Enanga *et al*., 2002). Interleukin-10 (IL-10), a Type II cytokine, has been suggested to be a critical immunomodulator of HAT that down-regulates a range of inflammatory and activate markers on macrophages, including TNF-α (Isaac *et al*., 2016). The role of Type II cytokines (IL-10 and IL-13) in conferring immunity to HAT positives is highly speculative because of the contrasting reports by authors who observed advanced deleterious (Uzonna *et al*., 1998), protective (Bakhiet *et al*., 1996), or null (Schopf *et al*., 1998) effects. For decades, the control of animal trypanosomosis relies principally on chemotherapy and chemoprophylaxis using the salts of three compounds; diminazene,homidium, and isometamidium. Most of these agents, however, do not provide total control of the infection and are associated with high rates of disease re-occurrence, severe toxicity and side-effects (Molefe *et al*., 2017; Al-Otaibi *et al*., 2019). In addition, the parasites have developed resistance towards these long-standing agents, used in the control of the disease (Molefe *et al*., 2017).

Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) (Figure 1.1) is a lipophilic reddish pigment, which occurs in certain marine animals and plants such as carapace of crustaceans, flesh of salmon, trout, shrimps and algae (Odeberg *et al*., 2003; Choi *et al*., 2011; Ambati *et al*., 2014). It contains a unique molecular structure in the presence of hydroxyl and keto-moieties on each ionone ring, which are responsible for the high antioxidant properties (Hussein *et al*., 2006; Liu and Osawa, 2007).

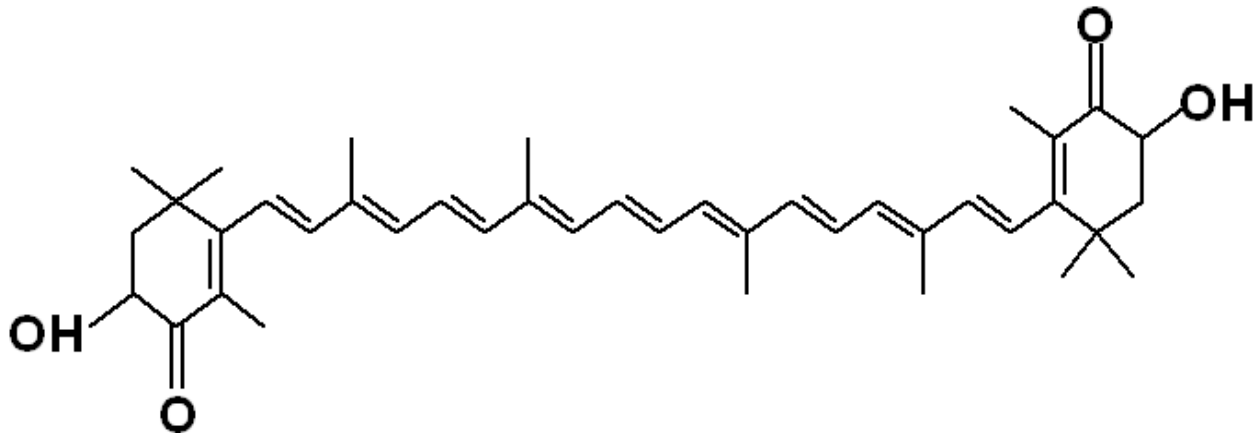

Figure 1.1: Structure of astaxanthin (Ambati *et al*., 2014)

The oxo-functional group in carotenoids has higher antioxidant activity, but without pro-oxidative contribution (Martin *et al*., 1999). The polyene chain in astaxanthintraps radicals in the cell membrane, while the terminal ring of astaxanthin scavenges ROS at the outer and inner parts of cell membrane (Augusti *et al*., 2012).

Astaxanthin is commercially available as a nutritional supplement in many countries, including USA, Sweden, Japan and South Korea (Choi *et al*., 2011; Ambati *et al*., 2014). Its products are available in the form of capsule, soft gel, tablet, powder, biomass, cream, energy drink, oil and extract in the market (Ambati *et al*., 2014). It is safe, with no side-effects when consumed with food. After feeding of astaxanthin to rats, it accumulates in animal tissues with no toxic effects (Stewart *et al*., 2008; Ambati *et al*., 2010, 2013a). Like other carotenoids, astaxanthin plays important functions in regulating immunity and disease aetiology (Park *et al*., 1998, 2010). The antioxidant activity of astaxanthin is 10 times more than zeaxanthin, lutein, canthaxanthin, β-carotene, and 100 times higher than α-tocopherol (Ambati *et al*., 2014; Park *et al*., 2010, 2014).It stimulates immune response in mice (Jyonouchi *et al*., 1994; Bennedsen *et al*., 1999; Chew *et al*., 1999). In addition to immunoregulatory activity, astaxanthin inhibits mammary tumour growth in mice (Chew *et al*., 1999), reduces bacterial load and gastric inflammation in *Helicobacter pylori*-infected mice (Bennedsen *et al*., 1999), and protects against UVA-induced oxidative stress (O'Conner and O'Brien, 1998).

1.1 Statement of Research Problem

African animal trypanosomes are the main parasitological constraints to livestock production in many sub-Saharan African countries, infested with tsetse flies. African trypanosomosis is responsible for about 55,000 human and 3 million livestock deaths annually, with over 60 million people and 48-55 million livestock at risk of the disease (Abenga *et al*., 2002; Obi *et al*.,

2013; Umar *et al*., 2014). Annual losses are approximately over 500 million US dollars in meat, milk, loss in traction power, control programme and annual administration of about 35 million doses of trypanocidal drugs in Africa (Geerts *et al*., 2001). According to Food and Agriculture Organisation of the United Nations (FAO, 2002), trypanosome-induced annual losses in cattle production alone are estimated in the range of 1.0-1.2 billion US dollars in sub-Saharan Africa. Reproductive organs are reportedly affected under trypanosome infection in both male and female animals. Sterility, menstrual disorders, and still-birth have been reported in humans during trypanosome infection (Maudlin, 2006). The disease causes abortion, premature birth and prenatal losses as well as cystic ovaries in female animals (Faye *et al.,* 2004, Silva *et al.,* 2013), with associated vertical transmission through the placenta (Batista *et al.,* 2012). In trypanosome-infected males, studies have indicated adverse effects of the disease on almost all fertility indices. Vaccine development has been hindered by the ability of the parasite to evade the host immune response via an elaborate mechanism, combining antigenic variation and immunosuppression (Donelson, 2003; Abdulazeez *et al*., 2013; Feyera *et al*., 2014). The rapid increase in drug resistance, drug counter-feiting, unpleasant side-effects of most trypanocidal drugs, vector resistance to insecticides, and affordability of the chemotherapeutic and chemoprophylactic agents have brought about increased incidence of the disease (Itty *et al*., 1995; Oluwafemi *et al*., 2007; Barrett *et al*., 2011). Therefore, there is the need to develop new, cheap, safe, accessible and affordable drugs for the treatment of African trypanosomosis in both humans and animals.

1.2 Justification of the Study

For the past four decades trypanosomosis, along with malaria, cancer and heart disease, was considered by the World Health Organisation (WHO, 1998) as one of the ten major health problems facing mankind. The disease has been a great challenge to the livestock industry, where the barrier imposed has been difficult, thusfar, to surmount by any form of chemotherapy, prophylaxis or control (Holmes *et al*., 2004; Ameen, 2013; Shittu *et al*., 2013). Astaxanthin is an effective inhibitor of oxidative damage (Naguib, 2000; Nakao *et al*., 2010), exhibiting immunomodulatory and anti-inflammatory actions (Chew and Park, 2009). It increases immunoglobulin production in human cells *in vitro* (Jyonouchi *et al*., 1995), enhances mouse splenic lymphocyte response, elevates mitogen-induced blastogenenesis and cytotoxic activity (Chew *et al*., 1999). It also increases the production of IL-1α and TNF-α (Okai and Higashi-Okai, 1996). In fact, astaxanthin pre-treatment prior to drug administration shows a protective effect by alleviating lipid oxidation during naproxen-induced gastric ulceration in rats (Kim *et al*., 2005). Activities of cellular enzymes involved in defence against oxidative stress including superoxide dismutase, catalase and glutathione peroxidase have also been shownto increase. Therefore, under conditions of increased oxidative stress, such as occur during disease states, dietary antioxidants may be critical in maintaining a desirable oxidant:antioxidant balance. It is conceivable that the administration of dietary astaxanthin alone or in combination with diminazene aceturate may prevent relapse of infection that occurs following treatment with diminazene aceturate, reduce the degree and rate of degeneration of tissues and organs,decrease the parasitaemia and anaemia in trypanosome-infected animals. The antioxidant may serve as a promising alternative to therapeutic intervention in animal trypanosomosis; thus, reducing the colossal economic losses incurred by livestock farmers due to trypanosomosis.

1.3 AIM AND OBJECTIVES

1.3.1 Aim of the Study

The aim of the study was to evaluate the effects of administration of asthaxanthin singly or in combination with diminazene aceturate on *Trypanosoma brucei brucei*-infection in Wistar rats.

1.3.2 Specific objectives of the study

The specific objectives of this study were to investigate in *T.brucei brucei*-infected Wistar rats, the effects of treatment with astaxanthin and/or diminazene aceturate on:

i. Body weight, survival rate and the level of parasitaemia

ii. Haematological parameters [erythrocyte (RBC), platelets, total (WBC) and differential leucocyte counts, packed cell volume (PCV), haemoglobin (Hb) concentration, erythrocytic indices (MCV, MCH, MCHC) and erythrocyte osmotic fragility(EOF)].

iii. Serum biochemical parameters including activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), concentrations of cholesterol, triglycerides and high density lipoproteins.

iv. Oxidative stress biomarkers (activities of superoxide dismutase (SOD), glutathione peroxidase (GPx),catalase (CAT)and 8-isoprostane).

v. Levels of interleukin-1α (IL-1α), interleukin-6 (IL-6),tumour necrosis factor-α (TNF-α)and immunoglobulin G (IgG).

1.4 Research Hypotheses

The null hypotheses for this study were:

i. Infection of Wistar rats with *Trypanosoma brucei brucei* does not induce significant changes on body weight, survival rate, level of parasitaemia, haemato-biochemical parameters,concentrations of IL-1α, IL-6, TNF-α, IgG and biomarkers of oxidative stress.

ii. Administration of astaxanthin alone or in combination with diminazene aceturate does not exert any significant effect on body weight, survival rate, level of parasitaemia, haemato-biochemical parameters, concentrations of IL-1α, IL-6, TNF-α, IgG and biomarkers of oxidative stress in *Trypanosoma brucei brucei*-infected Wistar rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background of the Study

African trypanosomosis affects both humans and livestock. In humans, the disease is known as sleeping sickness or human African trypanosomosis, while in livestock, it is referred to as *nagana* or African animal trypanosomosis (Wamwiri and Changasi, 2016). African animal trypanosomosis is widespread across most of the 38 countries of sub-Saharan Africa that are considered endemic for tsetse flies and the disease (Senait *et al*., 2016; Wamwiri and Changai, 2016). Tsetse flies are endemic in Africa and the occupy an area over 10 km^2between latitude 15° N and 29° S, from the southern edge of the Sahara desert (Figure 2.1) (Senait *et al*., 2016; Wamwiri and Changai, 2016; Omonona *et al*., 2020). African animal trypanosomosis is caused by *Trypanosoma congolense*, *T. vivax*, and *T. bruceispecies,*while *T. evansi* causes Surra in camels. The host preferences of each trypanosome species may differ, but *T. congolense*, *T. vivax*, and *T. brucei* have a wide host range among domesticated animals (Tsegaye *et al*., 2015; Senait *et al*., 2016). The disease is responsible for about 55,000 human and 3 million livestock deaths annually, with over 60 million people and 48-55 million livestock at risk of the disease (Abenga *et al*., 2002; Obi *et al*., 2013; Umar *et al*., 2014). Therefore, it remains a major constraint to livestock productivity that has a significant impact on the livelihood of millions of people in African developing countries, inflicting colossal economic losses of about several billion US dollars annually (Chitanga *et al*., 2011; Shiferaw *et al*., 2015).

The major pathological effects of the parasites are varying degrees of anaemia and degenerative changes in organs and tissues (Umar *et al*., 2010). The mechanism of pathogenesis is in part due

to the generation of ROS during trypanosome infection, which causes degenerative changes in vital tissues and organs of infected animals due to cellular injury and death (Igbokwe, 1994). Infection with African trypanosomosis is mainly characterised by immuno-suppression, which is a crucial factor in host-parasite relationship. The trypanosomes suppress the ability of the hosts to elicit a protective immune response by depressing the antibody, T-cell and macrophage responses (Taylor and Mertens, 1999). Some African cattle breeds, like *Bos taurus* are highly tolerant to trypanosome infection, but the more productive Zebu breeds are extremely susceptible. Increasing tolerance of Zebu cattle to trypanosomosis could make a significant impact on productivity of cattle (dairy and beef), in addition to crop cultivation (Kristjanson *et al*., 1999). N'Dama cattle are tolerant to infection with *T. congolense* and remain apparently healthy.

The ability to remain productive while harbouring pathogenic trypanosomes is known as trypanotolerance (Murray *et al*., 1984), which has been evolved as a mechanism of disease resistance, possibly through the lengthy co-existence of animals with the parasites. The animals can control parasitaemia and severe anaemia through an effective immune response to trypanosomes,although the mechanisms are yet to be well understood (Lutje *et al*., 1996; Yoshihara *et al*., 2007). It has been observed in mouse models that a balance between pro- and counter-inflammatory cytokines is central to the outcome of the disease (Kennedy, 2008). Cytokines play different roles in the control of infection, but overproduction of pro-inflammatory cytokines can lead to aggravated pathological changes (Magez *et al.*, 2004). Some studies have suggested that interferon-gamma (IFN-γ) stimulates parasite growth (Bakhiet *et al*., 1996), while TNF-α exerts trypanocidal effects (Lucas *et al*., 1993; Magez *et al*., 1999). Increased production

of regulatory cytokines such asIL-10 is associated with decreased pathology and resistance to infection (Sternberg *et al*., 2005). Currently, there is no effective vaccine against the disease. However, the available means to protect and maintain livestock despite the short-comings of the measures include tsetse-control strategies, chemotherapy and chemoprophylaxis and the rearing of trypanotolerant livestock (Chekwube *et al*., 2014; Eze *et al*., 2015a). In Africa, control relies mainly on chemotherapy and chemoprophylaxis using salts of three compounds – diminazene aceturate, homidium and isometamidum (Chekwube *et al*., 2014). In Nigeria, diminazene aceturate is the most commonly used therapeutic agent, while isometamidium chloride is the most commonly used prophylactic agent (Anene *et al*., 2001; Ezeokonkwo *et al*., 2010; Chekwube *et al*., 2014). Despite this, treatment of trypanosomosis is faced with challenges of drug resistance due to wrong use of drugs, and availaibility of only few trypanocides (Ezeokonkwo *et al*., 2007; Chitanga *et al*., 2011)). The inability of readily available drugs to cross the blood-brain barrier and toxicity is also major concerns in chemotherapy of trypanosomosis (Jennings *et al*., 1996; Anene *et al*., 1997). In view of these, the use of immunostimulants or immunomodulatory agents, such as vitamins, flavonoids and micronutrients have shown promising effects in the management of African trypanosomosis (Eze and Ochike, 2007; Umar *et al*., 2008; Eze *et al*., 2011; Kobo *et al*., 2014). The immune system is a highly proliferative, complex and integrated network of cells and organs, and may be strongly influenced by the micronutrients and vitamins (Wellinghausen and Rink, 1998).

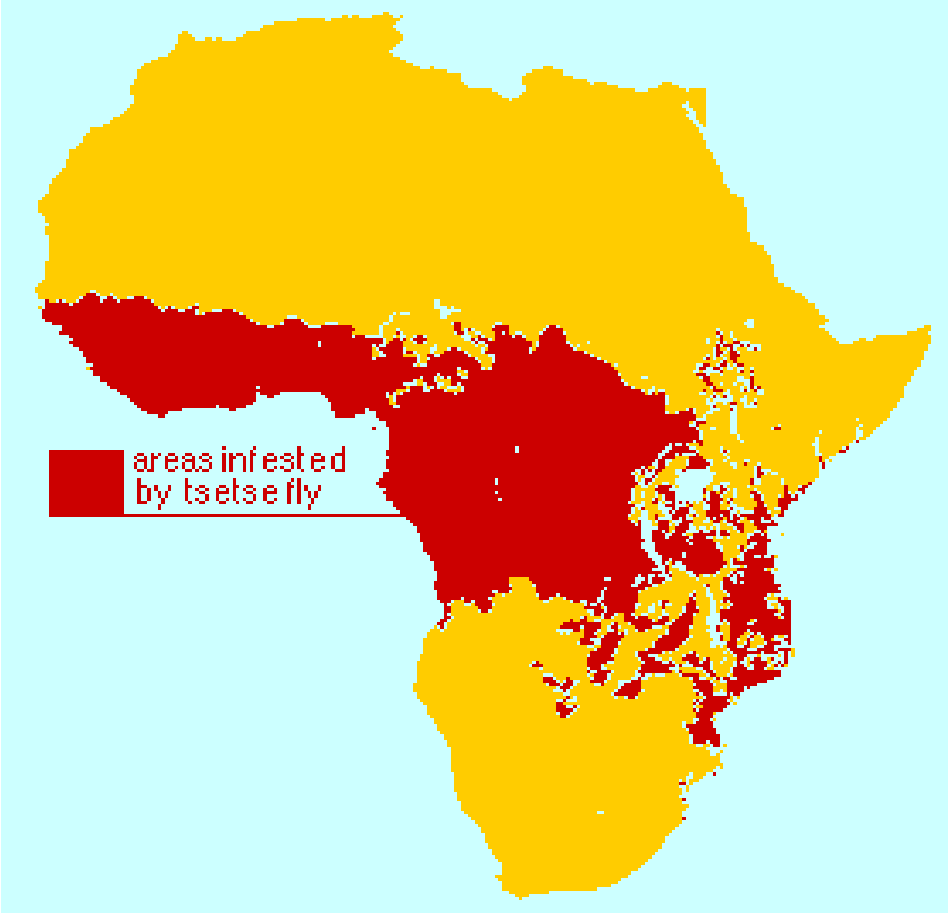

Figure 2.1: Map of Africa showing the distribution of tsetse flies(Adapted from FAO, 1998).

2.2 African Trypanosomes

Trypanosomes are unicellular organisms belonging to the Phylum *Sarcomastigophora*, class *Mastigophora*, Order *Kinetoplastida*, family *Trypanosomatidae* and genus *Trypanosoma* (MacLennan, 1980). Trypanosome species belong to three different subgenera: *Trypanozoon* (*Trypanosoma brucei brucei*, *T. brucei rhodesiense*, *T. brucei gambiense*, *T. evansi*, *T. equiperdum*), *Dutonella* (*T. vivax, T. uniforme*), and *Nanomonas* (*T. congolense, T. simiae, T. godfreyi*) (Uilenberg, 1998; Antoine-Moussiaux *et al*., 2009; Bezie *et al*., 2014). The *T. brucei* group of trypanosomes invades tissues (humoural), whereas *T. congolense* and to a lesser extent*T. vivax* predominantly restrict themselves to the blood circulation (Igbokwe, 1994). The trypanosomes are transmitted mainly by the tsetse flies of the genus *Glossina.* Some trypanosomes can also be transmitted mechanically by the blood sucking flies like the tabanids, horse flies and stomoxys (Baral, 2010). The species transmitted by the tsetse flies include:*T. brucei* subspecies, *T. congolense* and *T. vivax* and they undergo development in the tsetse fly vector (Welburn and Maudlin, 1999). *Trypanosoma evansi* is transmitted mechanically and, thus, the infection can be acquired outside the tsetse belt region (Baral, 2010). Trypanosomes that are transmitted by the tsetse flies can be grouped as haematic *(T.vivax, T. congolense, T. simiae)* causing anaemia; or humoural (*T.brucei* sub species) associated with inflammation and tissue damage (Losos and Ikede, 1972).

2.2.1 *Trypanosoma brucei*

Trypanosoma brucei belongs to the salivaria group in general and subgenus *Trypanozoon* in particular. The parasite was first described from Zulu land in 1914. Trypanosome species in this group are typically polymorphic with small sub-terminal kinetoplast, well-developed, undulating

membrane and undergo development in the mid-gut and salivary glands of tsetse fly. The *Trypanosoma brucei*complex comprises three morphologically identical subspecies: *T. bruceibrucei, T. b. rhodesiense*, and *T. b. gambiense*. *Trypanosoma brucei brucei*is pathogenic to animals, while the other subspecies cause acute sleeping sickness in East Africa and chronic sleeping sickness in West Africa. The other aberrant species, *T. evansi* and *T. equiperdum* are transmitted non-cyclically or by coitus (Odeyemi *et al*., 2015).

2.2.2 Life cycle of trypanosomes

The life cycle of trypanosomes (Figure2.2) includes, several phases both in the intermediate invertebrate and in the mammalian hosts (Bezie *et al*., 2014). Transmission by insects may be cyclical by tsetse flies, *Glossina* species, or mechanical by other biting flies. Apart from transmitting trypanosomes cyclically, tsetse flies can also act as mechanical vectors. In principle, any species may occasionally be transmitted congenitally (Uilenberg, 1998). The tsetse fly ingests the parasite when taking a blood meal from an infected animal. Blood stream forms (trypomastigotes) ingested by the fly undergo considerable changes in morphology and in their metabolism. They change into long slender forms called epimastigotes, multiply and finally give rise to the infective meta-trypanosomes. The meta-trypanosomes occur either in the biting mouthparts or in the salivary glands. Once infective meta-trypanosomes are present, the fly remains infective for the remaining part of its life. A new host will be infected with the parasite, when the fly takes another blood meal (Bezie *et al*., 2014). During the act of feeding, the fly penetrates the skin with its proboscis. Due to rupture of small blood vessels, a pool of blood is formed in the tissues and the fly injects saliva to prevent coagulation. Infection of the host takes place at this stage, with infective metacyclic trypanosomes (Uilenberg, 1998).

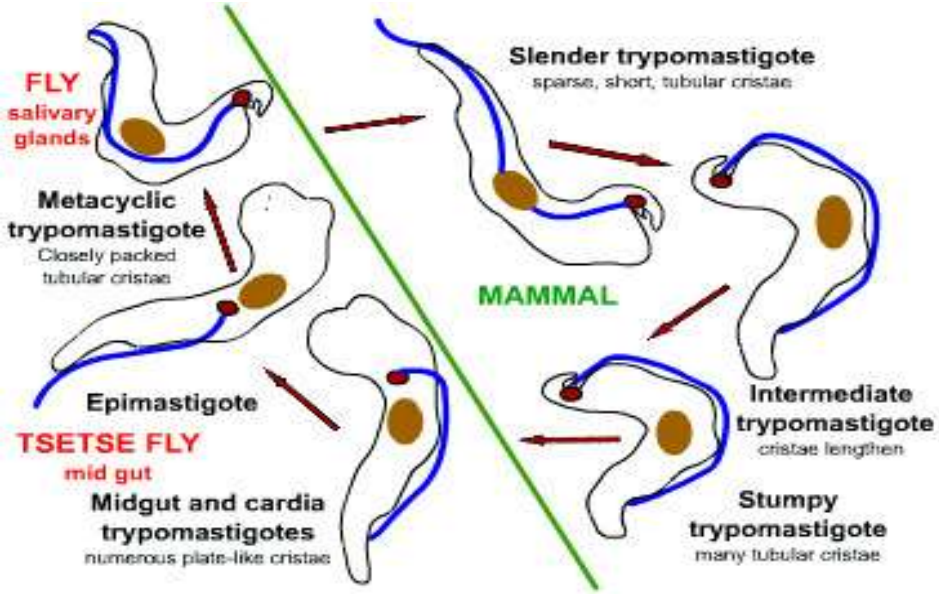

Figure 2.2 The life cycle of trypanosomes(Adapted from Vickerman, 1985).

2.2.3 The economic impact of African trypanosomosis

Tsetse-transmitted trypanosomosis is the main constraint to livestock production in the continent of Africa, preventing full use of land to feed the rapidly increasing human population (Shaw, 2004; Shiferaw *et al*., 2015). It affects 37 sub-Saharan African countries, extending over 10 million km^2 of land (Erkelens *et al*., 2000). Large areas of tropical Africa are unsuitable for livestock production due to presence of tsetse flies (Murray and Gray, 1984). African animal trypanosomosis is the single greatest health constraint to increased livestock production in sub-Saharan Africa, with direct annual production losses in cattle estimated at US$ 0.6-1.2 billion. Estimates of the overall annual loss potential in livestock and crop production have been as high as US$ 4.75 billion. An estimated 45 to 60 million cattle and tens of millions of small ruminants are at risk from trypanosomosis (Gilbert *et al*., 2001). FAO (2000) estimates that about three million cattle die each year due to African animal trypanosomosis. Furthermore, about 50 million people in Africa are exposed to the risk of contracting sleeping sickness (Franco *et al*., 2014).

Direct costs due to African animal trypanosomosis involve decreased livestock productivity (mortality, fertility, milk yield, ability to work as traction animals) to which can be added expenditure on controlling the disease (Shaw, 2004). Thirty five million doses of trypanocides are administered each year to protect livestock in tsetse-infected areas (Sones, 2001). Direct losses due to trypanosomiasis are estimated to be between US$ 1-1.2 billion each year whereas the indirect impact on agriculture in sub-Saharan Africa exceeds this amount. The overall impact extends to the restricted access to fertile and cultivable areas, imbalances in land use and exploitation of natural resources and compromised growth and diversification of crop-livestock production systems (Mattioli *et al*., 2004).

2.2.4 Drug treatment of African trypanosomosis

There is no effective vaccine against trypanosomes and in the absence of coherent environmentally-friendly and sustainable vector control strategies, the control of trypanosomosis continues to rely principally on chemotherapy and chemoprophylaxis using the salts of three compounds: diminazene, an aromatic diamidine; homidium, a phenanthridine; and isometamidium, a phenanthridine-aromatic amidine (Leach and Roberts, 1981; Anene *et al*., 2001). In addition, quinapyramine, suramin, and recently, melarsen oxide cysteamine (cymelarsan) are generally used for therapy and prophylaxis of *T. evansi* (Zhang *et al*., 1991; Ndoutamia *et al*., 1993). Of the six trypanocides, diminazene aceturate is the most commonly used therapeutic agent, while isometamidium chloride is most commonly used as prophylactic agent (Holmes *et al*., 2004). The drugs, with the exception of cymelarsan, have been in use for at least 30 years (Kinabo, 1993).

The therapeutic and prophylactic uses of trypanocides are beset by numerous limitations, including toxicity and the development of resistance by the parasites. The concept of "sanative pair" treatment prescribes the use of a pair of trypanocides (for examplediminazene and isometamidium), which are chemically unrelated and, therefore, are unlikely to induce cross-resistance is a practical method in using trypanocidal drugs (Machilla *et al*., 2001). One of the pair is used until resistant strains of trypanosomes appear and then the second is substituted and used until the resistant strains have disappeared from cattle and tsetse (Afewerk *et al*., 2000; Shiferaw *et al*., 2015). However, some strains of trypanosomes have shown their ability to develop multiple drug resistance to chemically unrelated trypanocidals, raising questions on the validity of "sanative pairs" (Delespaux *et al*., 2008). The emergence of drug-resistant strains is considered a very serious problem in trypanosomosis control, particularly for the resource-poor,

at-risk populations and farmers in Africa, and in the context of sustainable parasite control. Trypanosome resistance to trypanocides increases cost, reduces the efficiency of production and depletes the stock farmer of effective control tools (Donald, 1994). This increases the risk of environmental contamination due to progressive increase in frequency of use and dose rate of drugs with declining or little beneficial effects. Moreover, there is increased risk of toxicity from the use of large doses (Donald, 1994).

2.2.5 Drug resistance in African trypanosomosis

Currently, resistance to trypanocides has been reported from 15 countries in Africa (Geerts and Holmes, 1998). The exposure of trypanosomes to sub-therapeutic concentrations of trypanocidal drugs, the treatment frequency, the degree of drug exposure of the parasite population and immune competance of the host are important factors influencing the development of drug resistance (Geerts and Holmes, 1998; McDermott *et al*., 2003). Furthermore, some trypanocidal drugs such as homidium are well-known mutagenic compounds and may induce mutations, the most resistant of which may be selected under drug pressure (Holmes *et al*., 2004). The phenomenon of cross-resistance has now been clearly demonstrated. Quinapyramine usage has been shown to induce resistance to isometamidium, homidium and diminazene aceturate (Ndoutamia *et al*., 1993).

From a historical perspective, it may be pertinent to mention that relapses were reported immediately after the introduction of suramin, the preferred drug for the treatment of camel trypanosomosis (Knowles, 1927). Nevertheless, such relapses are occasional and since a better alternative does not exist, its use has been continued. However, there is now further field and

experimental evidence of diminished effectiveness and increased resistance of trypanosomes to suramin (Payne *et al*., 1992, 1994; El *et al*., 1999; Onah *et al*., 1999). This, coupled with the fact that commercial production of suramin has ceased, is a serious limitation in the treatment of surra, which can only worsen unless new anti-surra drugs are developed soon (Payne *et al*., 1994). Although quinapyramine was seriously affected by drug resistance, leading to its withdrawal from the market in 1976, it was later reintroduced for the treatment of surra exclusively (Schillinger and Rottcher, 1986).

Over the years, diminazene aceturate and isometamidium chloride have, respectively, been regarded as the best therapeutic and prophylactic trypanocides. The former was reputed as the only drug to which trypanosomes do not easily develop resistance because of its rapid elimination from the system, when compared with the more persistent prophylactic drugs such as isometamidium (Aliu *et al*., 1984; Rushigajiki *et al*., 1986). Unfortunately, this view is no longer accepted as field and laboratory isolates and strains of diminazene aceturate resistant trypanosomes have been reported;for example, some field isolates require up to 45 mg/kg diminazene aceturate as the minimum required dose to achieve cure (Chitambo and Arakawa, 1992; Peregrine and Mamman, 1993). Similarly, isometamidium treatment failures and shortened prophylactic intervals have been attributed to infections with drug-resistant trypanosome species (Peregrine *et al*., 1991; Sutherland *et al*., 1991). Homidium, which was previously used extensively as a prophylactic drug, was rendered almost useless by widespread development of resistant trypanosome strains (Clausen *et al*., 1992; Codja *et al*., 1993). Even for the new trypanocide, cymelarsan, there seems to be a bleak future for its field use as resistance has already been induced experimentally (Fairlamb *et al*., 1992; Osman *et al*., 1992; Ross and

Barnes, 1996). In order to efficiently combat and treat trypanosomosis, there is a need to research for drugs that have little or no resistance.

2.2.6 Host immune responses to trypanosomes

It is now clear that sera from humans and non-human primates have the ability to kill trypanosomes (Vanhollebeke *et al*., 2006; Molina-Portela *et al*., 2008). This ability to kill trypanosomes has been linked to the innate trypanosome lytic factors (TLF). Compelling evidencesuggests that apolipoprotein LI (ApoLI) and haptoglobin relatedprotein (Hpr) may be crucial elements of theTLF (Vanhollebeke *et al*., 2006, 2007). *Trypanosoma brucei brucei* has been shown to neutralise thetrypanolytic activity of normal human serum through theserum-resistance associated protein that binds toApoA1 (Van Xong *et al*., 1998). Trypanosomes escape host immune recognitionthrough antigenic variation of the membrane-boundvariant-specific surface glycoprotein, VSG (Pays, 2006). The VSGacts as a barrier preventing components of the immuneresponses from accessing the underlying plasma membrane (Ferrante and Allison, 1983). At peak parasitaemia, the parasite releases VSGinto circulation (Kato *et al*., 2016), thus inducing inflammatory responses.It has been shown that coat-switching trypanosomes failto activate B-cells until coat VSG homogeneity is achieved,thus evading recognition (Dubois *et al*., 2005). Previously, the main mechanisminvolved in controlling parasitaemia was through antibodyproduction (Mansfield, 1978; Pentreath, 1995; Magez *et al*., 2002), with trypanosome specific IgMand IgG reported in the cerebro-spinal fluid of late-stagepatients (Lejon *et al*., 1998). In a murine *T. b. brucei* model using B cell and IgM-deficient mice, the role of B-cells and IgMantibodies in parasitaemia control was investigated (Magez *et al*., 2008).The authors demonstrated that B-cells play a critical rolein peak parasitaemia clearance, while IgM

antibodies onlyplay a limited role.In another study, a T-cell independentanti-VSG IgM response was proposed as thefirst line of defence against proliferating parasites (Reinitz and Mansfield, 1990).However, gaps exist on how and whether antibodiesplay a significant role in parasite control (Stijlemans *et al*., 2007). Evidence isbuilding up, suggesting that cytokines are key playersin human African trypanosomosis pathogenesis (Sternberg *et al*., 2005; Ngotho *et al*., 2009).Most studies on cytokine dysregulations in human African trypanosomosis haveused experimental animal models, making it possible tofollow immunological responses with disease progression (Hertz and Mansfield, 1999; Namangala *et al*., 2001; Maina *et al*., 2004; Sternberg *et al*., 2005). In general, the early stageof infection is characterised by an elevation in pro-inflammatorycytokines (IFN-γ and TNF-α) with a switch to acounter-inflammatory response in late stage infection (Sternberg *et al*., 2005; Namangala *et al*., 2009).

It has been demonstrated that prolonged survival tomurine African trypanosomosis may be infection-stagedependent, with pro-inflammatory cytokine responsesplaying a critical role during early stage of infection whilecounter-inflammatory cytokines determine survival duringlate stage (Namangala *et al*., 2009). Furthermore, cytokines in the central nervous system (CNS)have been shown to revert to normal levels after treatment,making them biomarker candidates for CNS invasion (Rhind *et al*., 1997).

2.3 Cytokines

These are low-molecular weight polypeptides secreted by numerous cells, which regulate and mediate the immune system, also referred to as immunodulating agents (Dinarello, 2000). Cytokines act via cell surface receptors to control the magnitude and extent of the immune-

inflammatory responses by activating the macrophages, controlling growth and differentiation of T- and B-cells. Many cytokines are pleiotropic in nature and influence synthesis and action of other cytokines. Cytokines are, thus, immuno-regulatory and classified according to their immunological role as anti-inflammatory or pro-inflammatory cytokines. Pro-inflammatory cytokines enhance inflammation and, thus, their major role is to enhance systemic inflammation through attraction of inflammatory cells, in addition to stimulating increased production of secondary mediators. They are also known as Th 1 cytokines and examples include: interferon IFN-γ, TNF-α, IL-1, IL-8, IL-11, IL-12, IL-17, IL-18 (Dinarello, 2000; Luheshi *et al*., 2009). Anti-inflammatory or Th 2 cytokines counteract the role of inflammatory cells, thus regulating the extent of inflammation during infection. They include; IL-4, IL-10, IL-13, IL-16, IFN-alpha, TGF-beta (Dinarello, 2000).

2.3.1 Interleukin-1 alpha (IL-1α)

Trypanosome-derived products have been shown to activate the generation of pro-inflammatory mediators, including IL-1α by macrophages (Tachado and Schofield, 1994). Chronic secretion of macrophage-derived mediators is in part responsible for the pathogenic aspects of HAT (Okomo-Assoumon *et al*., 1995; Rhind *et al*., 1997). In laboratory infected mice with *T. b. brucei*, an increase in the level of IL-1α has been reported (Sileghem *et al*., 1998), including upregulation of granulocyte and macrophage due to IL-1α administration in mice (Hestdal *et al*., 1992). Synergistic role of IL-1α enhances cytokine production and haematopoietic recovery (Pang *et al*., 1994; Kovacs *et al*., 1998).

2.3.2 Interleukin-6 (IL-6)

Interleukin-6 is a multi-functional cytokine that possesses both pro- and counter-inflammatory effects with varied implications in pathophysiology of many neurological and inflammatory disorders. In other disorders, IL-6 was shown to possess beneficial effects, involving metabolic control (Pedersen and Fischer,2007), neuronal survival (Kushima and Hatanaka, 1992), neuro-protective and analgesic effects in rats (Oka *et al*., 1995). On the other hand, destructive properties have also been reported. IL-6 has been associated with neuronal degeneration and cell death in degenerative disorders (Gadient and Otten, 1997). Furthermore, in other neuropathological disorders, mice over expressing IL-6 are associated with increased blood-brain barrier permeability coupled with neuropathological abnormalities (Brett *et al*., 1995). In a murine *T. b. brucei*-model, high levels of IL-6 are observed in mice with less severe neuropathology (Sternberg *et al*., 2005). These findings are consistent with studies in a *T. b. rhodesiense* vervet monkey model, in which CSF IL-6 levels are up-regulated in late stage disease (Nyawira-Maranga *et al*., 2013). Similarly, in HAT patients CSF IL-6 has been up-regulated in late stage *T. b. rhodesiense* disease (MacLean *et al*., 2006, 2012) and in *T. b. gambiense* disease (Lejon *et al*., 2002; Courtin *et al*., 2006). A study comparing plasma levels of IL-6 in two HAT foci in Uganda reported higher levels in Soroti with mild disease as compared to Tororo with a more acute disease (MacLean *et al*., 2007). The implication for the elevated IL-6 levels in Soroti patients was not explained. However, IL-6 may play a protective role as reported in experimental animals. Consequently, although murine models point to a protective role, in humans the role of IL-6 cannot yet be clearly defined.

2.3.3 Tumour necrosis factor-α (TNF-α)

Tumour necrosis factor-α (TNF-α) is a pro-inflammatory cytokine, predominantly produced by macrophages and is involved in the innate immunity against intracellular pathogens. Soluble VSGs shed by live trypanosomes are thought to be the major TNF-α-inducing factors (Magez *et al*., 1998). To date, there is still controversy on the role of TNF-α in trypanosome infection. Some of the studies using animal models indicate that TNF-α may be a key mediator in the control of *T. brucei* infections (Magez *et al*., 1997, 1999; Naessens *et al*., 2005). In one study, a direct dose-dependent lytic effect of TNF-α on purified *T. b. gambiense* parasite was reported, suggesting an involvement in parasite growth control (Daulouede*et al*., 2001). However, detrimental roles of TNF-α have also been reported.

In a murine model, TNF-α knock-out mice exhibited trypanosome-mediated immunopathological features, such as, lymphnode-associated immunosuppression and lipopolysaccharide hypersensitivity (Magez *et al*., 1999). High levels of brain TNF-α are associated with moderate to severe neuropathy (Sternberg *et al*., 2005). In a *T. b. rhodesiense* vervet monkey model, CNS TNF-α level did not differ from controls and no association with clinical presentation was reported (Maina *et al*., 2004). Furthermore, it has been demonstrated in a murine model that TNF-α may be involved in anaemia, associated with *T. b. rhodesiense* infection and not in *T. congolense* (Naessens *et al*., 2005). This demonstrates the challenges in comparing studies utilising different trypanosome or host species. There is evidence pointing to a possible role of TNF-α in trypanosome penetration of the blood-brain barrier, especially through Toll-like receptor (TLR)–MyD88–mediated signalling (Amin *et al*., 2012).

Human *T. b. rhodesiense* studies testing predictions from experimental animal models have started to emerge. A study comparing plasma cytokine levels between geographically isolated HAT foci reported elevated levels of TNF-α in Ugandan patients as compared to their counterparts in Malawi (MacLean *et al*., 2004). The authors proposed that TNF-α might play a role in the rapid disease progression reported among Uganda patients. On the contrary, in another study in Uganda both plasma and CSF TNF-α levels remained at base-line (MacLean *et al*., 2001), just as previously reported among *T. b. gambiense* patients (Lejon *et al*., 2002). From available literature foranimal models and human studies, the role of TNF-α in trypanosomosis pathology remains largely unclear.

2.4 Oxidative Stress

The harmful effect of ROS and reactive nitrogen species (RNS) radicals causing potential biological damage is termed oxidative stress and nitrosative stress, respectively (Owoade *et al*., 2019).This is evident in biological systems when there is either an excessive production of ROS/RNS and/or a deficiency of enzymatic and non-enzymatic antioxidants. The redox stress/oxidative stress, is a complex process. Its impact on the organism depends on the type of oxidant, site and intensity of its production, composition and activities of various antioxidants, and ability of repair systems (Durackova, 2007; Rahal *et al*., 2014). The term "ROS" includes all unstable metabolites of molecular oxygen (O_2) that have higher reactivity than O_2 like superoxide radical ($O_2^{\cdot}$) and hydroxyl radical ($HO^{\cdot}$) and non-radical molecules like hydrogen peroxide (H_2O_2) (Rahal *et al*., 2014; Kurutas, 2016).

The ROS are generated as by-product of normal aerobic metabolism, but their level increases under stress, which proves to be a basic health hazard. Mitochondrion is the major cell organelle responsible for ROS production (Liu *et al*., 2002; Inoue *et al*., 2003; Rahal *et al*., 2014; Kurutas, 2016). It generates ATP through a series of oxidative phosphorylation processes. During this process, one- or two-electron reductions instead of four electron reductions of O_2can occur, leading to the formation of $O_2^{\cdot}$ or H_2O_2, and these species can be converted to other ROS. Other sources of ROS may be reactions, involving peroxisomal oxidases (Dvorakova *et al*., 2000), cytochrome P-450 enzymes (Geiszt *et al*., 1997), nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Cheng *et al*., 2001), or xanthine oxidase (Dorsam *et al*., 2000).

2.4.1 Oxidative stress and diseases

A major consequence of oxidative/nitrosative stress is damage to nucleic acids, lipids, and proteins, which can severely compromise cell health and viability or induce a variety of cellular responses through generation of secondary reactive species, finally leading to cell death by necrosis or apoptosis (Valko *et al*., 2007; Flora, 2009; Rahal *et al*., 2014). Oxidative/nitrosative damage of any of the biomolecules, if unchecked, can theoretically contribute to disease development. Actually, an increasing amount of evidence shows that oxidative/nitrosative stress is connected to either the primary or secondary pathophysiological mechanisms of multiple acute and chronic human and animal diseases (Finkel and Holbrook, 2000).

Oxidative/nitrosative stress-induced peroxidation of membrane lipids may be very damaging because it alters the biological properties of the cytomembrane, such as the degree of fluidity, and can lead to inactivation of membrane-bound receptors or enzymes, which in turn may impair

normal function of the cell and increase its permeability. Lipid peroxidation may also contribute to, and amplify cellular damage resulting from increased generation of some of which are chemically reactive and covalently modify critical macromolecules (Uchida, 2003). Products of lipid peroxidation have therefore commonly been used as biomarkers of oxidative/nitrosative stress/damage. Lipid peroxidation generates a variety of relatively stable decomposition end-products, mainly α, β-unsaturated reactive aldehydes, such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 2-propenal (acrolein) and isoprostanes (Cracowski *et al*., 2002; Carini *et al*, 2004), which can be measured in plasma, urine, and tissues as an indirect index of oxidative/nitrosative stress.

The aldehydes unlike free radicals, are relatively stable and can diffuse within or even escape from the cell and attack targets far from the site of the original event. They, therefore, are not only end-products and remnants of lipid peroxidation processes, but also may act as "second cytotoxic messengers" for the primary reactions (Valko *et al*., 2007). Some of the aldehydesexhibit facile reactivity with various biomolecules, including proteins, DNA, and phospholipids, generating stable products at the end of a series of reactions that may contribute to the pathogenesis of many diseases (Valko *et al*., 2007). At present, measurement of isoprostane isof the most reliable approaches for the assessment of oxidative/nitrosative stress status or free-radical–mediated lipid peroxidation *in vivo*. The quantification of isoprostane in plasma, urine or tissue gives a highly precise and accurate index of oxidative/nitrosative stress (Basu, 2004; Miraloglu *et al*., 2016).

2.4.2 Oxidative stress and alteredimmune function

The relationship between oxidative stress and immune function of the body is well established. The immune defence mechanism uses the lethal effects of oxidants in a beneficial manner, with ROS and RNS playing a pivotal role in the killing of pathogens. The skilled phagocytic cells (macrophages, eosinophils, neutrophils), as well as B and T lymphocytes, contain an enzyme, NADPH oxidase (Hampton *et al*., 1998; Babior, 1999), which is responsible for the production of ROS following an immune challenge. At the onset of an immune response, phagocytes increase their oxygen uptake as much as 10–20 folds; that is, they undergo respiratory burst. The $O^{\cdot-}$generated by NADPH oxidase serves as the starting material for the production of a suite of reactive species. Other powerful pro-oxidants produced by these cells include: hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxynitrite (ONOO–), and, possibly, hydroxyl ($OH^{\cdot}$) and ozone (O_3). Although the use of the highly reactive endogenous metabolites in the cytotoxic response of phagocytes also injures the host tissues, the non-specificity of the oxidants is an advantage since they take care of all the antigenic components of the pathogenic cell (Rice-Evans and Gopinathan, 1995).

There is an interdependency of oxidative stress, immune system, and inflammation. Increased expression of NO occurs in Dengue and in monocyte cultures infected with different types of viral infections. Increased production of NO has also been accompanied with enhancement in oxidative markers like lipid peroxidation and an altered enzymatic and non-enzymatic antioxidative response in Dengue-infected monocyte cultures (Valero *et al*., 2013). More specifically, the oxygen stress related to immune system dysfunction may play a key role in senescence, in agreement with the oxidation/inflammation theory of aging. The immune status

directly interplays with disease production process. The role of physical and psychological stressors contributes to incidences and severity of various viral and bacterial infections. Both innate as well as acquired immune responses are affected by the altered interferon-gamma (IFN-γ) secretion, expression of CD14, production of the acute-phase proteins, and induction of tumour necrosis factor-alpha (TNF-α). Fatal viral diseases produce severe oxidative stress leading to rigorous cellular damage. However, initiation, progress, and reduction of damages are governed by the redox balance of oxidation and antioxidation. The major pathway of pathogenesis for cell damage is via lipid peroxidation, particularly in microsomes, mitochondria, and endoplasmic reticulum due to oxidative stress (Stehbens, 2004; Hodgson *et al*., 2012). All the factors responsible for the oxidative stress directly or indirectly participate in immune system defense mechanism. Any alteration leading to immunosuppression may trigger the disease production.

2.4.3 Oxidative stress and altered susceptibility to bacterial, viral, and parasitic infections

All pathogens, irrespective of their classification, bacterial, viral, or parasitic, with impaired antioxidant defences show increased susceptibility to phagocytic killing in the host tissues, indicating a microbicidal role of ROS (Halliwell and Gutteridge, 2007). *Vice versa* to this, different studies have proven that individuals deficient in antioxidative mechanism are more susceptible to severe bacterial and fungal infections (Nathan and Shiloh, 2010). Reactive species are important in killing pathogens, but as a negative side-effect can also injure the host tissues, resulting in immunopathology. This is particularly apparent during chronic inflammation, which may cause extensive tissue damage with a subsequent burst in oxidative stress (Sorci and Faivre, 2009).The ROS produced by macrophages and neutrophils are used to combat the invading

microbes. The whole of the process is performed in host cells during the activation of phagocytes or the effect of bacteria, virus, parasites, and their cell product reactivity with specific receptors. The multi-component flavoprotein, NADPH oxidase plays vital role in inflammatory processes by catalysing the production of superoxide anion radical O_2^-, and excessive ROS production leads to cellular damage. The cellular damages in general alter immune response to microbes and ultimately the susceptibility to bacterial, viral, and parasitic infections (Puertollano *et al*., 2011).

2.5 Antioxidants

The animal body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced *in situ* (endogenous) or externally supplied through foods and/or supplements (exogenous). Endogenous and exogenous antioxidants act as "free radical scavengers" by preventing and repairing damages caused by ROS; and, therefore, can enhance the immune defence and lower the risk of disease (Valko *et al*., 2006; Chatterjee *et al*., 2007).

2.5.1 Classification of antioxidants

Animal and humans have developed highly complex antioxidant systems (enzymatic and non-enzymatic) which work synergistically and together with each other to protect cells and organ systems of the body against free radical damage (Kurutas, 2016) (Figure 2.3).

2.5.1.1 Endogenous antioxidants

Endogenous antioxidant compounds in cells can be classified as enzymatic antioxidants and non-enzymatic (metabolic and nutrient) antioxidants. The major enzymatic antioxidants directly

involved in the neutralization of ROS are: SOD, CAT, GPx, and glutathione reductase (Halliwel, 2007; Pacher *et al*., 2007; Pham-Huy *et al*., 2008; Kurutas, 2016).

2.5.1.2 Enzymatic antioxidants

Glutathione peroxidase

Glutathione peroxidases catalyse the oxidation of glutathione at direction of a hydroperoxide, which may be hydrogen peroxide or another species such as a lipid hydroperoxide (Equation 1):

$$ROOH + 2GSH \rightarrow GSSG + H_2O + ROH$$ ---------- (Equation 1)

Other peroxides, including lipid hydroperoxides, can also act as substrates for these enzymes, which may hence play a role in repairing damage, resulting from lipid peroxidation. There are two forms of this enzyme; one which is selenium-dependent (GPx); and the other, which is selenium-independent (glutathione-S-transferase, GST). The differences rise from the number of subunits, catalytic mechanism, and the bonding of selenium at the active centre, and glutathione metabolism is one of the most important antioxidative defence mechanisms present in the cells. There are four different Se-dependent glutathione peroxidases, and these are known to add two electrons to reduce peroxides by forming selenole (Se-OH).The antioxidant properties of these selenoenzymes allow them to eliminate peroxides as potential substrates for the Fenton reaction. Selenium-dependent glutathione peroxidase acts in association with tripeptide glutathione (GSH), which exists in high concentrations in cells and catalyses the conversion of hydrogen peroxide or organic peroxide to water or alcohol, while simultaneously oxidising GSH. It also competes with catalase for hydrogen peroxide as a substrate and is the major source of protection against low levels of oxidative/nitrosative stress (Molavian *et al*., 2015).

Catalase

Catalase was the first antioxidant enzyme to be characterised and catalyses the conversion of hydrogen peroxide to water and oxygen (Equation 2):

$$2H_2O_2 \rightarrow 2H_2O + O_2 \text{…….. (Equation 2)}$$

Catalase consists of four subunits, each containing a haem group and a molecule of NADPH. The rate constant for the reactions described above is extremely high (~10^7 M/sec), implying that it is virtually impossible to saturate the enzyme *in vivo*. This enzyme is present in the peroxisome of aerobic cells and is very efficient in promoting the conversion of hydrogen peroxide to water and molecular oxygen. Catalase has one of the highest turn-over rates for all enzymes: one molecule of catalase can convert approximately 6 million molecules of hydrogen peroxide to water and oxygen each minute. The greatest activity is present in the liver and erythrocytes, but some catalase is found in all tissues (Sies, 2017).

Superoxide dismutase

Superoxide dismutase is one of the most potent intracellular enzymatic antioxidants and it catalyses the conversion of superoxide anions to dioxygen and hydrogen peroxide (Equation 3):

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \text{…… (Equation 3)}$$

The hydrogen peroxide is removed by catalase or glutathione peroxidase, as described above. The SOD exists in several isoforms, which differ in the nature of active metal centre, amino acid composition, co-factors and other features. There are three forms of SOD present in humans: cytosolic CuZn-SOD, mitochondrial Mn-SOD, and extracellular-SOD. Superoxide dismutase neutralises superoxide ions by going through successive oxidative and reductive cycles of transition metal ions at its active site. This enzyme has two similar subunits and each of the

subunits includes an active site, a dinuclear metal cluster, comprising copper and zinc ions, and it specifically catalyses the dismutation of the superoxide anion to oxygen and water (Al-Dalaen and Al-Qtaitat, 2014; Kurutas, 2016). The mitochondrial Mn-SOD is a homotetramer with a molecular weight of 96 kDa and contains one manganese atom per subunit, and it vacillates from Mn (III) to Mn (II), and back to Mn (III) during the two-step dismutation of superoxide anion (Al-Dalaen and Al-Qtaitat, 2014; Kurutas, 2016). Extracellular SOD contains copper and zinc, and is a tetrameric secretary glycoprotein having a high affinity for certain glycosaminoglycans (Sheng *et al*., 2014).

2.5.1.3 Non-enzymatic antioxidants

The non-enzymatic antioxidants are also divided into metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants, belonging to endogenous antioxidants, are produced by metabolism in the body, such as lipoid acid, glutathione, L-arginine, co-enzyme Q_{10}, melatonin, uric acid, bilirubin, metal-chelating proteins and transferrin (Willcox *et al*., 2004; Pham-Huy *et al*., 2008).Nutrient antioxidants, belonging to exogenous antioxidants, are compounds which cannot be produced in the body and must be provided through foods or supplements, such as vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids. Nutrient antioxidants are involved in detoxification ofROS (Gupta and Singh, 2013) and play an important role in enhancing endogenous antioxidants for the neutralisation of oxidative stress (Donaldson, 2004). The nutrient antioxidant deficiency is one of the causes of numerous chronic and degenerative pathologies (Willcox *et al*., 2004).

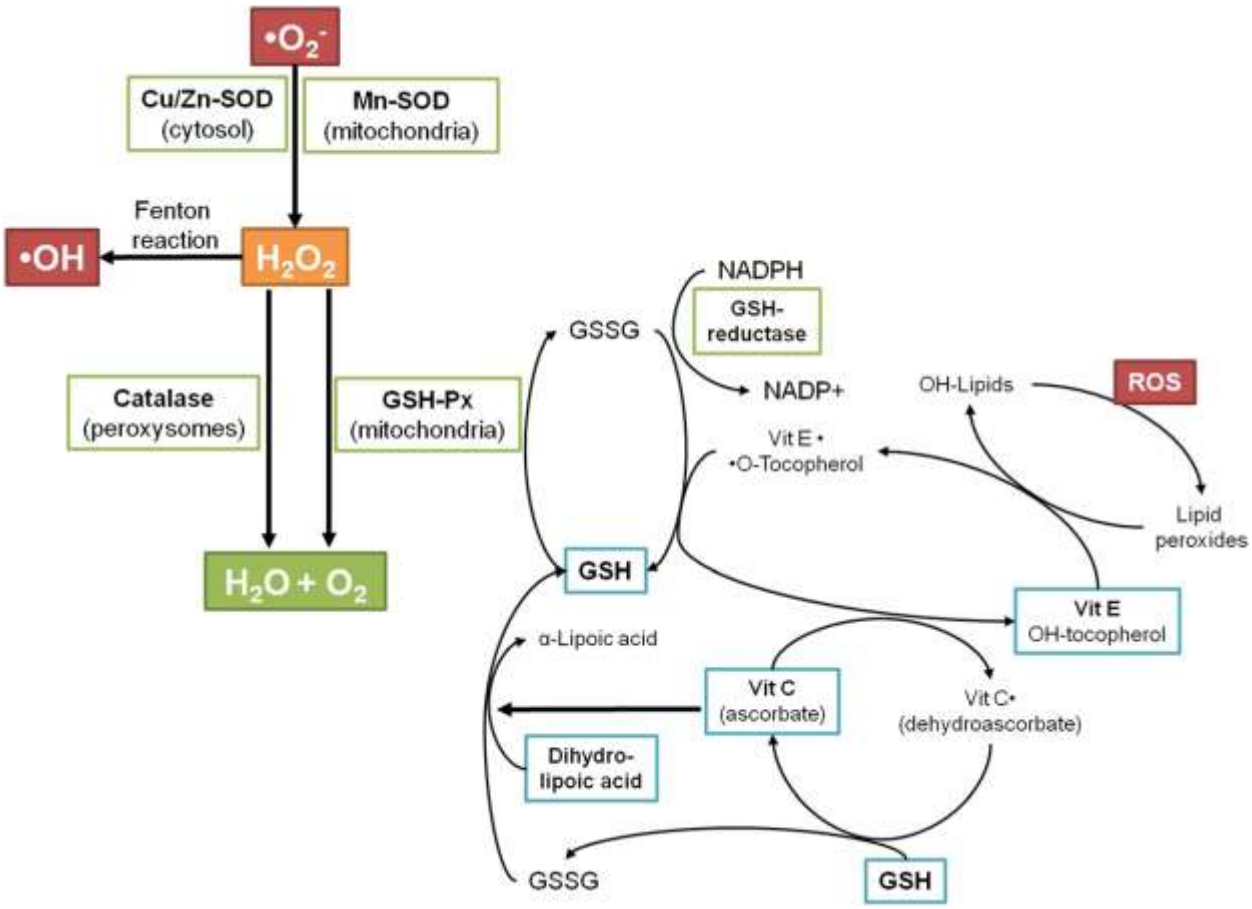

Figure 2.3: Antioxidant defences and their interrelationships in animals (Adopted from Kurutas, 2016).

2.5.2 Antioxidant process

When an antioxidant destroys a free radical, the antioxidant itself becomes oxidised. Therefore, the antioxidant resources must be constantly restored in the body. Thus, while in one particular system, an antioxidant is effective against free radicals; in other systems, the same antioxidant could become ineffective. In certain circumstances, an antioxidant may even act as a pro-oxidant; for example, it can generate toxic ROS (Young and Woodside, 2001; Gupta *et al*., 2014). The antioxidant process can function in one of two ways: chain-breaking or prevention. For the chain-breaking process, when a radical releases or steals an electron, a second radical is formed. The last one exerts the same action on another molecule and continues until either the free radical formed is stabiliSed by a chain-breaking antioxidant (vitamin C and E, carotenoids) or it simply disintegrates into an inoffensive product. The classic example of such a chain-breaking reaction is lipid peroxidation. For the preventive way, an antioxidant enzyme like SOD, catalase, and GPx can prevent oxidation by reducing the rate of chain initiation; for example, either by scavenging the initiated free radicals or by stabilising transition metal radicals, such as copper and iron (Young and Woodside, 2001; Gupta *et al*., 2014).

2.5.3 Phytochemical antioxidants: oxidative stress, disease, and epigenome

Cellular antioxidant defence machinery has been unequivocally established as an oxidative stress-counteracting entity. Antioxidant supplementation/treatment has been adopted for either prevention of, or protection against several disorders and pathophysiological states; wherein, oxidative stress has been established as a causative mechanism (Arts and Hollman, 2005; Gupta *et al*., 2014). Naturally occurring phytochemical antioxidants have occupied a prominent position as effective antioxidants for the prevention and/or treatment of several disorders and diseases among humans (Hollman *et al*., 2011; Spatafora and Tringali, 2012). The premise for this has

been the antioxidant actions of the phytochemicals as free-radical scavengers, oxidative stress relievers, and lipoperoxidation inhibitors (Scalbert *et al*., 2005). Phytochemical antioxidants include simple molecule antioxidants such as vitamins C, E, and K; plant pigments such as carotenoids (β-carotene), xanthophylls, lycopene, anthocyanins, and phaeophytins; and secondary plant metabolites, from simple phenolics to more complex polyphenols (Bors and Michel, 2002; Gupta *et al*., 2012). Animals and humans obtain these phytochemical antioxidants from diet or nutritional supplementation. In recent times, phytochemical antioxidants have been the attraction as effective antioxidants (from diet or supplementation) in prevention and treatment of several diseases, including cardiovascular diseases, cerebrovascular diseases, Alzheimer's disease, respiratory disease, and cancer, with a focus to alleviate the oxidative stress as the causative mechanism in the diseases (Wood *et al*., 2010; Choi *et al*., 2012).

2.6 Carotenoids

Carotenoids are ubiquitous in nature and present in plants, algae and microorganisms. However, humans and other animals are unable to manufacture carotenoids and, hence, require them in their diet (Sandmann, 1994). There are two types of carotenoids based on their chemical composition; carotenes and xanthophylls. Carotenes include β-carotene and lycopene and, xanthophylls carotenoids include lutein, canthaxanthin, zeaxanthin, violaxanthin, capsorubin and astaxanthin (Jackson *et al*., 2008; McNulty *et al*., 2008; Grimmig *et al*., 2017). Carotenoids exert different effects based on their interactions with membranes (McNulty *et al*., 2008). Non-polar carotene carotenoids such as lycopene and β-carotene cause disorders of model membranes and resulting in lipid peroxidation; unlike the polar xanthophylls carotenoid astaxanthin,that preserves the structure of the model membrane (McNulty *et al*., 2007). The differing effects of

the carotenoids may account for the different biological effects observed with these agents in clinical trials.

2.6.1 Astaxanthin as a carotenoid

Astaxanthin is a xanthophyll carotenoid, and, unlike other carotenoids, it contains two additional oxygenated groups on each ring structure (Figure 1.1), resulting in enhanced antioxidant properties (Guerin *et al*., 2003; Grimmig *et al*., 2017). It is found in living organisms, particularly in the marine environment where it is present in microalgae, plankton, krill and seafood. It gives salmon, trout, and crustaceans such as shrimp and lobster their distinctive reddish coloration (Hussein *et al*., 2006; Fassett and Coombes, 2011). It is also present in yeast, fungi, complex plants and the feathers of some birds, including flamingos and quail (Hussein *et al*., 2006; Fassett and Coombes, 2011). The US food and drug agency (FDA) approved astaxanthin in 1987 as a feed additive for the aquaculture industry and in 1999 approved its use as a dietary supplement (nutraceutical) (Guerin *et al*., 2003). Astaxanthin is not synthesised in mammals and, therefore, must be consumed in the diet (Jyonouchi *et al*., 1995).

2.6.2 Food sources of astaxanthin

Astaxanthin is biosynthesised by microalgae, bacteria, and fungi, and concentrates higher up the food chain. Humans commonly consume astaxanthin from seafood; such as salmon, trout, shrimp, lobster, crab, and fish eggs. Astaxanthin is also fed as farmed seafood to add red colour. The content of astaxanthin is 6–8 mg/kg flesh in farmed Atlantic salmon, and 6 mg/kg flesh and 25 mg/kg flesh in large trout in the European and Japanese markets, respectively (Ambati *et al*., 2014; Kishimoto *et al*., 2016). The highest known level of astaxanthin in nature is in the

chlorophyte alga,*Haematococcus pluvialis*, which has become a primary source of the astaxanthin used in the food industry (Lorenz and Cysewski, 2000; Grimmig *et al*., 2017). Both and the composition of isomers of astaxanthin differ among organisms. For example,*H. pluvialis* produces the all trans-geometric form 3S, 3'taxanthin, and therefore this type is most largely ingested by humans (Ambati *et al*., 2014). Humans cannot synthesise astaxanthin, and the ingested astaxanthin cannot be converted to vitamin A; excessive intake of astaxanthin will, thus, not cause hypervitaminosis A (Hussein *et al*., 2006; Fassett and Coombes, 2012). The use of astaxanthin as a dietary supplement has been rapidly growing in many countries. Japan is one of the global pioneers in astaxanthin research and production (Kishimoto *et al*., 2016).

2.6.3 Biochemistry of astaxanthin

Astaxanthin (3,3'-dihydroxy-beta, beta-carotene-4,4'-dione) has oxygen in its molecular structure, which differentiates it from β-carotene and other molecules of the carotene subclass (Fassett and Coombes, 2011). The astaxanthin molecule has an extended shape, with a polar structure at either end of the molecule and a non-polar zone in the middle (Figure 1.1). The polar structures are ionone rings that have potent capacity for quenching free radicals or other oxidants; primarily in an aqueous environment, but possibly also in the absence of water (Britton, 1995; Goto *et al*., 2001). The non-polar middle segment of astaxanthin molecule is a series of carbon-carbon double bonds, which alternate with carbon-carbon single bonds, termed “conjugated”. This series of conjugated double bonds gives the molecule a further antioxidant dimension, with a capacity to remove high energy electrons from free radicals and “delocalise” their electronic energy via the carbon-carbon chain analogous to a lightening rod on the molecular level (Pashkow *et al*., 2008).

2.6.4 Safety of astaxanthin

Astaxanthin is safe, with no side-effects when it is consumed. When fed to rats, it accumulates in the tissues with no toxic effects (Stewart *et al*., 2008; Ambati *et al*., 2010, 2013a). Excessive astaxanthin consumption leads to yellow to reddish pigmentation of the skin in animals. Astaxanthin is incorporated into fish feed, resulting in the fish skin becoming reddish in colour. Antioxidant enzymes such as SOD, catalase, and GPx activities significantly increase in rats after oral dosage of astaxanthin (Ambati *et al*., 2013 a,b). A study has shown that blood pressure reduces in stroke-prone and hypertensive rats, fed 50 mg/kg astaxanthin for five weeks and 14 days, respectively (Hussein *et al*., 2006). Astaxanthin also shows significant protection against naproxen-induced gastric, antral ulcer and inhibits lipid peroxidation levels in gastric mucosa (Kim *et al*., 2005; Augusti *et al*., 2012). Astaxanthin extracted from *Paracoccus carotinifaciens* exhibits potential antioxidant and anti-ulcer properties in murine models(Murara *et al*., 2012). Supratherapeutic concentrations of astaxanthin exerts no adverse effects on platelet, coagulation and fibrinolytic function (Serebruany *et al*., 2010). Research has so far reported no significant side-effects of astaxanthin consumption in animals and humans.

2.6.5 Bioavailability and pharmacokinetics of astaxanthin

Astaxanthin is digested and absorbed similar to lipids and other carotenoids, although the bioavailability is heavily influenced by other dietary components. When administered orally, a higher proportion is absorbed when taken with a meal or delivered in an oil-based formulation (Odeberg *et al*., 2003; Ambati *et al*., 2014; Grimmig *et al*., 2017). After release from the food matrix, astaxanthin accumulates in the lipid droplets within the gastric juices and then incorporates into micelles, when they encounter bile acids, phospholipids, and lipases in the

small intestine. The micelles passively diffuse into the plasma membrane of enterocytes. Astaxanthin, like the more polar xanthophylls, is transported in the circulation by high-density lipoprotein (HDL) and low-density lipoprotein (LDL), after being liberated from chylomicrons that are formed in the intestinal cells (Kishimoto *et al*., 2016). It has been reported that after a 100-mg/kg dose, plasma concentrations peak to 1 μg/mL at approximately 9 hours after dosing. Astaxanthin is taken up into many tissues, including the brain, but primarily accumulates in the liver (Choi *et al*., 2011).

2.6.6 Underlying basis to bioactivity of astaxanthin

Astaxanthin is a totally unique antioxidant, because it possesses three novel distinctions at once:

2.6.6.1 Powerful antioxidant

The inhibitory activity of astaxanthin on the peroxyl radical-mediated lipid peroxidation is more than 100 times greater than that of α-tocopherol in the homogenate of rat mitochondria (Miki, 1991). Among 27 common hydrophilic and lipophilic antioxidants such as polyphenols, tocopherols, carotenoids, ascorbic acid, co-enzyme Q10 and α-lipoic acid, astaxanthin shows the strongest singlet oxygen ($^1O_2$) quenching activity under the same test condition of the chemiluminescence detection system for direct $^1O_2$ counting using the thermodissociable endoperoxides of 1,4-dimethylnaphthalene as $^1O_2$ generator in DMF:CDCl3 (9:1) (Nishida *et al*., 2007). Hydroxyl radical scavenging ability of astaxanthin encapsulated in liposomes is more potent than that of α-tocopherol (Hama *et al*., 2012).

2.6.6.2 Mechanism of action

Astaxanthin provides cell membranes withpotent protection against free radical or otheroxidative attack. Experimental studies have shown thatthis nutrient has a large capacity to neutralise freeradical or other oxidant activity in the non-polar("hydrophobic") zones of phospholipid aggregates,as well as along their polar (hydrophilic) boundaryzones (Fassett and Coombes, 2011). The membrane systems of cells are particularly vulnerable to free radical or other oxidative attack, owing to their higher content of polyunsaturated fatty acids and metabolic activities, whichendogenously generate free radicals and other oxidants (Hulbert *et al*., 2007).In its position spanning the membrane, astaxanthin provides versatile antioxidant actions, including:

i. donating electrons to unpaired electrons to neutralise free radicals;
ii. pulling away ("abstracting") an unpaired electron, which also can neutralise a radical
iii. bonding with the radical to form an unreactive "adduct"
iv. conducting electrons or electronic energy out of the membrane
v. neutralising radical species of nitrogen, sulfur, or carbon, in addition to those of oxygen
vi. carrying very low net molecular energy, therefore, providing resistance to transformation into a pro-oxidant molecule (Goto *et al*., 2001; McNulty *et al*., 2007).

2.6.6.3 Safe antioxidant

Carotenoids have been divided into three classes; i) carotenoids without significant antioxidative properties, ii) carotenoids with anti- and pro-oxidants and, iii) carotenoids that are pure anti-oxidants. Astaxanthin is classified as "pure anti-oxidants" because it does not possess any pro-oxidative properties like β-carotene and lycopene (Martin *et al*., 1999; Eiji, 2013). Non-polar

carotenoids, such as lycopene and β-carotene, impair the membrane bilayer, enriched with polyunsaturated fatty acids and exert a potent pro-oxidant effect [>85% increase in lipid hydroperoxide (LOOH) levels] while astaxanthin preserves membrane structure and exhibits significant antioxidant activity (40% decrease in LOOH levels) (McNulty *et al*., 2007). The photostability of the three classes of carotenoids in the human dermal fibroblasts is as follows: astaxanthin > canthaxanthin > β-carotene. Only astaxanthin efficiently abrogates the apoptotic response to UVA. β-carotene dose-dependently induces caspase-3 activity following UVA exposure (Camera *et al*., 2009).

2.6.7 Superior position in cell membrane

Astaxanthin traps radicals not only at the conjugated polyene chain, but also in the terminal ring moiety, in which the hydrogen atom at the C3 methine may be a radical-trapping site. Owing to the equivalent amounts of the hydrophobic, intramolecular hydrogen-bonded ring and intermolecular hydrogen bonding with phospholipid polar heads; and the interconversion between the two hydrogen bond formations, the terminal ring of astaxanthin is able to scavenge radicals both at the surface and in the interior of the phospholipid membrane, although its unsaturated polyene chain traps radicals only in themembrane (Goto *et al*., 2001). Figure 2.4 shows astaxanthin's unique ability to span through the double- layer cell membrane. β-carotene and vitamin C, unlike astaxanthin only reside inside and outside the lipid bilayer membrane, respectively. The astaxanthin molecule is exposed both in- and outside of the cell to offer better overall protection.

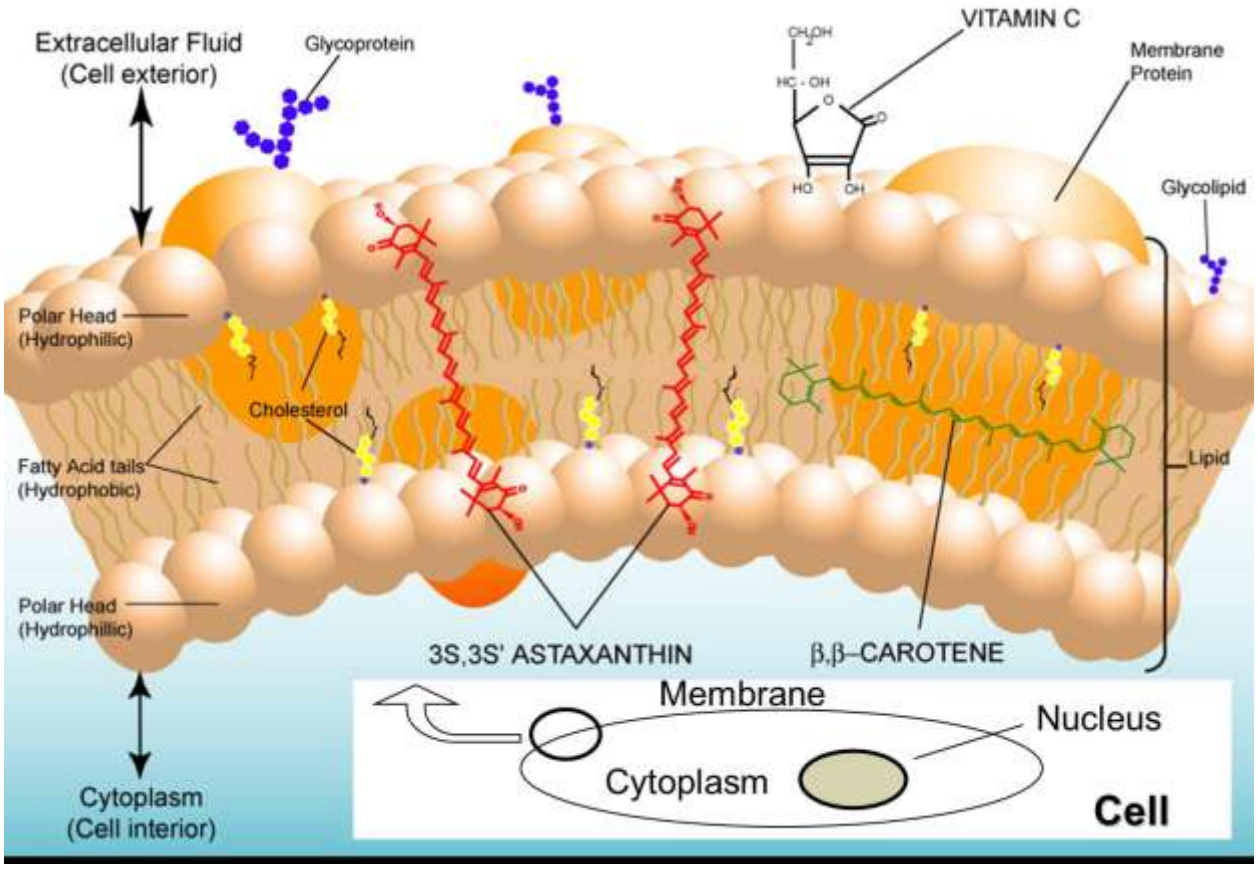

Figure 2.4 Superior position of astaxanthin in the cell membrane that offers a unique free radical scavenging effect (Adopted from Eiji, 2013).

No other antioxidants possess the three unique characters at once, associated with potent bioactivity of astaxanthin

2.6.8 Pharmacological effects of astaxanthin

Numerous studies have shown that astaxanthin has potential health-promoting effects in the prevention and treatment of various diseases, such as cancers, chronic inflammatory diseases, metabolic syndrome, diabetes, diabetic nephropathy, cardiovascular diseases, gastrointestinal diseases, liver diseases, neurodegenerative diseases, eye diseases, skin diseases, exercise-induced fatigue, male infertility (Higuera-Ciapara *et al*., 2006; Khan *et al*., 2010; Fassett and Coombes, 2011; Ishiki *et al*., 2013).

2.6.8.1 Antioxidant effect

Carotenoids contain long conjugated double bonds in a polyene chain that are responsible for antioxidant activities by quenching singlet oxygen and scavenging radicals to terminate the chain reaction. Astaxanthin contains a conjugated polyene chain at the centre and hydroxyl- and keto-moieties on each ionone ring. Owing to its unique molecular structure, astaxanthin shows better biological activity than other antioxidants because it can link with the cell membrane from the inside to the outside (Pashkow *et al*., 2008; Ambati *et al*., 2014). The polyene chain in astaxanthin traps radicals in the cell membrane, while the terminal ring of astaxanthin can scavenge radicals both at the surface and in the interior of the cell membrane (Goto *et al*., 2001). Astaxanthin is reported to be more effective than β-carotene in preventing lipid peroxidation in solution (Terao, 1989), and in various biomembrane models such as egg yolk phosphatidylcholine liposomes (Lim *et al*., 1992) and rat liver microsomes (Palozza and Krinsky, 1992). Goto *et al*. (2001) reported that astaxanthin was approximately two-fold more effective than β-carotene in the inhibition of liposome peroxidation induced by ADP and Fe^{2+}.

Their report was the first to demonstrate that astaxanthin could trap radicals not only at the conjugated polyene chain, but also in the terminal ring moiety.

The antioxidant enzymes, catalase, SOD, GPx and thiobarbituric acid reactive substances (TBARS) are high in rat plasma and liver after feeding *Haematococcus* biomass as source of astaxanthin (Ambati *et al*., 2010). Astaxanthin in *H. pluvialis* offers the best protection from free radicals in rats followed by β-carotene and lutein (Ambati *et al*., 2010, 2013a). Antioxidant enzyme activities have been evaluated in the serum after astaxanthin supplementation in the diet of rabbits, showing enhanced activity of SOD and thioredoxin reductase whereas paraoxonase is inhibited in the oxidative-induced rabbits (Augusti *et al*., 2012). Antioxidant enzyme activities increase when astaxanthin is fed to ethanol-induced gastric ulcer in rats (Kamath *et al*., 2008).

2.6.8.2 Anti-lipid peroxidation activity

Astaxanthin has a unique molecular structure which enables it to stay both in and outside the cell membrane. It gives better protection than β-carotene and vitamin C, which can be positioned inside the lipid bilayer. It serves as a safe-guard against oxidative damage by various mechanisms, like quenching of singlet oxygen; scavenging of radicals to prevent chain reactions; preservation of membrane structure by inhibiting lipid peroxidation; enhancement of immune system function and regulation of gene expression. Astaxanthin and its esters show 80% anti-lipid peroxidation activity in ethanol-induced gastric ulcer and skin cancer in rats (Liu and Osawa, 2007; Ambati *et al*., 2013a). Astaxanthin inhibits lipid peroxidation in biological samples reported by various authors (Goto *et al*., 2001; Kamath *et al*., 2008; Ambati *et al*., 2010, 2013a, b).

2.6.8.3 Anti-inflammation

Astaxanthin is a potent antioxidant terminating the induction of inflammation in biological systems. It acts against inflammation. Algal cell extracts of *Haematococcus* and *Chlorococcum* significantly reduced bacterial load and gastric inflammation in *H. pylori*-infected mice (Liu and Lee, 2003; Ambati *et al*., 2010). Park *et al.* (2010) reported that astaxanthin reduces the DNA oxidative damage, concentration of biomarker of inflammation, thus enhancing immune response in young healthy, adult female human subjects. Haines *et al.* (2011) reported lowered bronchoalveolar lavage fluid and inflammatory cell numbers, and enhanced cAMP, cGMP levels in lung tissues after feeding astaxanthin with *Ginkgo biloba* extract and vitamin C. Astaxanthin esters and total carotenoids from *Haematococcus* exert a dose-dependent gastro-protective effect on acute, gastric lesions in ethanol-induced gastric ulcers in rats,apparently due to inhibition of H1, K1 ATPase, upregulation of mucin content and an increase in antioxidant activities (Kamath *et al*., 2008). Astaxanthin exerts protective effect on high glucose-induced oxidative stress, inflammation and apoptosis in proximal tubular epithelial cells. It is a promising molecule for the treatment of ocular inflammation (Ohgami *et al*., 2003; Suzuki *et al*., 2006). Astaxanthin prevents skin thickening and reduces collagen reduction against UV-induced skin damage (Hama *et al*., 2012; Santos *et al*., 2012).

2.6.8.4 Anti-diabetic Activity

Generally, oxidative stress levels are very high in diabetes mellitus patients. It is induced by hyperglycaemia, due to the dysfunction of pancreatic β-cells and tissue damage in patients. Astaxanthin may reduce the oxidative stress caused by hyperglycaemia in pancreatic β-cells and improve glucose and serum insulin levels (Uchiyama *et al*., 2002). Astaxanthin

protectspancreatic β-cells against glucose toxicity. It is a good immunological agent in the recovery of lymphocyte dysfunctions, associated with diabetic rats (Otton *et al*., 2010). In another study, the combination of astaxanthin and α-tocopherol ameliorates oxidative stress in streptozotocin-diabetic rats (Nakano *et al*., 2008). Astaxanthin inhibits glycation and glycated protein-induced cytotoxicity in human umbilical vein endothelial cells by preventing lipid/protein oxidation (Nishigaki *et al*., 2010). Improved insulin sensitivity in both spontaneously hypertensive corpulent rats and mice on high-fat plus high-fructose diets was observed after feeding with astaxanthin (Hussein *et al*., 2007; Bhuvaneswari *et al*., 2010, 2014). The urinary albumin level was lower in astaxanthin treated diabetic mice (Otton *et al*., 2010). Astaxanthin prevents diabetic nephropathy by reducing oxidative stress and renal cell damage (Naito *et al*., 2004; Manabe *et al*., 2008; Kim *et al*., 2009).

2.6.8.5 Cardiovascular Disease Prevention

Astaxanthin is a potent antioxidant with anti-inflammatory activity. Oxidative stress and inflammation are pathophysiological features of atherosclerotic cardiovascular disease (Fassett and Combes, 2011). The efficacy of disodium disuccinate astaxanthin in protecting the myocardium using myocardial ischaemia perfusion model in animals has been evaluated. Myocardial infarct size reduces in Sprague Dawley rats, and improves in myocardial salvage in rabbits after four days of pre-treatment with disodium disuccinate astaxanthin at 25, 50 and 75 mg/kg body weight (Gross and Lockwood, 2005; Lauver *et al*., 2005). Astaxanthin was found in rat myocardial tissues after pretreatment with disodium disuccinate astaxanthin at 150 and 500 mg/kg/day for seven days (Gross *et al*., 2006). Astaxanthin effects on blood pressure in spontaneously hypertensive rats, normotensive Wistar Kyoto rats, and stroke prone

spontaneously hypertensive rats have also been reported (Monroy-Ruiz *et al*., 2011). Human umbilical vein endothelial cells and platelets treated with astaxanthin show increased nitric oxide levels and decrease in peroxynititrite levels (Khan *et al*., 2010).Mice fed with 0.08% astaxanthin have higher heart mitochondrial membrane potential and contractility index, compared to the control group (Nakao *et al*., 2010). The effects of astaxanthin on paraoxonase, thioredoxin reductase activities, oxidative stress parameters and lipid profile in hypercholesterolaemic rabbits have been evaluated. Astaxanthin was found to inhibit the activities of enzymes from hypercholesterolaemia-induced protein oxidation at the dosages of 100 mg and 500 g (Augusti *et al*., 2012).

2.6.8.6 Immuno-modulation

Immune system cells are very sensitive to free radical damage. The cell membrane contains poly unsaturated fatty acids. Antioxidants, in particular astaxanthin, offer protection against free radical damage to preserve immune-system defences. There are reports on astaxanthin and its effect on immunity in animals however, clinical research is lacking in humans. Astaxanthin shows higher immune-modulating effects in mouse model, when compared to β-carotene (Jyonouchi *et al*., 1991). Enhanced antibody production and decreased humoral immune response in older animals occur after dietary supplementation with astaxanthin (Jyonouchi *et al*., 1991, 1994). Eight-week supplementation with astaxanthin in humans increases blood levels of astaxanthin and improves activity of natural killer cells that destroy cells infected with viruses (Park *et al*., 2010). Supplementation with astaxanthin also shows increase in T- and B-cells, decreased DNA damage and low C-reactive protein (Chew and Park, 2004; Park *et al*., 2011; Augusti *et al*., 2012)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental Animals

Eighty (80) adult male Wistar rats, weighing between 180 and 210 g were used for the experiment. The animals were obtained from the Animal House of the Nigerian Institute of Trypanosomiasis Research (NITR), Vom, Plateau State. They were allowed to acclimatise to laboratory conditions for 14 days. The rats were housed in plastic cages with wood shavings as bedding, which was changed weekly. They were given access to commercial feed and water *ad libitum*.

3.2 Ethical Clearance

Ethical approval for the study was sought from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC), Ahmadu Bello University, Zaria and was obtained with approval number ABUCAUC/2021/029.

3.3 Trypanosome Parasites

Trypanosoma brucei brucei (Federe strain) was obtained from Nigerian Institute for Trypanosomosis Research, Kaduna. The parasite was maintained by serial passages in other donor rats. Parasitaemia was monitored daily by preparing a wet mount and viewed under the light microscope (Olympus® CH23, Germany) at 400 × magnification.

3.4 Inoculation of Rats

The infected blood from a donor rat (when the parasitaemia was 2.5×10^5) was collected and diluted with physiological saline. Each rat was inoculated with a suspension containing 3 or

4trypanosomes per microscopic field at × 100 magnification (approximately 10^6 trypanosomes/ml of blood) as described by Adeyemi *et al*. (2012).

3.5 Experimental Design

Eighty (80) experimental rats were randomly allotted into eight groups (I, II, III, IV, V, VI, VII and VIII) of 10 animals each and treated as follows:

Group I: Rats in this group were administered with distilled water (2mL/kg) only.

Group II: Rats were given soya oil (2mL/kg) only.

Group III: Rats were infected with 10^6 trypanosomes intraperitoneally.

Group IV: Rats were pre-treated with astaxanthin (Source Naturals, Santa Cruz, California, USA) at 100 mg/kg (Choi *et al*., 2011) once daily by oral gavage for 3 weeks and then intraperitoneally infected with 10^6 trypanosomes/mL of blood. Astaxanthin (100 mg/kg) administration continued for the remaining part of the experiment.

Group V: Rats were pre-treated with astaxanthin at 100 mg/kg once daily by oral gavage for 3 weeks and then intraperitoneally infected with 10^6 trypanosomes/mL of blood. Diminazene aceturate (Berenil®, Hoechst, Germany) was administered once when infection was established at 3.5 mg/kg body weight intraperitoneally, followed by oral administration of astaxanthin (100 mg/kg) once daily by oral gavage for the rest of the experiment.

Group VI: Rats were intraperitoneally infected with 10^6 trypanosomes/mL of blood. Once infection was established, astaxanthin was administered at 100 mg/kg, once daily by oral gavage, till the end of the experiment.

Groups VII: Rats were infected with 10^6 trypanosomes/mL of blood. Diminazene aceturate was administered once, when infection was established at 3.5 mg/kg intraperitoneally, followed by

astaxanthin (100 mg/kg) administration once daily by oral gavage for the remaining part of the experiment.

Group VIII: Rats were infected with 10^6 trypanosomes/mL of blood and administered with a single dose of diminazene aceturate (3.5 mg/kg) intraperitoneally.

All animals were monitored daily for clinical signs, weekly body weight changes, and death throughout the experimental period. Parasitaemia was monitored in all the infected rats daily using the rapid matching method described by Herbert and Lumsden (1976). The groups treated with diminazene aceturate alone or its combination with astaxanthin were monitored for relapse of infection after treatment.

3.6 Determination of Body Weight

The weight of the rats were monitored from the first day and later on weekly basis using automated electronic scale (Sensor Disc Technology, London). To weigh a rat, a round plastic container was placed on the scale and tarred to zero following which the rat was dropped inside the container and subseguently weighed. The weights of the rats were measured in grams (g).

3.7 Determination of Survivability of Rats

Survivability of the rats was determined by calculating the difference between the day of infection and the day the animal died (Aremu *et al*., 2018).

3.8 Determination of Parasitaemia in the Experimental Rats

Parasites in the blood were estimated according to the rapid "matching" method of Herbert and Lumsden (1976). The method employed a matching technique in which microscopic fields were

compared with a range of standard logarithmic values. To count the number of parasites in blood, a drop of blood was placed on a glass slide from the rat's tail by cutting the tip of the tail with a scissors after it had been cleaned with cotton wool soaked in ethanol. The drop of blood on the slide was immediately covered with a cover slip. The wet mount on the slide was observed under x 400 magnification (x 40 objective lens and x 10 eye piece). The number of trypanosomes per microscopic field was then compared with the table of logarithmic values. The logarithm values which matched the microscopic observation were then converted to antilogarithm from where the absolute number of trypanosomes per ml of blood was obtained.

3.9Collection of Blood

At the end of the five-week experimental period, the rats were sacrificed by jugular venisection after light chloroform anaesthesia. Blood sample (5 mL) was collected from each rat into sample bottles, containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant for the evaluation of haematological parameters.Blood samples were also collected into test tubes without anticoagulant addition, and allowed to clot. They were centrifuged at 1000 *g* for 10 min. The serum harvested was stored at 4^{o}C and used for evaluation of biochemical parameters.

3.10 Determination of Haematological Parameters

3.10.1 Determination of blood cellular components

Haematological parameters such as packed cell volume (PCV), haemoglobin (Hb) concentration, red blood cell (RBC), platelet, absolute and differential leucocyte counts were determined using the automated haematologic analyser (Sysmex, KX-21, Japan) as described by Dacie and Lewis (1991). Erythrocytic indices of mean corpuscular volume (MCV), mean corpuscular

haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated from the value of PCV, Hb concentration and RBC count as described by Schalm *et al*. (1975):

$$\text{Mean corpuscular volume (MCV)} = \frac{\text{Haematocrit (\%)} \times 10}{\text{RBC } (\times 10^{12}/\text{L})}$$

$$\text{Mean corpuscular haemoglobin (MCH)} = \frac{\text{Hb (g/dL)} \times 10}{\text{RBC } (\times 10^{12}/\text{L}}$$

$$\text{Mean corpuscular haemoglobin concentration (MCHC)} = \frac{\text{Hb (g/dL)} \times 100}{\text{Haematocrit (\%)}}$$

3.10.2 *In vitro* determination of erythrocyte osmotic fragility

Erythrocyte osmotic fragility was determined as described by Faulkner and King (1970)andas modified by Oyewale (1991). Briefly, sodium chloride solution (pH 7.4) was prepared at varying concentrations, 0.0%, 0.1%, 0.3%, 0.5%, 0.7%, 0.9%. Five millilitres (5 mL) of each NaCl concentration was placed in labelled test tubes serially in a rack. Exactly 0.02 mL of the blood sample was pipetted into each of the tubes. The contents of the tubes were gently mixed by inverting the tubes and allowing them to stand at room temperature (24–26°C) for 30 min. Thereafter, the tubes were centrifuged at 1580 x*g* for 5 min using a centrifuge model IEC HN-SII (Damon/IEC Division, UK). The supernatant obtained from each tube were transferred to a clean glass cuvette and the absorbance of the supernatant measured spectrophotometrically with Spectronic 20 (Bausch and Lomb, USA) at a wavelength of 540 nm. The percentage haemolysis for each sample was calculated using the following formula:

$$\text{Percentage (\%) haemolysis} = \frac{\text{Optical density of test solution}}{\text{Optical density of standard solution}} \times 100$$

3.11 Determination of Serum Biochemical Parameters

3.11.1 Determination of activities of liver enzymes

Serum samples were evaluated for the activities of alanine aminotranferase (ALT) and aspartate aminotransferase (AST) using Randox Glutamic-pyruvate transaminase test kit and Randox Glutamic-oxaloacetic transaminase test, respectively as described by Reitman and Frankel (1957) (Randox Laboratories, County Antrim, United Kingdom). The activity of alkaline phosphatase (ALP) was determined using Quinica clinica test kits (QCA, CN-340km 1081-P.O.Box 20-E43870 AMPOSTA/Spain) as described by Klein *et al*. (1960).

3.11.2 Determination of lipid profile

Serum concentrations of triglycerides were determined using colorimetric method after enzymatic hydrolysis with lipases. Concentrations of cholesterol were analysed using colorimetric enzymatic end-point, while high-density lipoprotein cholesterols (HDL-C) were determined using precipitant method. All the determinations were carried out using standard commercial test kits (Randox Laboratories Ltd., Ardmore, Diamond Road, Co. Antrim, United Kingdom) according to the methods of Allain *et al*. (1974) and Rifai *et al*. (1999). The manufacturer's instructions were strictly followed.

3.11.3 Determination of immunoglobulin G

Determination of immunoglobin G (IgG) was carried out using rat enzyme immunoassay kit (Abcam®, Cambridge Science Park, Cambridge, United Kingdom) as described by Stein *et al*. (1977) and according to the manufacturer's instruction. The SimpleStep ELISA™ employed an affinity tag-labeled capture antibody and a reporter conjugated-detector antibody, which

immunocaptured the sample analyte in solution in a microtitre well. Briefly, 50 µL of standard or sample was added to appropriate wells. An antibody cocktail (capture antibody and detector antibody) was then added to all wells and incubated at room temperature. Each well was washed followed by addition of 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate and incubated for 3 minutes in the dark on a plate shaker. One hundered (100) µL of stop solution was added to each well and the optical density recorded at 450 nm using a microtitre plate reader.

3.11.4Determination of cytokines

The concentrations of TNF-α, IL-1α and IL-6 cytokines were determined by ELISA kits (Abcam® Cambridge Science Park, Cambridge, United Kingdom) as described by Brennan and Mclnnes (2008). The kit employed an antibody specific for rat TNF-α, IL-1α and IL-6 coated on a 96-well plate. Briefly, standards and samples (100 µL) were pipetted into the wells and incubated over night at 4°C. The wells were washed and 100 µL of biotinylated anti-rat TNF-α, IL-1α and IL-6 antibodies were added and incubated at room temperature for 1 h. After washing away unbound biotinylated antibodies, 100 µL of horseradish peroxidase conjugated streptavidin was pipetted into the wells and incubated for another 45 min at room temperature. The wells were washed again, and TMB substrate solution was added to the wells. The development of yellow colouration from the initial blue colour on adding the stop solution depended on the amount of bound TNF-α, IL-1α and IL-6. The intensity of the colour was measured at 450 nm using a 96-well plate spectrometer. Calculation of the concentrations of the cytokines was done in a log-log linear regression according to the instruction in the protocol.

3.12Preparation of Tissues

The activities of catalase, SOD, GPx, and concentration of 8-isoprostane in tissues were determined using commercial kits as described by the manufacturers. Freshly-obtained liver, kidney, and heart samples were sonicated, suspended in ice-cold phosphate-buffered saline (PBS) and then vortexed for 5-10 s. The mixture was centrifuged at 1000 g for 15 min, and the supernatant transferred into sterile test tubes and stored on ice and immediately used for the analysis of the activities of SOD, catalase, GPx, and concentration of 8-isoprostane.

3.13 Determination of Oxidative Stress Biomarkers

3.13.1 Determination of 8-isoprostane

The competitive *in vitro* enzyme-linked immunosorbent assay(ELISA) kit was used to determine the level of 8 isoprostane tissue samples, and was done according to the method of Wang *et al*. (1995). Briefly, 100 µL of the standards and samples were added into appropriate wells, followed by addition of 100 µL of the horseradish peroxidase (HRP) conjugate into each well and incubated at room temperature for 2 h. The plates were washed 3 times with 400 µL of wash buffer per well. The TMB substrate (200 µL) was then loaded into each well and the plates were incubated for 15-30 min at room temperature before the addition of 50 µL of stop solution. The absorbance of each well was read immediately at 450 nm.

3.13.2 Determination of catalase activity

The activity of catalase was determined spectrophotomerically according to the method of Beers and Sizer (1952), with modifications to increase robustness and convenience using the Northwest Life Science Specialties (NWLSS™) catalase activity assay kit protocol. Catalase activity was

measured by monitoring the consumption of H_2O_2 substrate at 240 nm. Briefly, 15 µL of diluted sample was added to clean UV microplate well. Thereafter, 290 µL of assay cocktail was added to each well and mixed as quickly as possible using a reader shaker. The absorbance was recorded immediately at 240 nm, every 2 s using a microtitre plate reader.

3.13.3 Determination of superoxide dismutase activity

The activity of SOD was assessed using the NWLSS™ superoxide dismutase activity assay, which provided a simple, rate method for determining SOD activity. This method was based on monitoring the auto-oxidation rate of haematoxylin as originally described by Martin *et al*. (1987), with modifications to increase robustness and reliability. In the presence of SOD at specific assay pH of 7.4, the rate of auto-oxidation was inhibited and the percentage of inhibition was linearly proportional to the amount of SOD present within a specific range. Sample SOD activity was determined by measuring ratios of auto-oxidation rates in the presence and absence of the sample, and was expressed as traditional McCord-Fridovich "cytochrome c" units. Briefly, 230 µL of assay buffer was added to each well, followed by addition of 10 µL of assay buffer (for blank) or 10 µL to the wells. The wells were shaken to mix, and incubated for 2 minutes. Haematoxylin reagent (10 µL) was added to begin the reaction. It was mixed quickly using the instrument's shaker function, and immediately the absorbance was recorded at 560 nm every 10 s for at least 5 min using a microtitre plate reader.

3.13.4 Determination of glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was determined using spectrophotometry method of Paglia and Valentine (1967), andbased on the NWLSS™ glutathione peroxidase assay

kits'protocol. The GPx catalysed the reduction of hydrogen peroxide (H_2O_2), oxidising reduced glutathione (GSH) to form oxidised glutathione (GSSG). The GSSG was then reduced by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH), forming NADP+ (resulting in decreased absorbance at 340 nm) and recycling the GSH. The decrease in absorbance at 340 nm was directly proportional to the GPx concentration. Briefly, 50 µL of diluted sample was added to each well followed by the addition of 50 µL of working NADPH to the wells. Fifty (50) µL of working H_2O_2 was added to each well and the absorbance was read at 340 nm for 5 min using a spectrophotometer with a recording interval of 30 s. The GPx activity was then calculated from the net rate.

3.14Data Analyses

Values obtained were expressed as mean ± standard error of the mean (mean ± SEM). Data were subjected to one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. All the data were analysed using Graphpad Prism, version 5.0 for windows (Graphpad Software, San Diego, California, USA). Values of $P < 0.05$ were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Effect of Treatments on the Dynamics of Body Weight Changes in Rats

There was a significant ($P < 0.05$) increase in the body weight of rats treated with DW, S/oil, PreA+T, PreA+T+DZ, T+A+DZ and T+DZ at week 4, compared to the corresponding values obtained at week 1. However, the body weight of rats in T and T+A groups did not differ at week 4 in comparison to the corresponding values, recorded at week 1(Figure 4.1).

4.2 Effect of Treatment on Survivability of Rats

There was a significant ($P < 0.05$) decrease in the survival rate of rats treated with T and T+A, when compared to the values obtained in those administered with DW, S/oil, PreA+T+DZ, T+A+DZ and T+DZ, respectively. The survival rate of rats in T+A group was significantly ($P < 0.05$) lower than PreA+T group; relatively higher in PreA+A group when compared to the T group, although the difference in the values was insignificant (Figure 4.2).

4.3 Effect of Treatments on the Level of Parasitaemia

Parasitaemia was first observed on day 2 post-infection; and by day 4 post-infection, parasitaemia was observed in all the infected groups, but did not differ ($P > 0.05$) between the groups. The level of parasitaemia dropped to zero at day 6 post-infection in all groups (PreA+T+DZ, T+A+DZ and T+DZ) treated with diminazene aceturate alone or in combination with astaxanthin. However, there was resurgence of parasitaemia in 10% of the rats in the T+A+DZ group at day 7 post-treatment (Table 4:1). The level of parasitaemia in T, PreA+T, and T+A groups progressively increased till the termination of the experiment; but was significantly

($P < 0.05$) lower in PreA+T group than in T or T+A group at both days 6 and 8 post-infection (Figure 4.3).

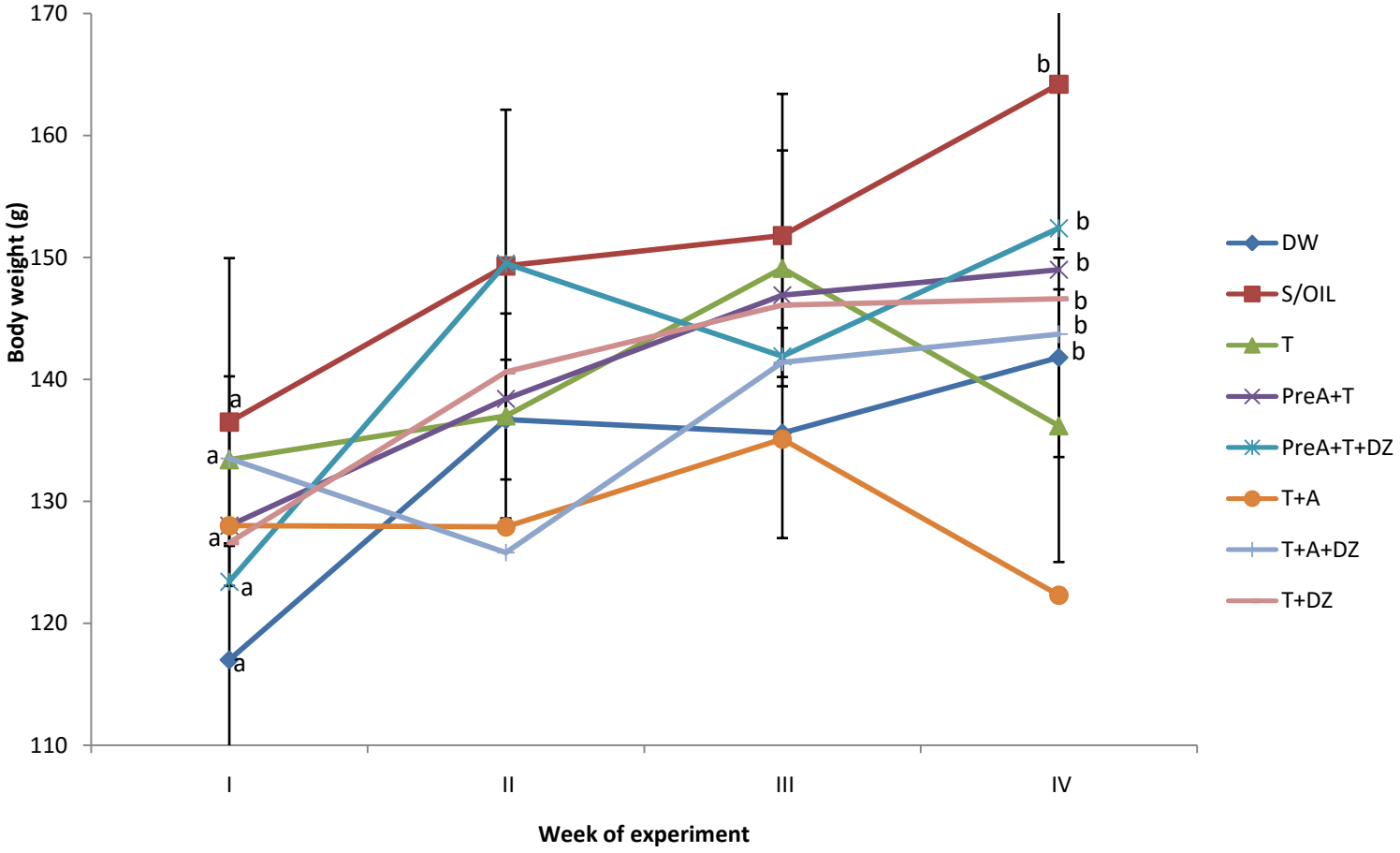

Figure 4.1 Effect of astaxanthin and diminazene aceturate on the dynamics of body weight changes in Wistar rats experimentally infected with *Trypanosoma brucei brucei*

[a, b] = Means in the same series with different superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

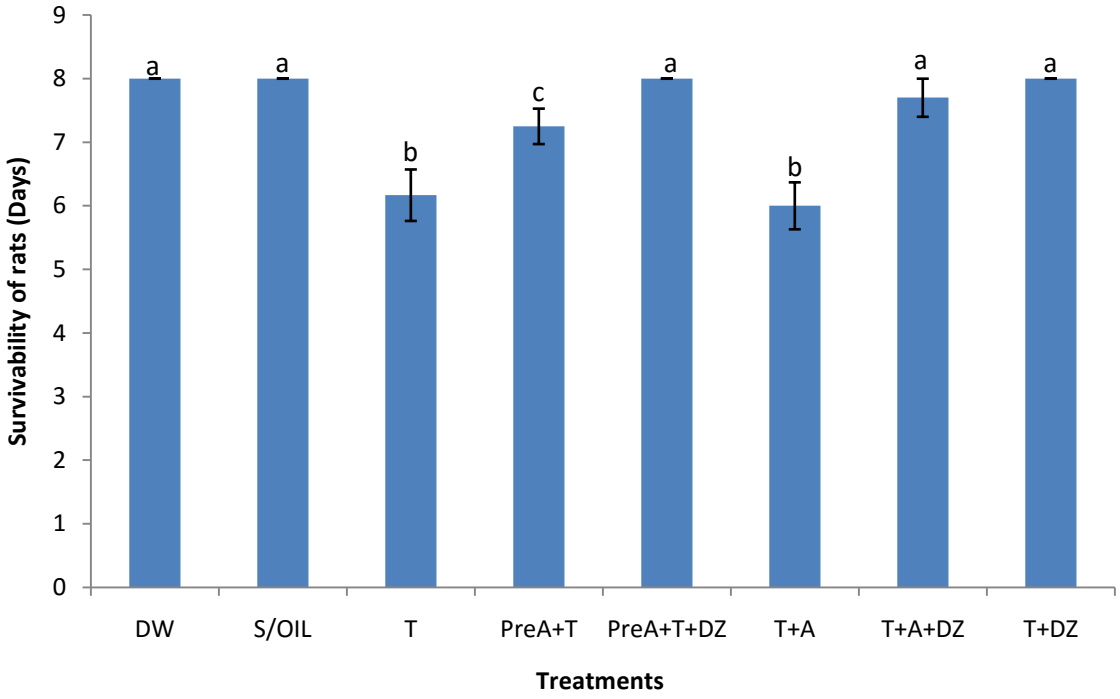

Figure 4.2 Effect of astaxanthin and diminazene aceturate on the survivability of Wistar rats experimentally infected with *Trypanosoma brucei brucei*

[a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/oil = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

Table 4.1: Parasitaemia, clearance and resurgence of parasitaemia in rats infected with *T. b. brucei* and treated with astaxanthin and diminazene aceturate

Days post-infection	DW	S/OIL	T	PreA+T	PreA+T+DZ	T+A	T+A+DZ	T+DZ
0	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
2	0/10	0/10	3/10	4/10	3/10	5/10	2/10	5/10
4	0/10	0/10	10/10	10/10	10/10	10/10	10/10	10/10
6	0/10	0/10	5/5	7/7	0/9	4/4	0/8	0/10
8	0/10	0/10	5/5	6/6	0/9	3/3	0/8	0/10
10	0/5	0/5	0/0	1/1	0/4	0/0	1/3	0/5
12	0/5	0/5	0/0	0/0	0/4	0/0	0/2	0/5

Numerator – Number of parasitaemic animals in the group, Denominator - Total number of rats surviving in the group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

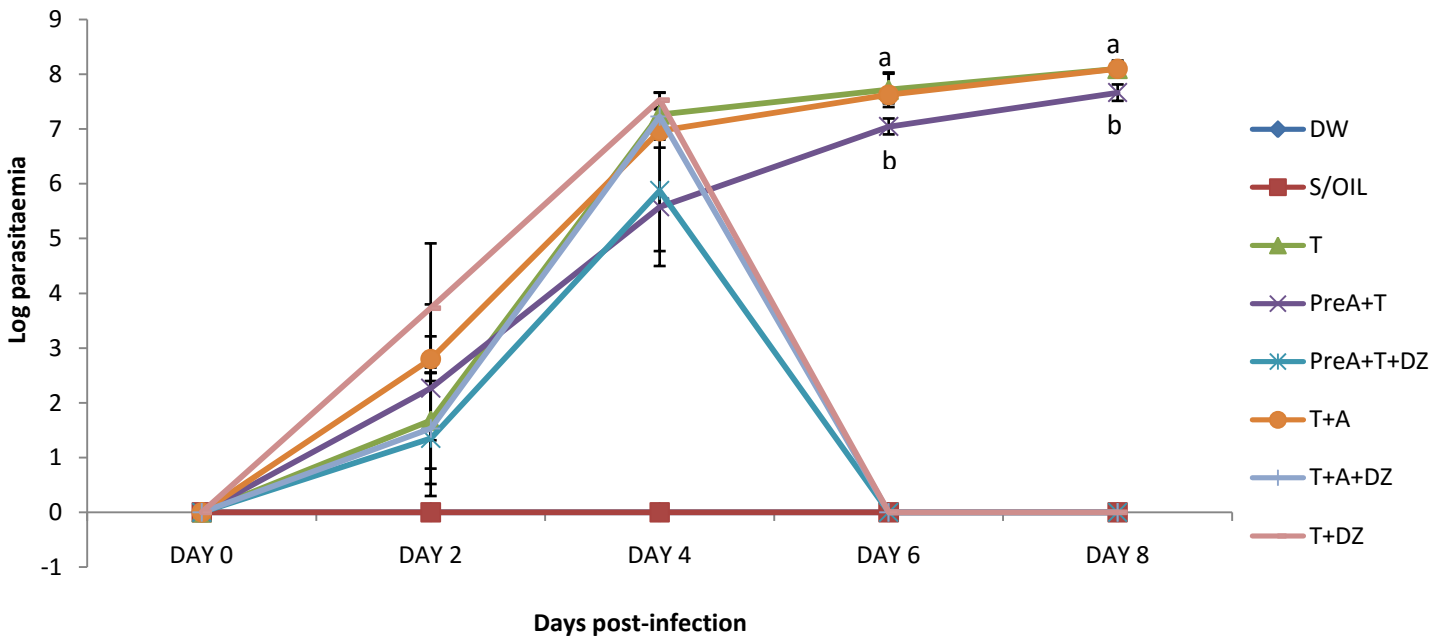

Figure 4.3 Effect of astaxanthin and diminazene aceturate on the level of parasitaemia of Wistar rats experimentally infected with *Trypanosoma brucei brucei*.

[a, b] = Means on the same day with different superscript letters are significantly different ($P < 0.05$). Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

4.4 Effect of Treatments on Haematological Parameters

4.4.1 Effect of treatments on total red blood cell count

The RBC count decreased ($P < 0.05$) in T group, when compared to that of PreA+T group. Rats in T and PreA+T groups showed no difference in their RBC counts, when compared to the corresponding counts obtained in T+A, and PreA+T+DZ. The RBC count in PreA+T group was significantly ($P < 0.05$) higher in comparison to that of T+A.The RBC countin PreA+T+DZ group was higher ($P < 0.05$) than in T+A+DZ group. Group T+A+DZ rats had higher ($P < 0.05$) RBC count compared to that of T+DZ group (Figure 4.4).

4.4.2 Effect of treatments on packed cell volume

The PCV was significantly ($P < 0.05$) lower in T group compared to the value obtained in PreA+T group. The difference in PCV of rats in T and T+A groups was not significant. Rats in PreA+T group had higher ($P < 0.05$) PCV compared to those in T+A group. There was a significant ($P < 0.05$) decrease in PCV of rats in PreA+T group in comparison to the value obtained in PreA+T+DZ group. The PCV significantly ($P < 0.05$) increased in PreA+T+DZ group, compared to the value recorded in T+A+DZ group. Rats in T+A+DZ showed no significant difference in PCV when compared to T+DZ rats (Figure 4.5).

4.4.3 Effect of treatments on haemoglobin concentration

The Hb concentration of rats in T group was significantly ($P < 0.05$) lower than that of PreA+T group, and concentrations of Hb in T and T+A groups, did not differ significantly. There was a significant ($P < 0.05$) decrease in Hb concentration in PreA+T group in comparison to the concentration obtained in PreA+T+DZ group. The astaxanthin pretreated groups (PreA+T and PreA+T+DZ) had higher ($P < 0.05$) Hb concentration, when compared to the

correspondingastaxanthin post-treated group alone or in combination with DZ (T+A, and T+A+DZ). The Hb concentration of rats in T+A+DZ group was significantly ($P < 0.05$) higher than in T+DZ group (Figure 4.6).

4.4.4 Effect of treatments on erythrocytic indices

There was a significant ($P < 0.05$) increase in MCV of rats in T group in comparison to the value obtained in PreA+T group. The MCV of rats in T group did not differ from that of T+A group. Rats in PreA+T group had higher ($P < 0.05$) value of MCV than in PreA+T+DZ group. A significant ($P < 0.05$) decrease in MCV was recorded in PreA+T rats compared to the value obtained in T+A rats. There was a significant ($P < 0.05$) decrease in the MCV of PreA+T+DZ rats in comparison to the corresponding value recorded in T+A+DZ rats. The MCV in T+A+DZ and T+DZ groups did not differ.

The MCH increased significantly ($P < 0.05$) in T group, when compared to the corresponding values recorded in PreA+T and PreA+T+DZ groups. The MCH values in T and T+A groups were not different. Similarly, the MCH in PreA+T and PreA+T+DZ rats did not differ. The MCH in the group pretreated with astaxanthin in trypanosome group alone and/or with diminazene aceturate (PreA+T and PreA+T+DZ) was lower than in the corresponding group, infected with *Trypanosoma brucei brucei* and treated with astaxanthin alone and/or with diminazene aceturate (T+A and T+A+DZ). Rats in T+A+DZ treated group had lower ($P < 0.05$) MCH value in comparison to those of T+DZ group.

The MCHC in T group was significantly ($P < 0.05$) higher when compared to that of PreA+T or PreA+T+DZ group. Rats in groups T and PreA+T showed no difference in their MCHC when compared respectively to the values recorded in T+A, or PreA+T+DZ group. The MCHC of rats

in PreA+T group was lower than that of T+A group. The MCHC in PreA+T+DZ and T+A+DZ groups did not differ. A significant ($P < 0.05$) decrease occurred in the MCHC of rats in T+A+DZ group in comparison to that of T+DZ group (Figure 4.7).

4.4.5 Effect of treatments on platelet count.

The platelet count of rats in the T group was significantly ($P < 0.05$) lower than in the PreA+T and T+A rats. There was a significant ($P < 0.05$) decrease in platelet count of rats in PreA+T group in comparison to PreA+T+DZ group. Rats in PreA+T and PreA+T+DZ treatment groups had higher ($P < 0.05$) platelet counts, when compared to their corresponding astaxanthin post-treated groups (T+A, and T+A+DZ). The platelet counts in T+A+DZ and T+DZ groups did not differ (Figure 4.8).

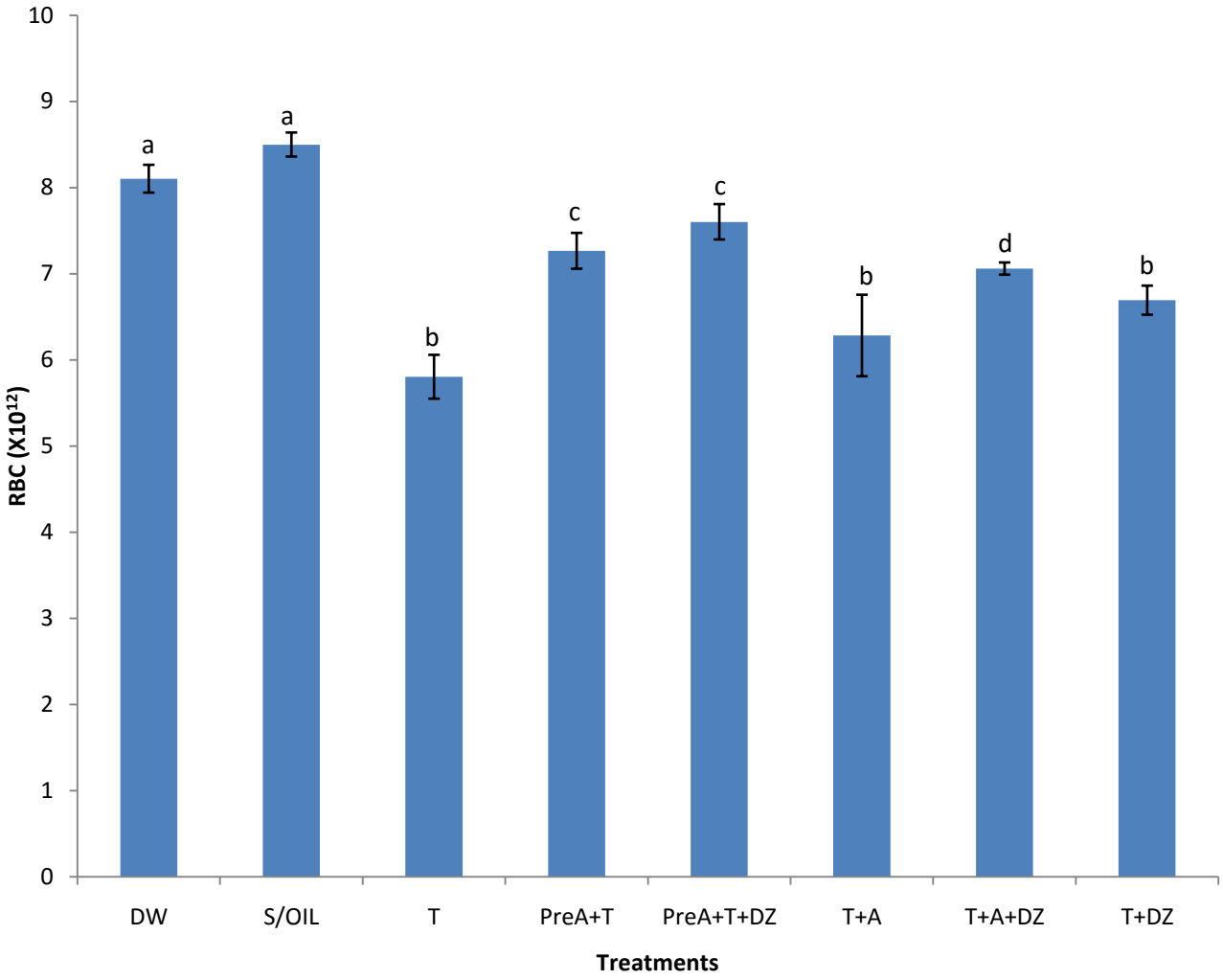

Figure 4.4 Effect of treatments with astaxanthin and diminazene aceturate on erythrocyte count of Wistar rats experimentally infected with *Trypanosoma brucei brucei*.

[a, b, c, d] = Means with different superscript letters are significantly ($P < 0.05$)different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

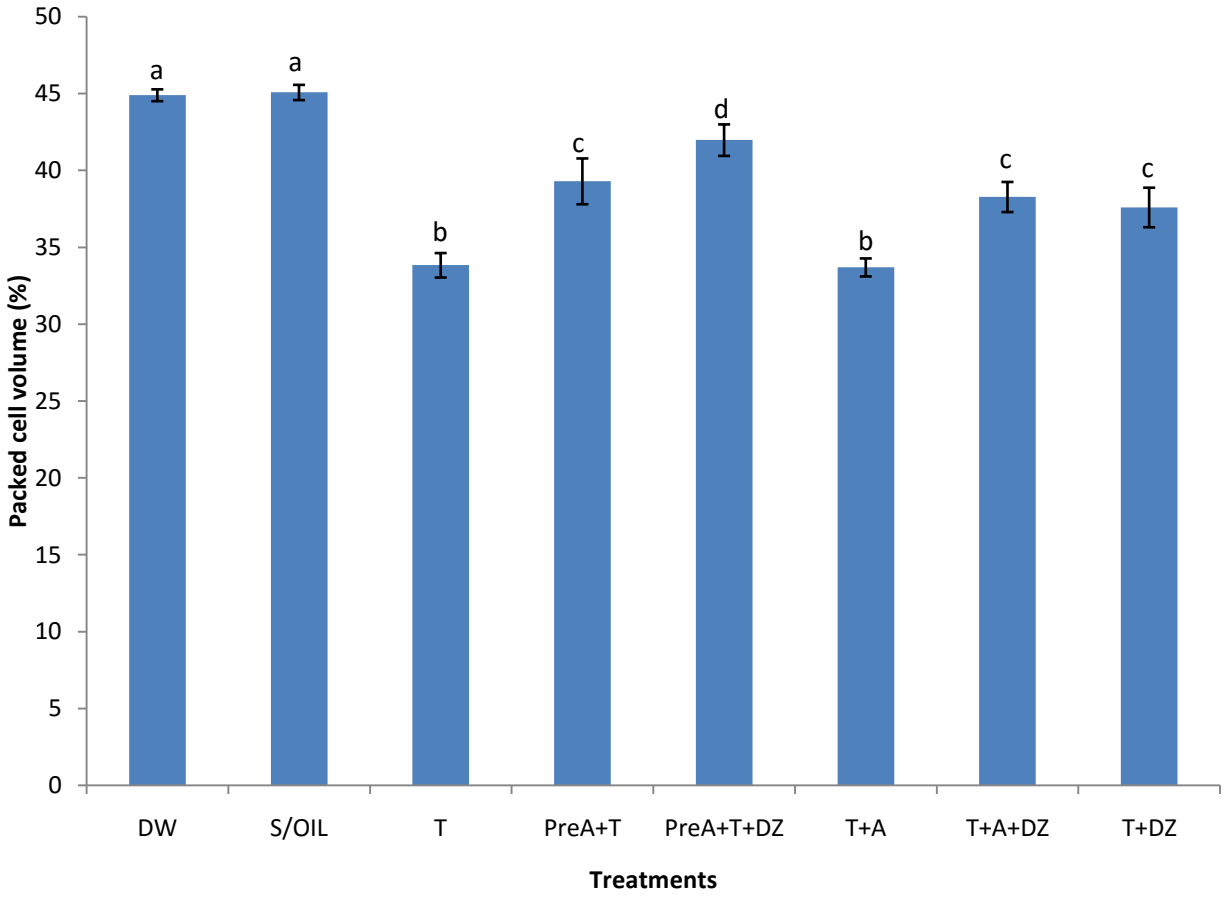

Figure 4.5 Effect of treatments with astaxanthin and diminazene aceturate on packed cell volume of Wistar rats experimentally infected with *Trypanosoma brucei brucei*.

[a, b, c, d] = Means with different superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

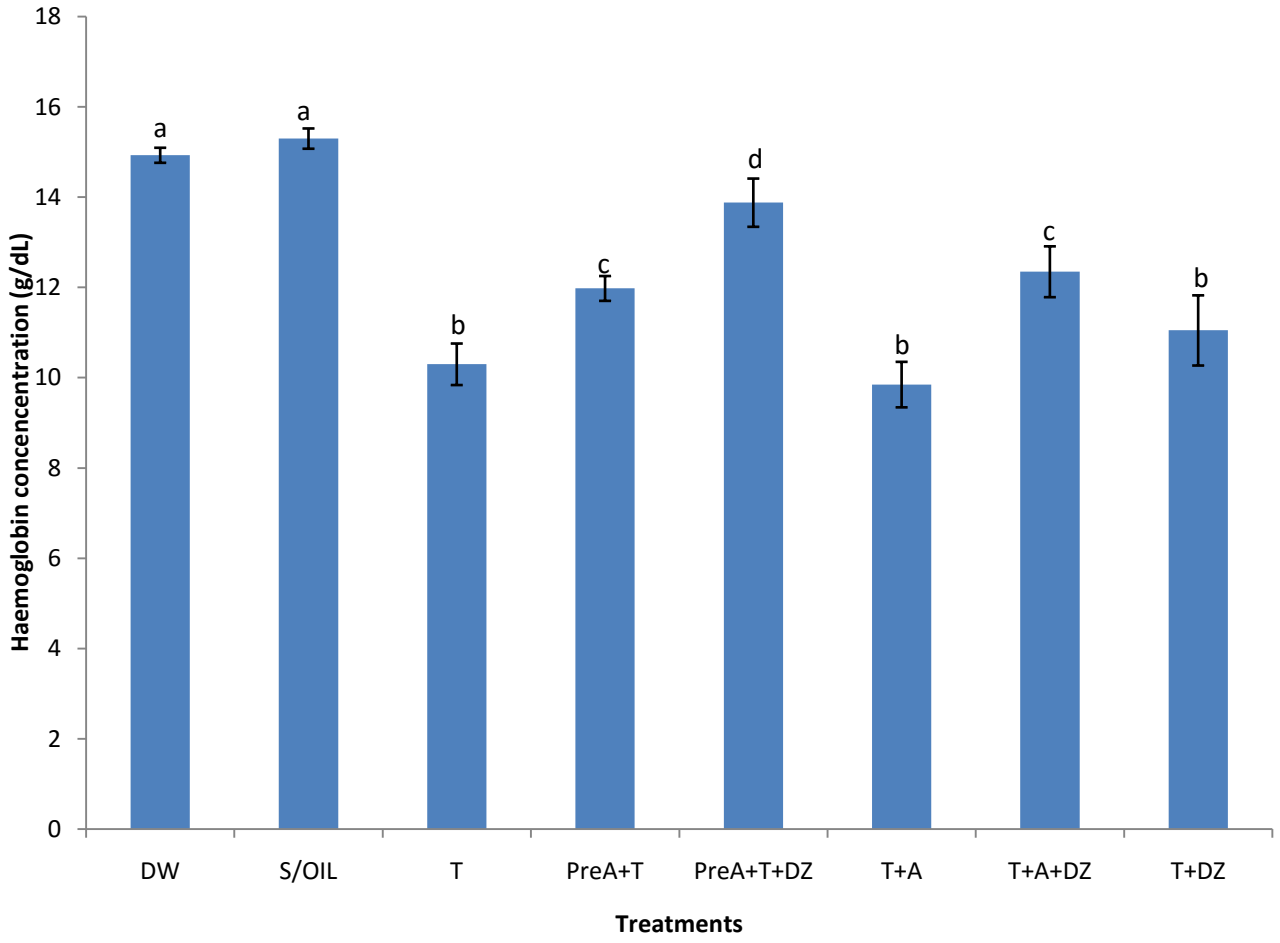

Figure 4.6 Effect of treatments with astaxanthin and diminazene aceturate on haemoglobin concentration of Wistar rats experimentally infected with *Trypanosoma brucei brucei*.

$^{a, b, c, d}$ = Means with different superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

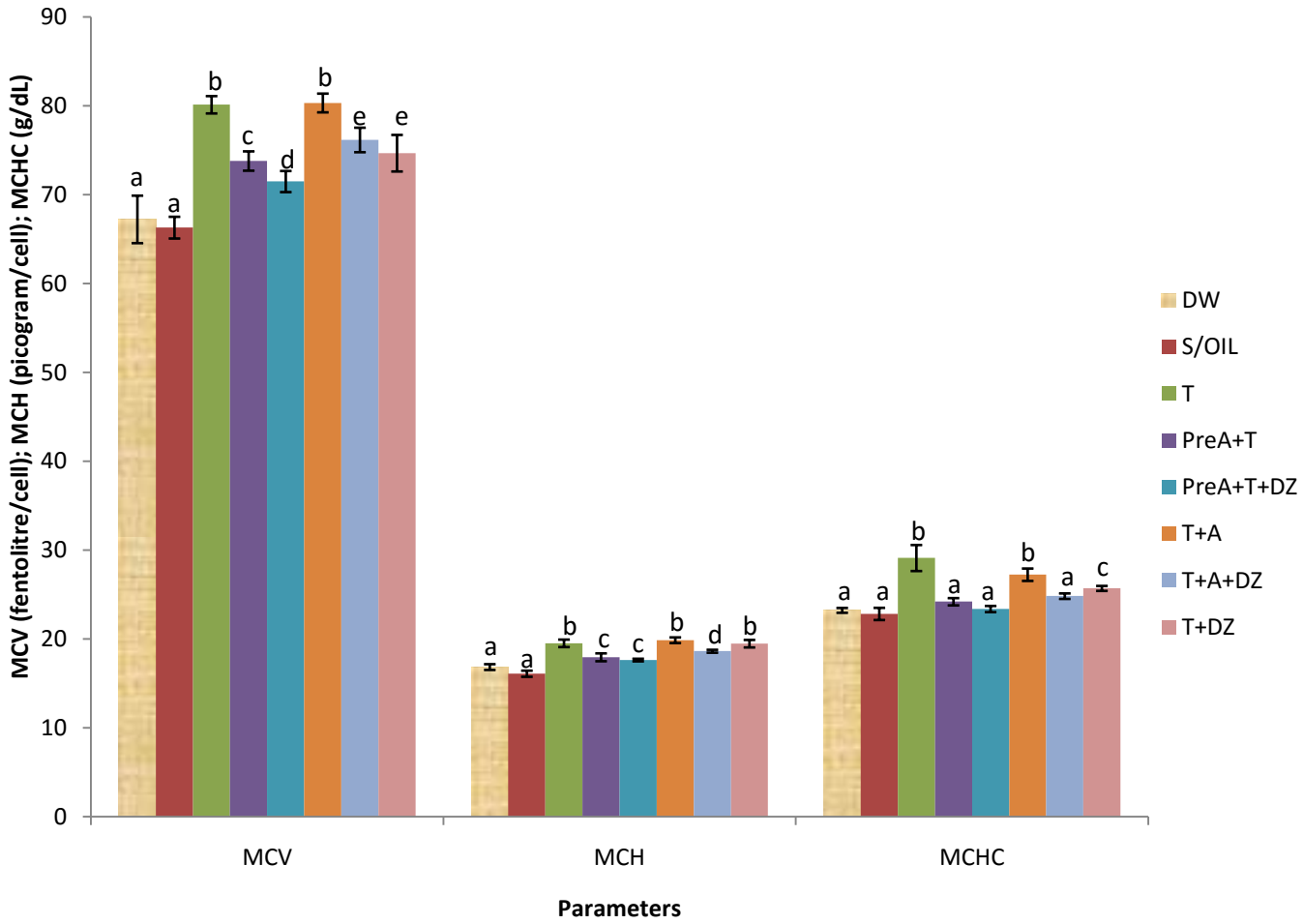

Figure 4.7 Effect of treatments with astaxanthin and diminazene aceturate on erythrocytic indices of Wistar rats experimentally infected with*Trypanosoma brucei brucei*.

[a, b, c, d, e] = Means with different superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

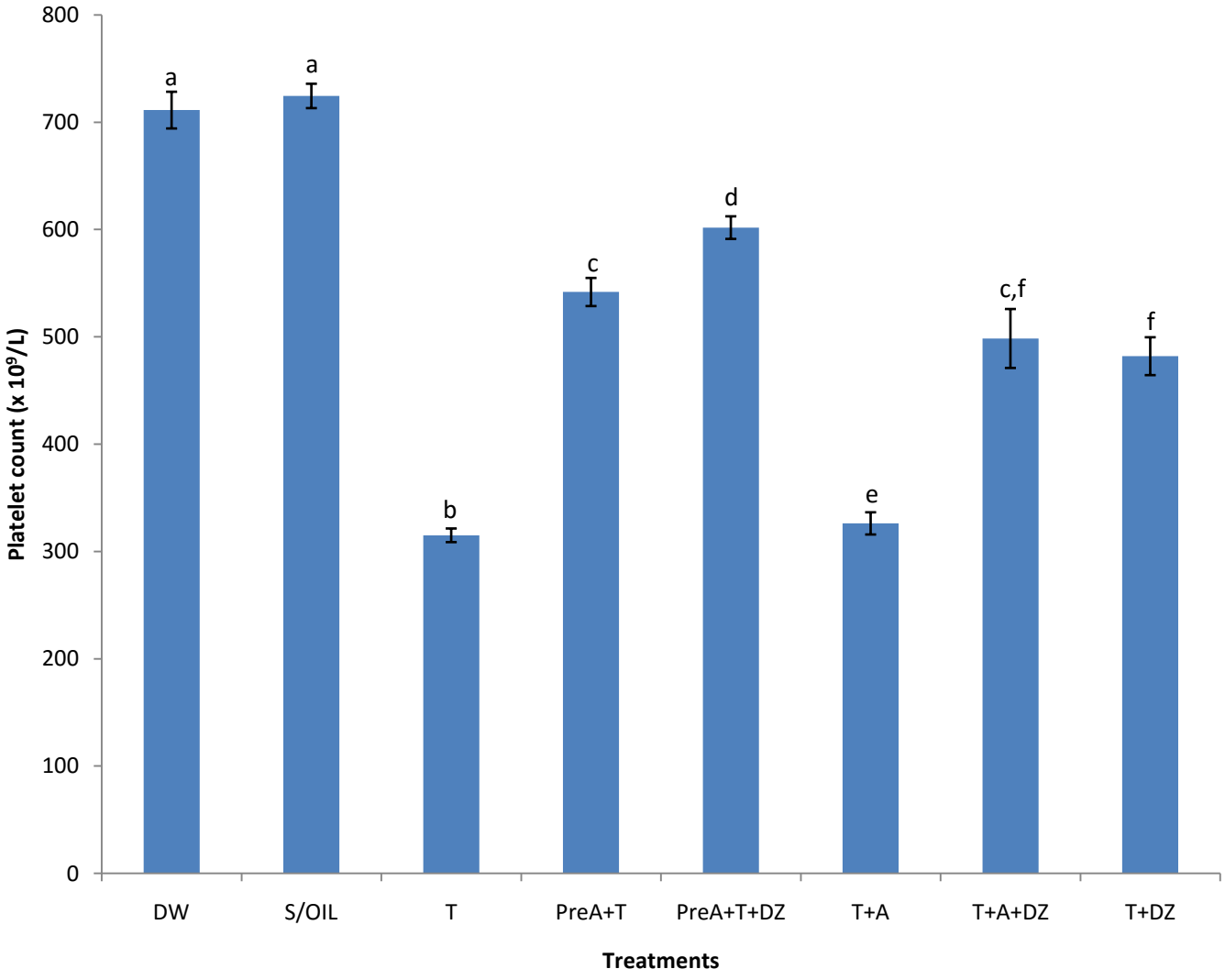

Figure 4.8 Effect of treatments with astaxanthin and diminazene aceturate on platelet count of Wistar rats experimentally infected with*Trypanosoma brucei brucei*.

$^{a, b, c, d, e, f}$ = Means with different superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

4.4.6 Effect of treatments on total leucocyte count

The WBC countin the T group was higher ($P < 0.05$) than thecount recorded inPreA+T group, butthe counts in T and T+Aratswere not different. There was a significant ($P < 0.05$) increase in the WBC countof rats in PreA+T group, when compared withthe count in PreA+T+DZ group. Rats in PreA+T group had lower ($P < 0.05$) WBC count in comparison to those of T+A group. The WBC count in PreA+T+DZ groupwas lower ($P < 0.05$) than the count recorded in T+A+DZ group. There was no difference in the WBC counts of rats in T+A+DZ and T+DZ groups(Figure 4.9).

4.4.7 Effect of treatments on differential leucocyte count

There was no difference in lymphocyte counts of rats in all the treatment groups.

Rats in T group had significantly ($P < 0.05$) higher neutrophil count in comparison to those of PreA+T group. The neutrophil counts of rats in T and T+A groups were not significantly different. The neutrophil count in PreA+T rats was higher ($P < 0.05$) than the count recorded in PreA+T+DZ rats. Rats in PreA+T group had lower ($P < 0.05$) neutrophil count when compared to that of T+A group. The neutrophil count in PreA+T+DZ group was lower ($P < 0.05$) than the count in T+A+DZ group. The neutrophil counts in T+A+DZ and T+DZ groups did not differ significantly (Figure 4.10).

4.4.8 Effect of treatments on erythrocyte osmotic fragility

The percentage haemolysis at 0.9% NaCl concentration was significantly ($P < 0.01$) higher in T group, when compared to PreA+T group. At 0.9% NaCl concentration, the percentage haemolysis of rats in T group was not different from that of T+A group. There was a significant

($P < 0.05$) increase in percentage haemolysis at 0.9% NaCl concentration in PreA+T group, when compared to the value recorded in PreA+T+DZ group. At 0.9% NaCl concentration, the percentage haemolysis decreased significantly ($P < 0.05$) in PreA+T group when compared to that of T+A group; and in PreA+T+DZ rats, the percentage haemolysis was similarly lower ($P < 0.05$) than that of T+A+DZ rats. The percentage haemolysis at 0.9% NaCl concentration in T+A+DZ and T+DZ groups did not differ.

At 0.7% NaCl concentration, the percentage haemolysis in T group increased significantly ($P < 0.05$) in comparison to that of PreA+T group. There was no significant difference in percentage haemolysis at 0.7% NaCl concentration in T and T+A groups. The percentage haemolysis of rats at 0.7% NaCl concentration was significantly ($P < 0.05$) higher in PreA+T treated group, when compared to the corresponding value obtained in group PreA+T+DZ. There was a significant ($P < 0.05$) decrease in percentage haemolysis of rats at 0.7% NaCl concentration in PreA+T group, when compared to that of T+A group. The percentage haemolysis recorded in PreA+T+DZ group was lower ($P < 0.05$) than in T+A+DZ group. At 0.7% NaCl concentration, the percentage haemolysis in T+A+DZ and T+DZ did not differ.

There was a significant ($P < 0.05$) increase in percentage haemolysis at 0.5% NaCl concentration in T group, compared to the value recorded in PreA+T group. The percentage haemolysis at 0.5% NaCl concentration in T group was not significantly different from that of T+A group. At 0.5% NaCl concentration, the percentage haemolysis in PreA+T rats rose significantly ($P < 0.05$) in comparison to the corresponding value recorded in PreA+T+DZ rats. The percentage haemolysis at 0.5% NaCl concentration was lower ($P < 0.05$) in PreA+T and PreA+T+DZ groups, when compared compared to T+A and T+A+DZ groups. There was no difference in

percentage haemolysis at 0.5% NaCl concentration in T+A+DZ group, when compared to that of T+DZ group (Figure 4.11).

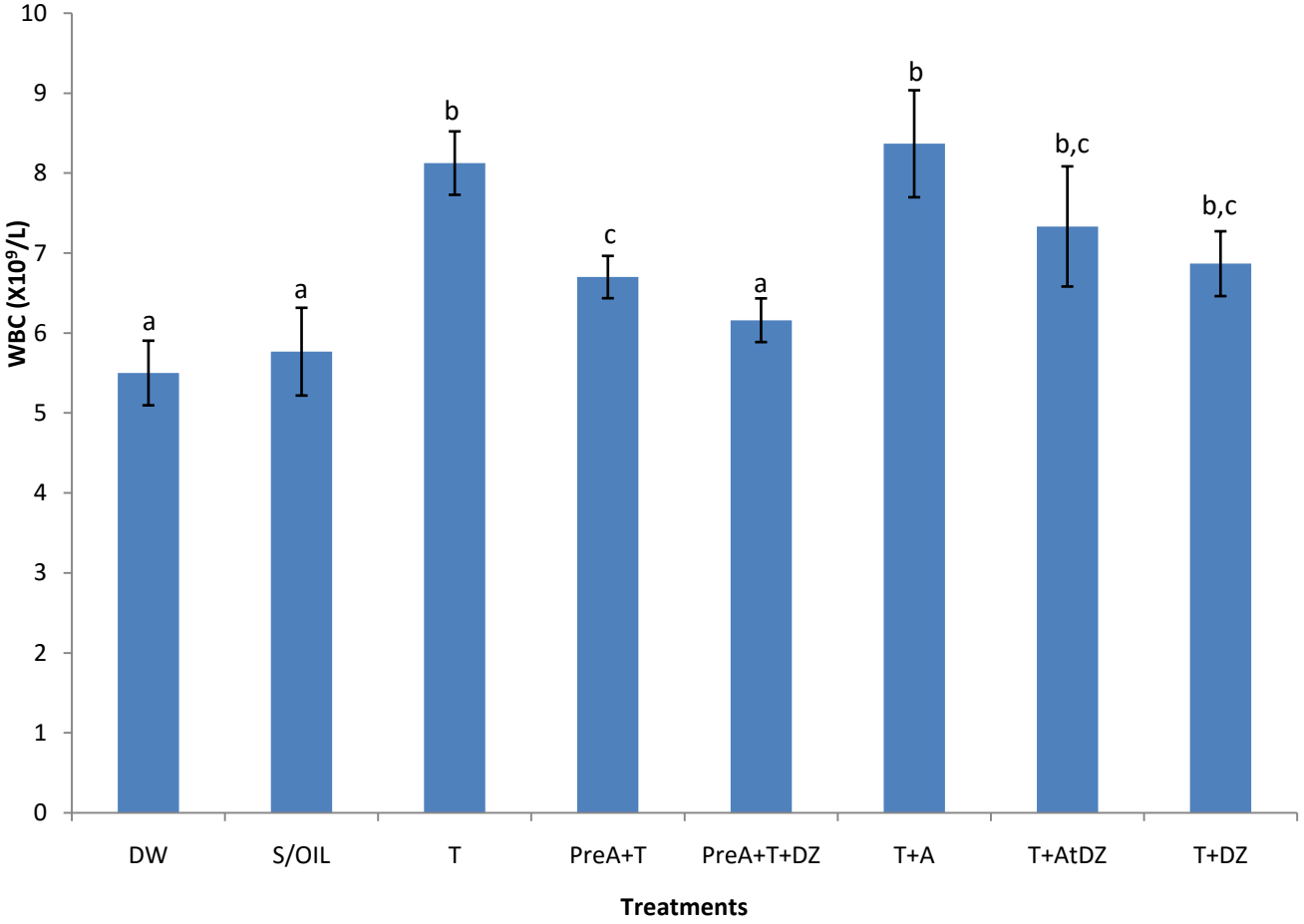

Figure 4.9 Effect of treatments with astaxanthin and diminazene aceturate on absolute leucocyte count of Wistar rats experimentally infected with *Trypanosoma brucei brucei*

[a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

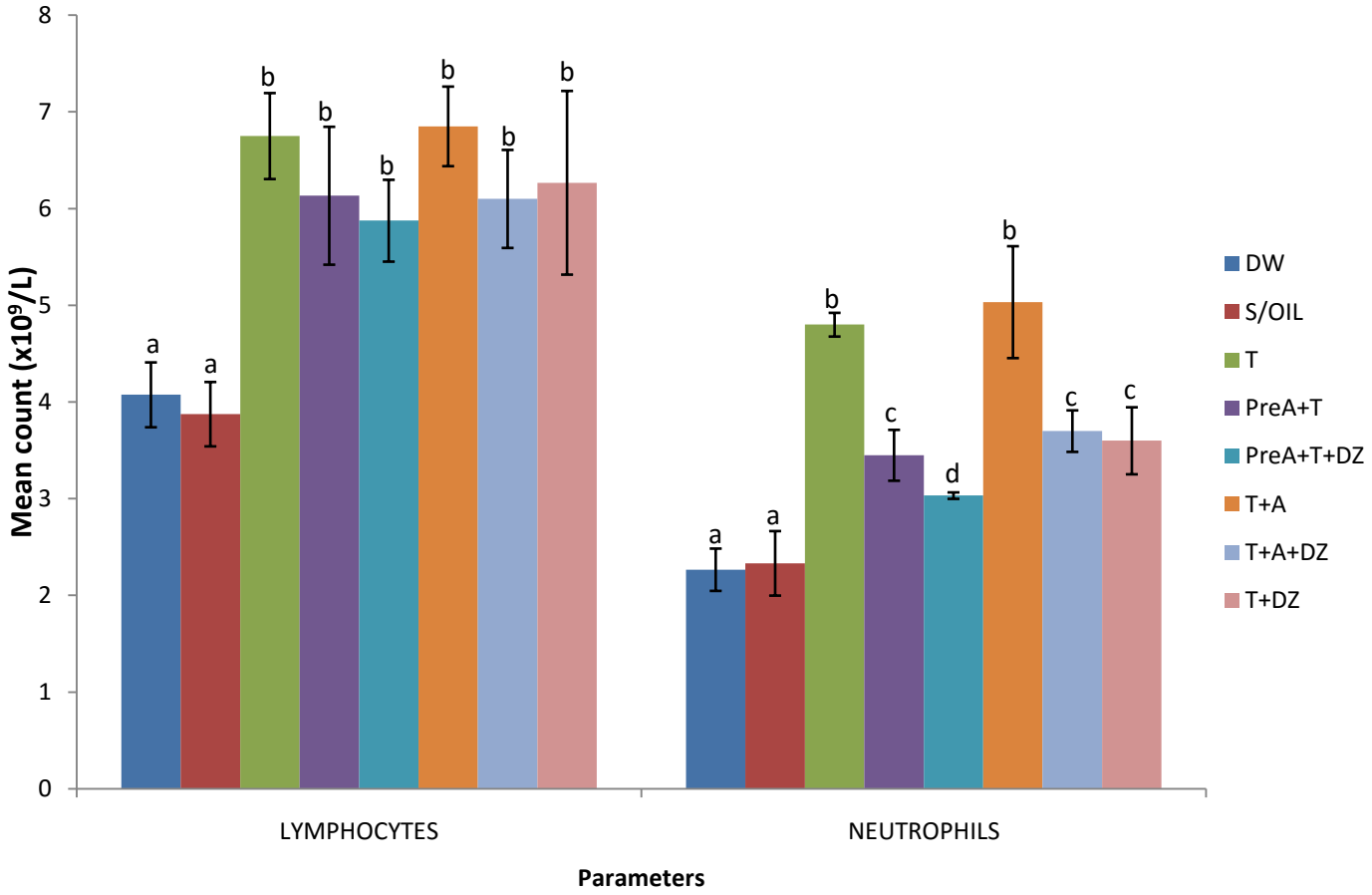

Figure 4.10 Effect of treatments with astaxanthin and diminazene aceturate on lymphocyte and neutrophil counts of Wistar rats experimentally infected with *Trypanosoma brucei brucei*

$^{a, b, c, d}$ = Means with different superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

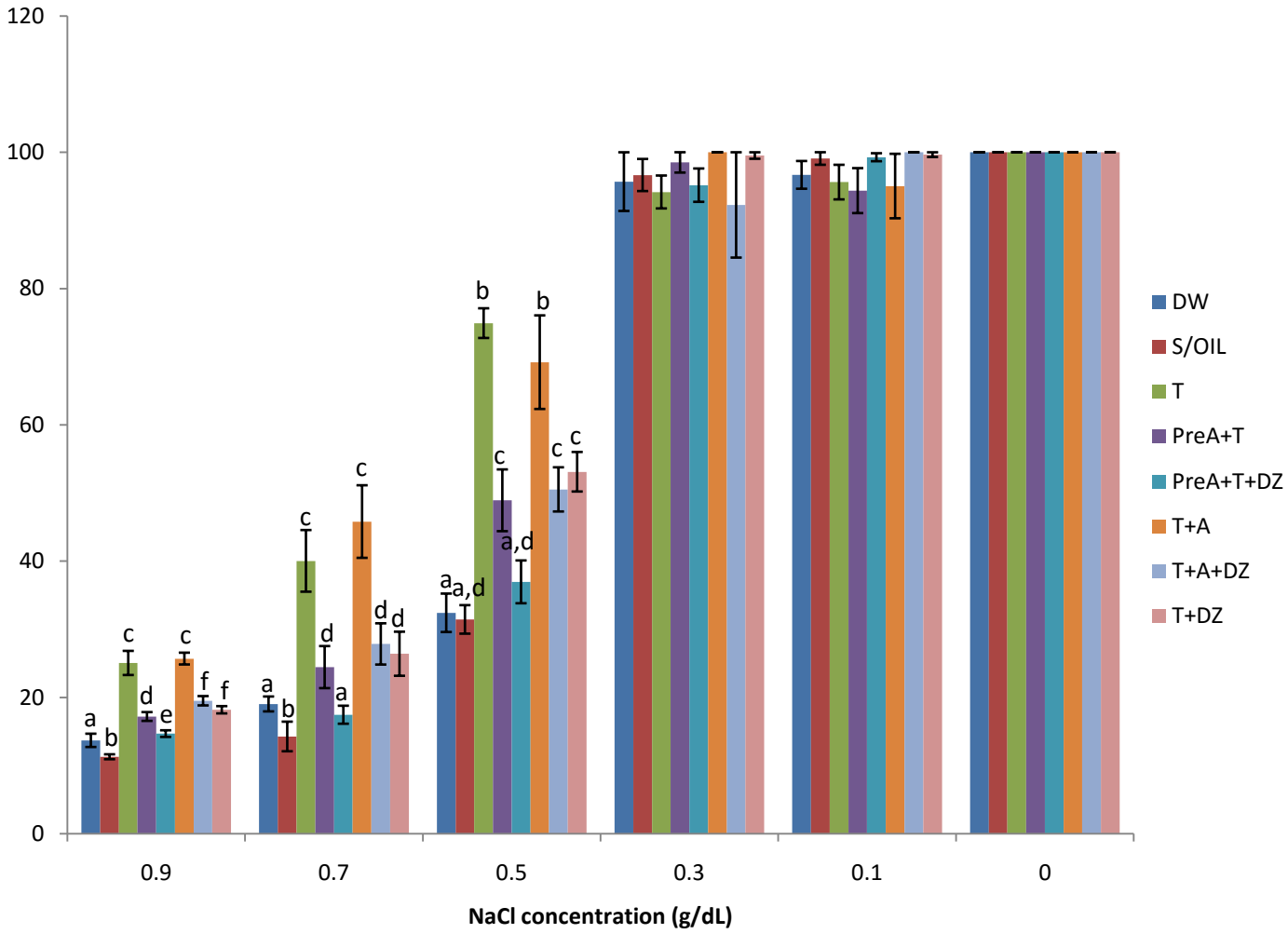

Figure 4.11 Effect of treatments with astaxanthin and diminazene aceturate on erythrocyte osmotic fragility of Wistar rats experimentally infected with *Trypanosoma brucei brucei*.

$^{a, b, c,d,e,f}$ = Means with different superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

4.5 Efect of Treatments on Serum Biochemical Parameters

4.5.1 Liver enzymes

The activity of AST in Trats was higher ($P < 0.05$) than in PreA+T rats,but did not differ in Tand T+A groups.Similarly, the AST activities of rats in PreA+T and PreA+T+DZ groups were not different. Rats in PreA+T group had lower ($P < 0.05$) AST activity in comparison to those of T+A group. The AST activities in PreA+T+DZ andT+A+DZ groups did not differ. Rats in T+A+DZgroup showed no difference in AST activity when compared to the activity obtained in T+DZ group.

The activity of ALT in T group was significantly higher ($P < 0.05$)in comparison to that ofPreA+T group. There was no difference in ALT activity of rats in T group,when compared to the activity recorded in T+Agroup. The ALT activity rose significantly ($P < 0.05$) in PreA+T group, compared to the activity obtained in PreA+T+DZ group. There was a significant ($P < 0.05$) decrease in ALT activity of rats in PreA+T group, when compared to that of the T+A group. Rats in PreA+T+DZ group recorded lower ($P < 0.05$) ALT activity than those in T+A+DZ group. The activity of ALT in T+A+DZ and T+DZ groups were not significantly different.

The ALP activity of rats in T group rose ($P < 0.05$) in comparison to that recorded inPreA+Tgroup. There was no difference in ALP activities in T and T+A groups. Similarly, ALP activities in PreA+T and PreA+T+DZ groups did not differ.A significant ($P < 0.05$) decrease occurred in ALP activity of PreA+T rats, when compared to that of T+A rats. The ALP activities in PreA+T+DZ and T+A+DZ rats were not significantly different. The difference in ALP activities in T+A+DZ and T+DZ rats was insignificant(Figure 4.12).

4.5.2 Effect of treatments on lipid profile

4.5.2.1 Effect of treatments on cholesterol level

The level of cholesterol in T group was significantly ($P < 0.05$) higher, when compared to that obtained in PreA+T group. Rats in T and T+A groups showed no difference in cholesterol level. There was a significant ($P < 0.05$) increase in the level of cholesterol in PreA+T group in comparison to PreA+T+DZ group. The level of cholesterol in PreA+T group was lower ($P < 0.05$) than the level in T+A group. Rats treated with PreA+T+DZ had lower ($P < 0.05$) cholesterol level compared to those in T+A+DZ group (Figure 4.13).

4.5.2.2 Effect of treatments on the level of triglycerides

The level of triglyceride in T treated rats was higher ($P < 0.05$) than that of PreA+T rats. There was no difference in triglyceride levels in groups T and PreA+T, when compared respectively to T+A and PreA+T+DZ groups. The triglyceride level in PreA+T rats was significantly ($P < 0.05$) lower than the corresponding value recorded in T+A. The level of triglyceride in PreA+T+DZ rats did not differ from that of T+A+DZ rats, and similarly, those of T+A+DZ and T+DZ rats did not differ (Figure 4.14).

4.5.2.3 Effect of treatments on the level of high-density lipoprotein

The HDL significantly increased ($P < 0.05$) in T group, compared to that of PreA+T group. The HDL levels in T, T+A, T+A+DZ andT+DZ groups did not differ. Rats in PreA+T group had lower ($P < 0.05$) HDL level, when compared to that of T+A. Similarly, the level of HDL in PreA+T+DZ was lower than that of the T+A+DZ group (Figure 4.15).

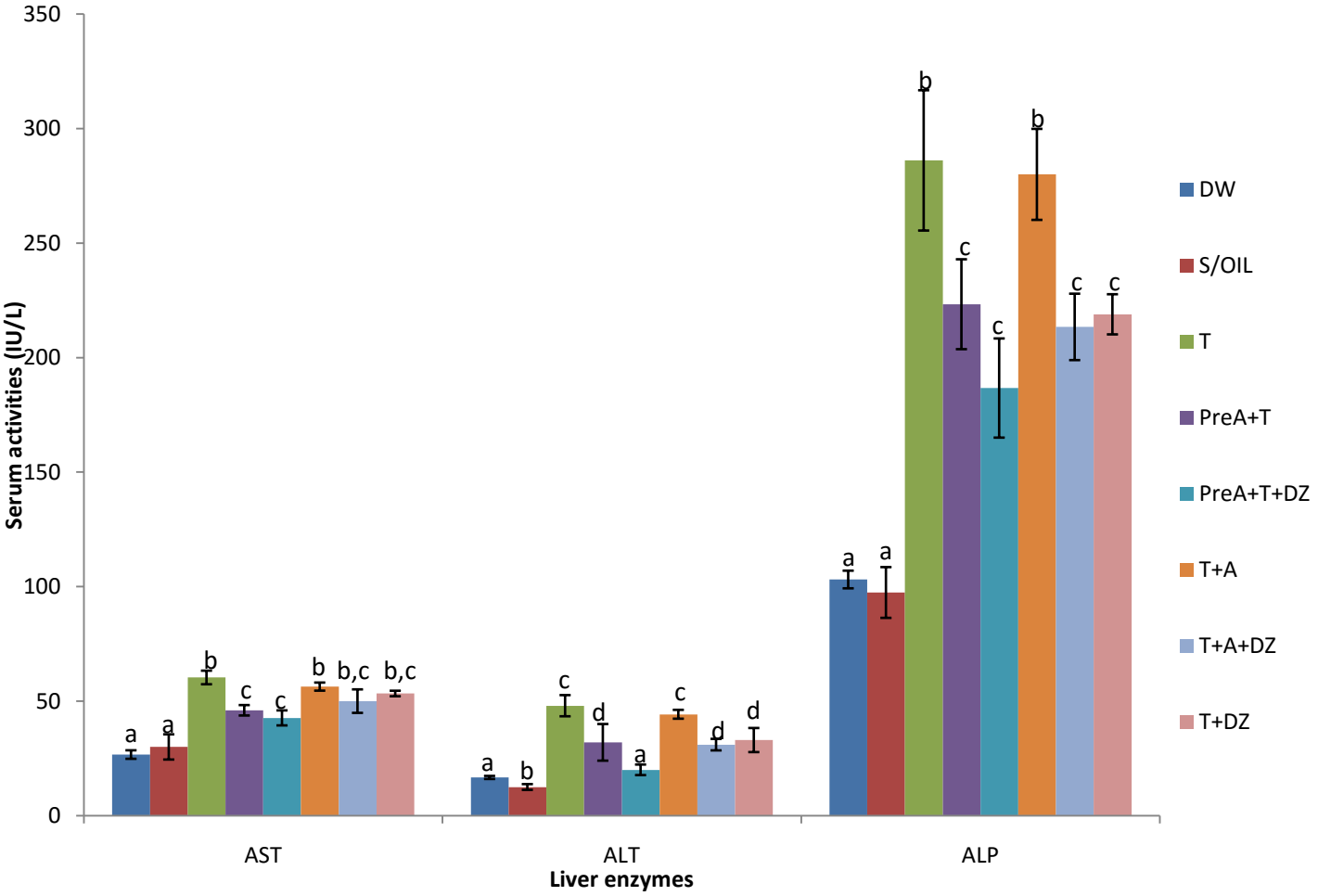

Figure 4.12: Effect of treatments with astaxanthin and diminazene aceturate on activities of liver enzymes in Wistar rats experimentally infected with *Trypanosoma brucei brucei*.

[a, b, c, d] = Means with different or not sharing superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALP = alkaline phospharase.

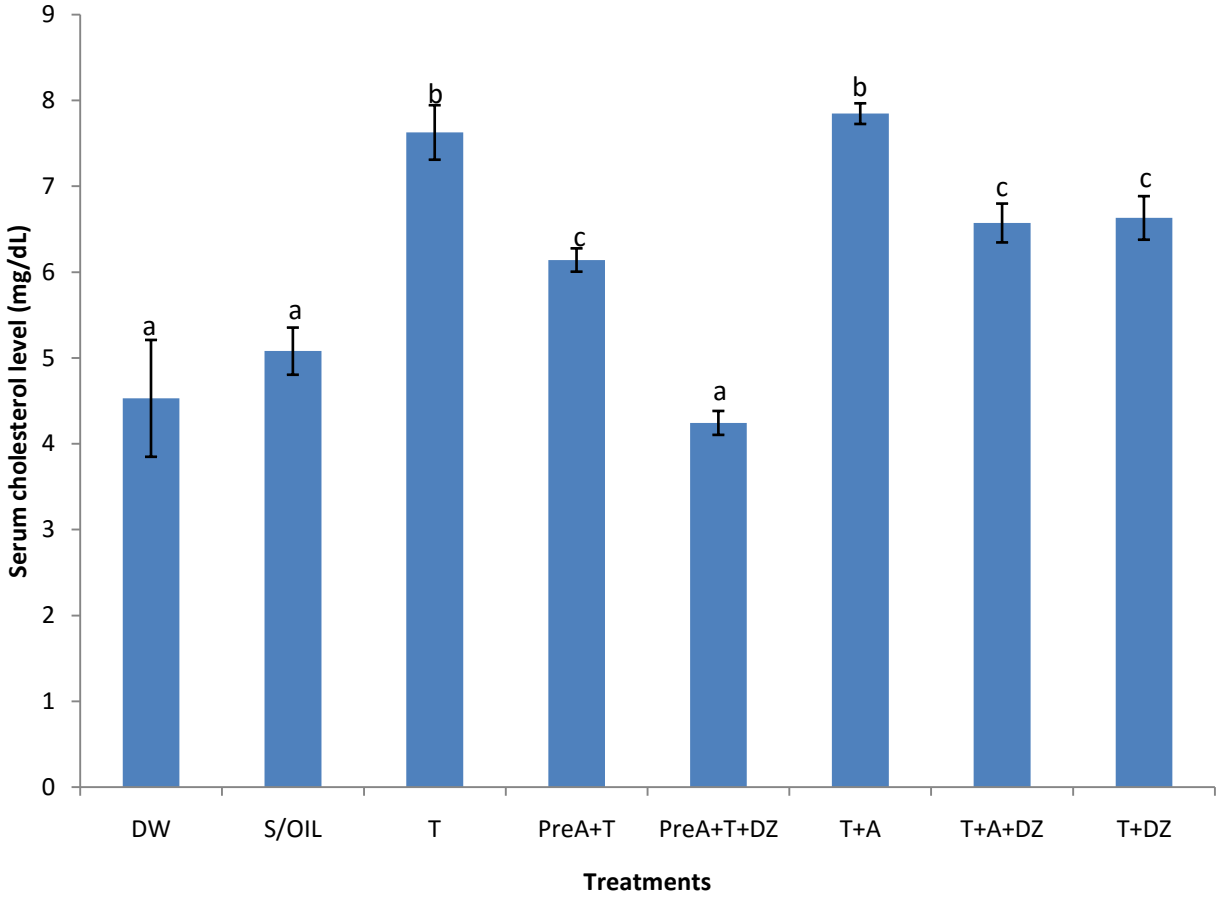

Figure 4.13: Effect of treatment with astaxanthin and diminazene aceturate on the level of cholesterol inWistar rats experimentally infected with *Trypanosoma brucei brucei*.

[a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

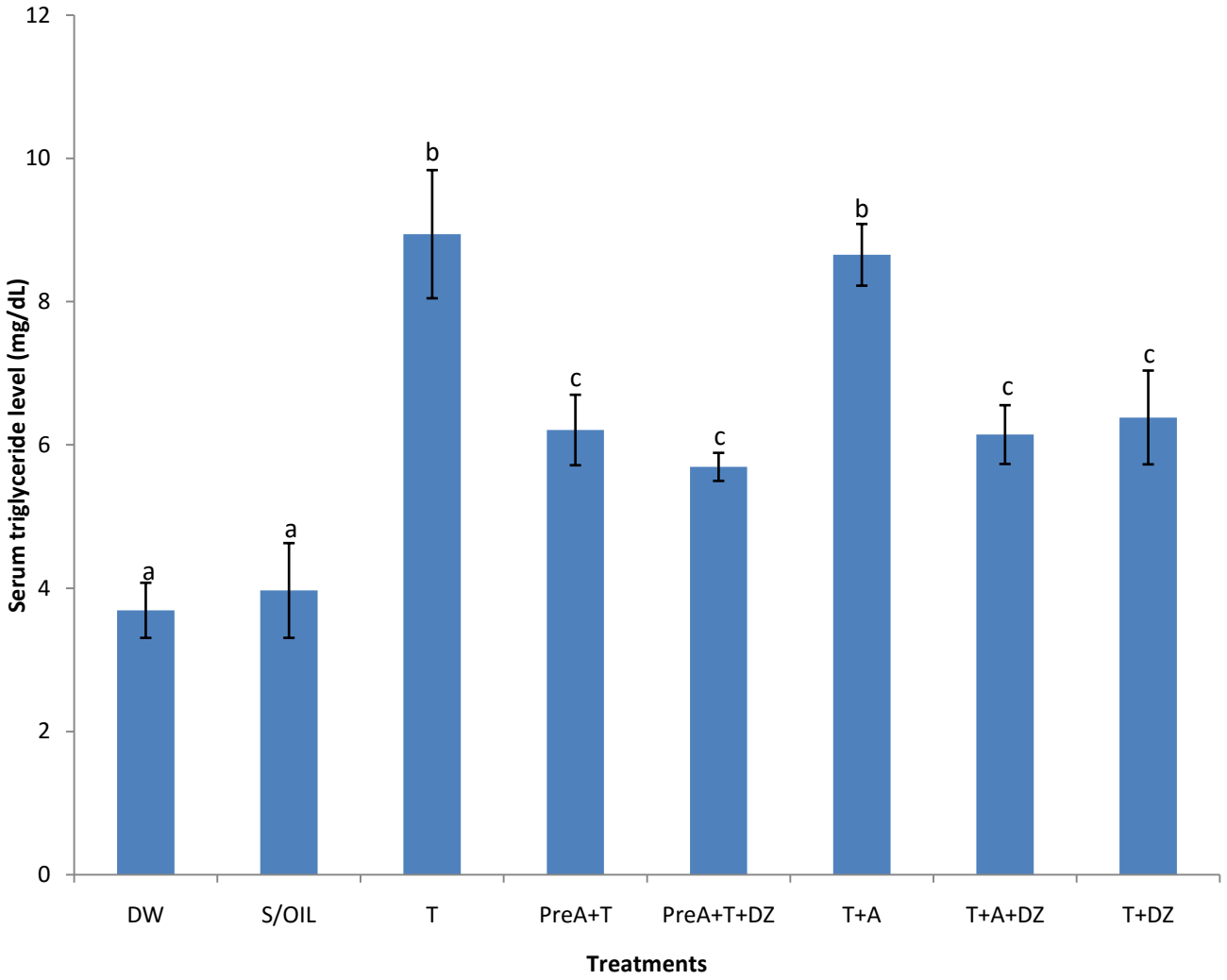

Figure 4.14: Effect of treatment with astaxanthin and diminazene aceturate on the level of triglycerides inWistar rats experimentally infected with *Trypanosoma brucei brucei*.

[a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

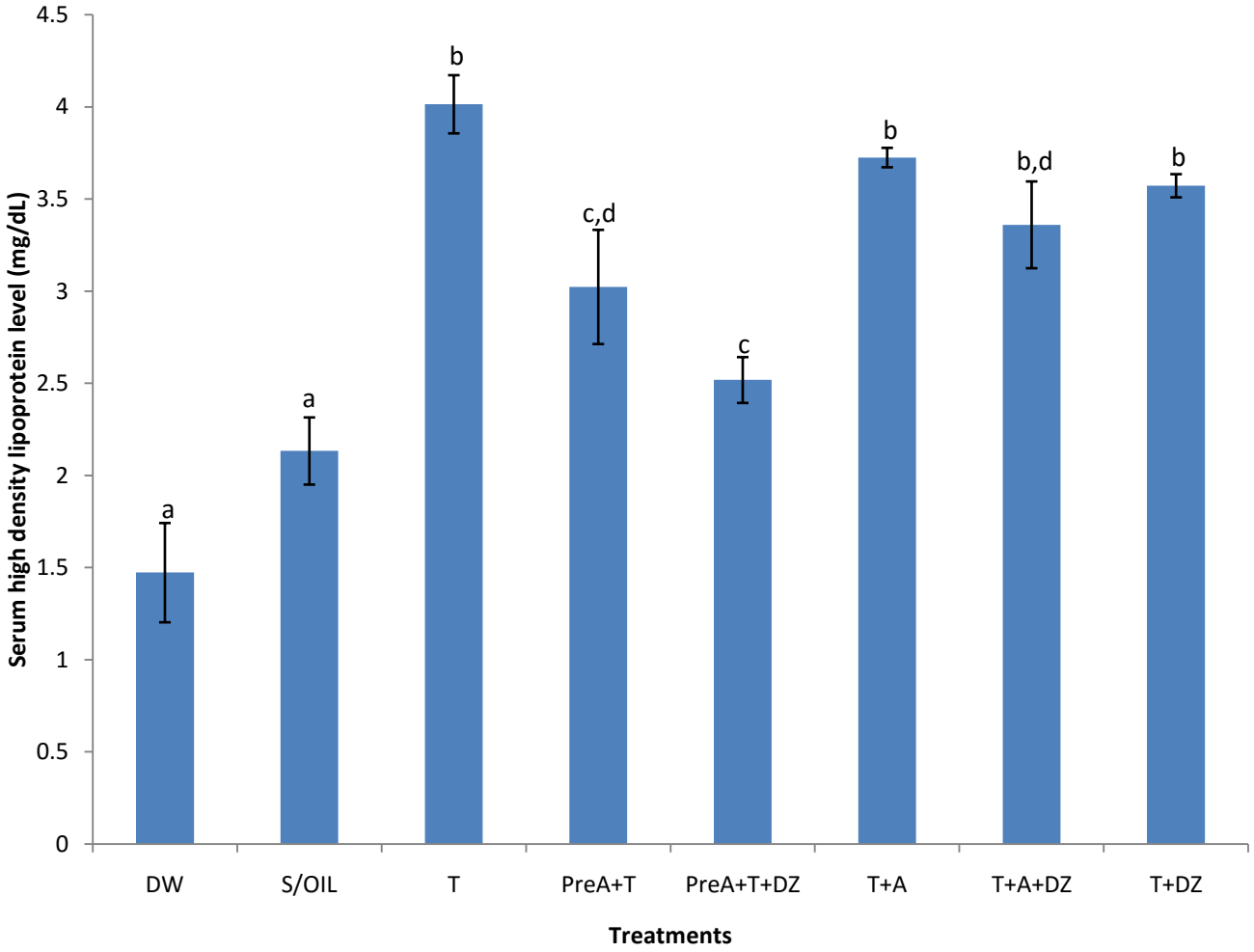

Figure 4.15: Effect of treatment with astaxanthin and diminazene aceturate on the level of high density lipoprotein in Wistar rats experimentally infected with *Trypanosoma brucei brucei*

[a, b, c] = Means with different or not sharing superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

4.5.3 Effect of treatment on immunoglobulin G

The level of IgG significantly ($P < 0.05$) decreased in T group, compared to the value obtained in PreA+T group. There was a significant increase ($P < 0.05$) in the levels of IgG in PreA+T and PreA+T+DZ groups, when compared to that of T+A and T+A+DZ, respectively. The IgG levels in T, T+A, T+A+DZ and T+DZ groups did not differ. Similarly, the levels in PreA+T and PreA+T+DZ rats were not different (Figure 4.16).

4.5.4. Effect of treatments on cytokines

4.5.4.1 Interleukin-1 alpha

The concentration of IL-1α was higher ($P < 0.05$) in T group, compared to that of PreA+T group. The difference in concentration of IL-1α in groups T and T+A, was not significant. Similarly, the concentrations of IL-1α in PreA+T, PreA+T+DZ, T+A, T+A+DZ and T+DZ groups did not differ (Figure 4.17).

4.5.4.2 Interleukin-6

The concentration of IL-6 was significantly higher ($P < 0.05$) in T group than in PreA+T group. There was no difference in the concentrations of IL-6 in T and T+A groups. The concentration of IL-6 was lower ($P < 0.05$) in PreA+T group, when compared to that of T+A group. There was no significant difference in the concentrations of IL-6 in PreA+T, PreA+T+DZ, T+A+DZ and T+DZ groups (Figure 4.18).

4.5.4.3 Effect of treatment on tumour necrosis-factor alpha

The concentration of TNF-α was significantly ($P < 0.05$) higher in T group than in PreA+T group. There was no difference in the concentrations of TNF-α in T and T+A groups. The concentration of TNF-α in PreA+T group was lower ($P < 0.05$) in comparison to that of T+A group. The difference in the concentrations of TNF-α in PreA+T, PreA+T+DZ, T+A+DZ and T+DZ groups was not significant (Figure 4.19).

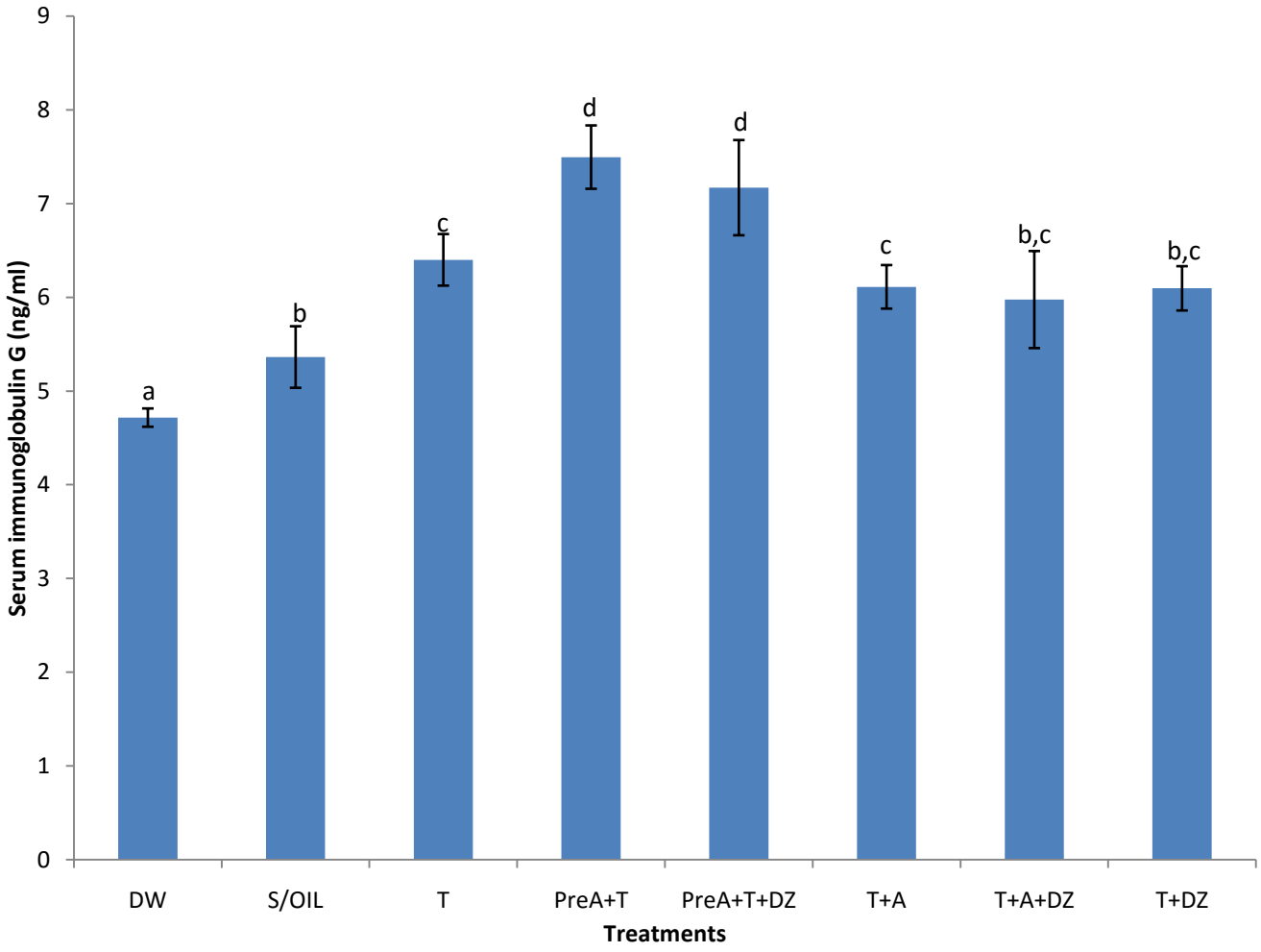

Figure 4.16: Effect of treatments with astaxanthin and diminazene aceturate on the level of immunoglobulin G inWistar rats experimentally infected with *Trypanosoma brucei brucei*

[a, b, c, d] = Means with different or not sharing superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

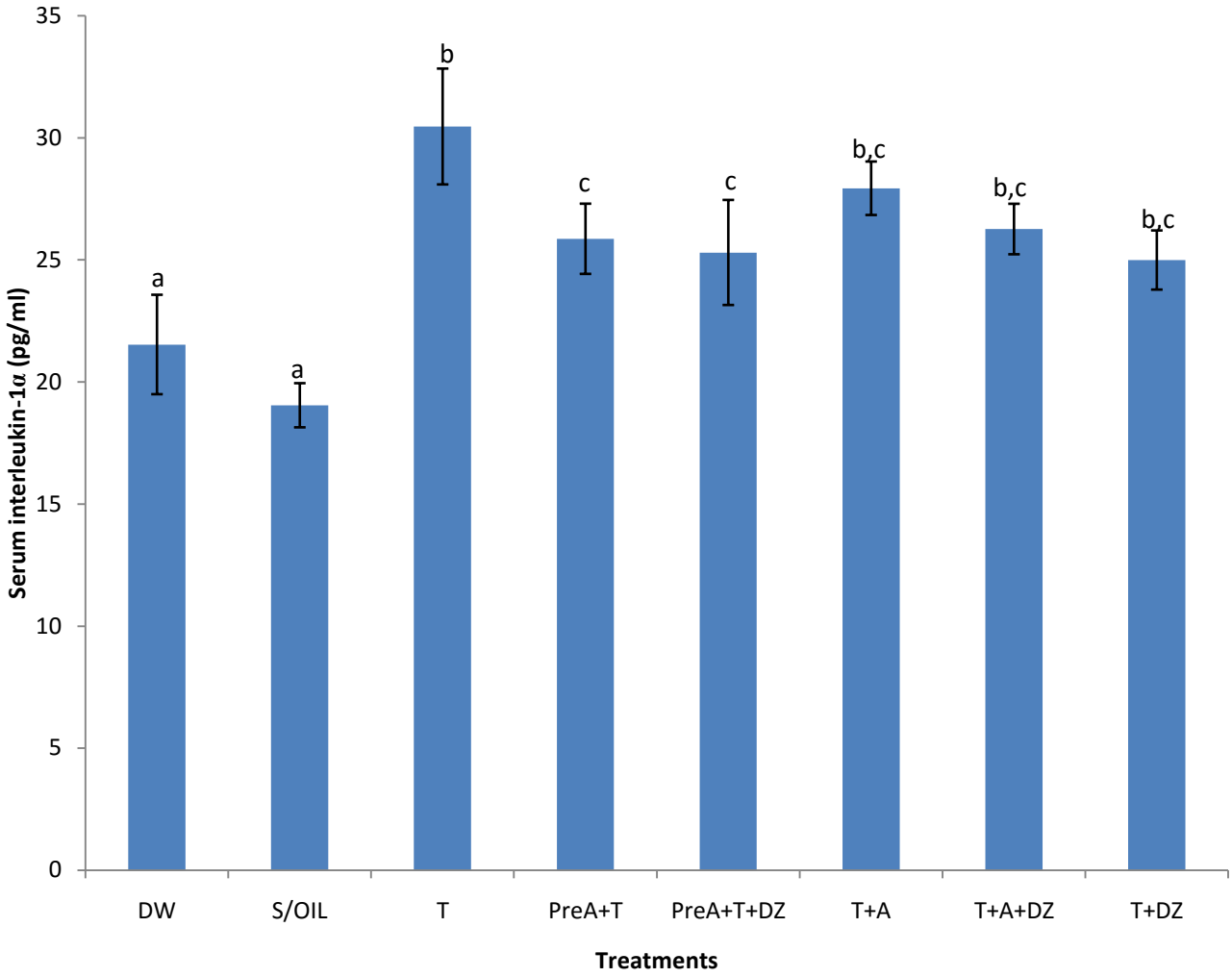

Figure 4.17: Effect of treatment with astaxanthin and diminazene aceturate on the level of IL-1α in Wistar rats experimentally infected with *Trypanosoma brucei brucei*.

[a, b, c] = Means with different or not sharing superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

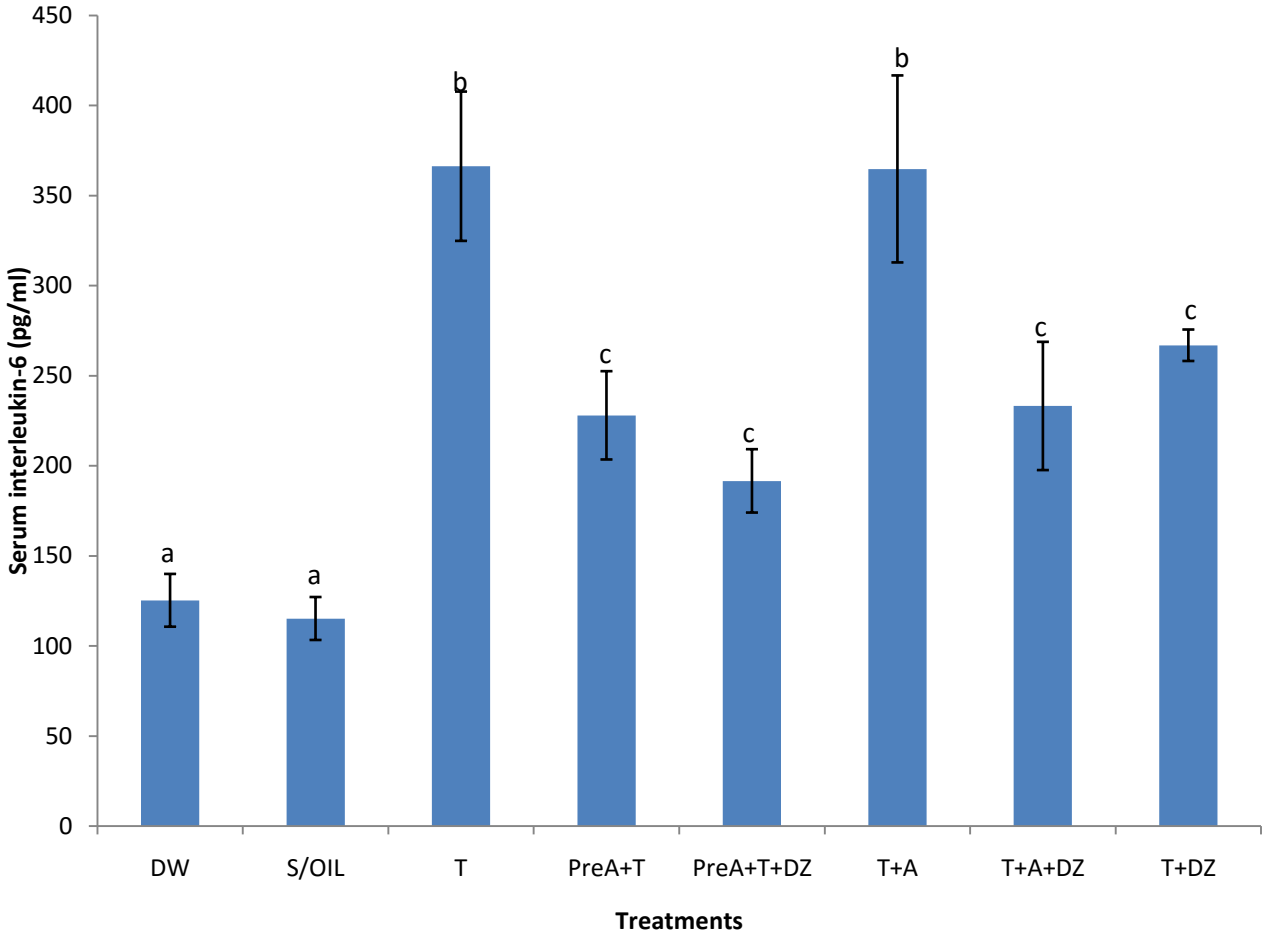

Figure 4.18: Effect of treatment with astaxanthin and diminazene aceturate on the level of IL-6 in Wistar rats experimentally infected with *Trypanosoma brucei brucei*.

[a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

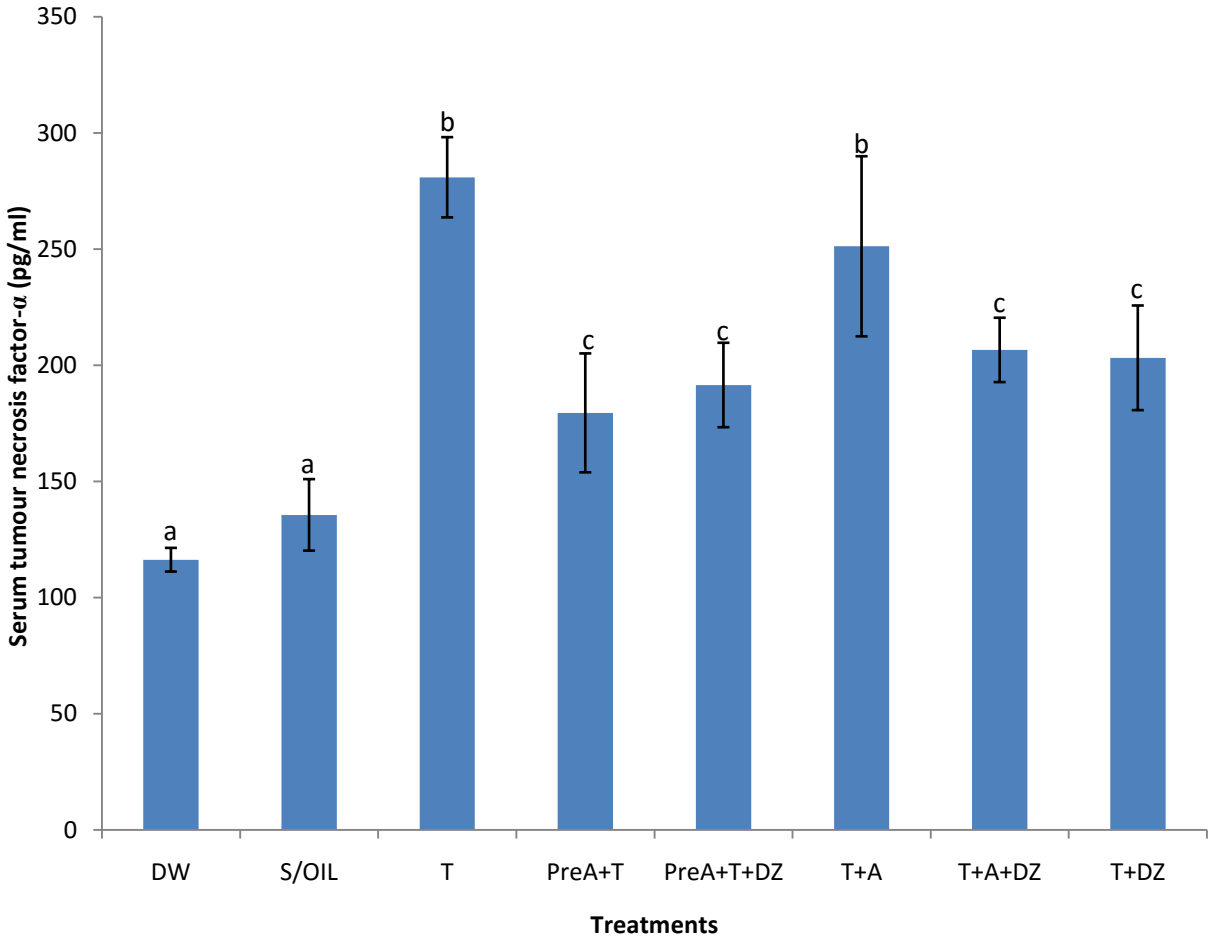

Figure 4.19: Effect of treatment with astaxanthin and diminazene aceturate on the level of TNF-α in Wistar rats experimentally infected with *Trypanosoma brucei brucei*.

[a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different. Values are mean ± SEM of 5 animals per group.

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

4.6 Effect of Treatments on Oxidative Stress Biomarkers

4.6.1 Effect of treatments on tissue 8-isoprostane

The level of 8-isoprostane in the liver of rats in T group rose ($P < 0.05$) compared to the value obtained in PreA+T rats, but did not differ when compared to T+A. Similarly, the level of 8-isoprostane in the liver of PreA+T group was not different from that of PreA+T+DZ group. There was a significant ($P < 0.05$) decrease in 8-isoprostane level in the liver of rats in PreA+T group in comparison to T+A group. The liver level of 8-isoprostane of rats in PreA+T+DZ group was significantly ($P < 0.05$) lower, when compared to that of T+A+DZ rats. There was no difference in the level of 8-isoprostane in the liver of rats in T+A+DZ and T+DZ groups.

The level of 8-isoprostane in the kidneys of rats was significantly ($P < 0.05$) higher in T group than in PreA+T group. There was no difference in 8-isoprostane levels in the kidneys of T and T+A rats, but the level increased ($P < 0.05$) in the kidneys of rats in PreA+T in comparison to that recorded in PreA+T+DZ rats. The kidney of PreA+T rats had a significant decrease ($P < 0.05$) in 8-isoprostane level, when compared to that of T+A rats. Similarly, PreA+T+DZ group had lower ($P < 0.0$) level of 8-isoprostane than T+A+DZ group. The level of 8-isoprostane in the kidneys of rats in T+A+DZ and T+DZ groups were not significantly different.

There was a significant ($P < 0.05$) increase in the level of 8-isoprostane in the heart of rats in T group in comparison to that of PreA+T group. The 8-isoprostane level in the heart of rats in T and T+A groups were not different; and similarly, those of PreA+T and PreA+T+DZ groups did not differ. The heart of rats in PreA+T group showed a significant ($P < 0.05$) decrease in 8-isoprostane level, compared to that of T+A group. The level of 8-isoprostane was lower ($P < 0.05$) in the heart of rats in T+A+DZ group than in T+DZ group (Table 4.2).

Table 4.2:Mean ± SEM of tissue activity of 8-isoprostane (Pg/mL) in the liver, kidney and heart of rats infected with *Trypanosoma brucei brucei* and treated with astaxanthin and Diminazene aceturate.

Liver		Kidney	Heart
Groups			
DW	2540±186.0[a]	3797±105.8[a]	2768±190.7[a]
S/OIL	2401±301.2[a]	3353±232.1[b]	2630±200.5[a]
T	5629±229.6[b]	5854±61.07[c]	5002±468.9[b]
PreA+T	3240±215.3[a]	4922±242.5[d]	4063±432.6[c]
PreA+T+DZ	3579±383.4[a]	4610±98.99[e]	3838±372.3[c]
T+A	5074±144.9[b]	5776±39.08[c]	5347±416.2[b]
T+A+DZ	4633±257.7[c]	5290±190.5[d]	4207±184.2[c]
T+DZ	4553±352.1[c]	5123±117.7[d]	4660±197.0[d]

[a,b,c,d] = Different superscript in a row indicates significant difference between the group mean at ($P < 0.05$).

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

4.6.2 Effect of treatments on tissue superoxide dismutase activity in the heart, lungs and liver

The activity of SOD in the heart of rats in T group was significantly ($P < 0.05$) higher when compared to that of PreA+T group. TheSOD activities in the heart of rats in T and T+A groups were not significantly different. Similarly, the difference in SOD activities in PreA+T and PreA+T+DZ rats was insignificant, but the activity decrease ($P < 0.05$) in the heart of rats in PreA+T group compared to that recorded in T+A group. The SOD activities in the heart of rats in PreA+T+DZ, T+A+DZ and T+DZ groups were not different.

There was a significant ($P <0.05$) increase in SOD activity in the lungs of Trats, when compared tothat ofPreA+Tor T+A rats. The activities of SOD in the lungs of rats in PreA+T and PreA+T+DZ groups were not different. The lungs of PreA+T rats had lower ($P < 0.05$) SOD activity than that of T+A rats. There was no difference in SOD activities in the lungs of PreA+T+DZ, T+A+DZ and T+DZrats.

The SOD activity in the liver of rats in T group was higher ($P < 0.05$) in comparison to PreA+T group.The SOD activities in the liver of rats in T and T+A groups did not differ. Similarly, the difference in SOD activities in the liver of PreA+T and PreA+T+DZrats was insignificant.There was a significant ($P <0.05$) decrease in SOD activity in the liver of rats in PreA+T group compared to that obtained in T+A group. The SOD activities in the liver of PreA+T+DZ, T+A+DZ and T+DZ rats were not different (Table4.3).

Table 4.3:Mean ± SEM of tissue activity of superoxide dismutase(IU/L) in the heart, lungs, and liver of rats infected with *Trypanosoma brucei brucei* and treated with astaxanthin and diminazene aceturate

Heart		Lungs	Liver
GROUPS			
DW	0.3692 ± 0.13^{a}	2.187 ± 0.04^{a}	0.2582 ± 0.02^{a}
S/OIL	0.4492 ± 0.21^{a}	2.124 ± 0.04^{a}	0.3035 ± 0.05^{a}
T	1.711 ± 0.22^{b}	2.904 ± 0.09^{b}	1.842 ± 0.29^{b}
PreA+T	1.352 ± 0.07^{c}	2.543 ± 0.07^{c}	1.170 ± 0.04^{c}
PreA+T+DZ	1.347 ± 0.12^{c}	2.552 ± 0.03^{c}	1.056 ± 0.07^{c}
T+A	1.778 ± 0.11^{b}	2.667 ± 0.02^{d}	$1.529 \pm 0.08^{b,d}$
T+A+DZ	1.486 ± 0.19^{c}	2.612 ± 0.10^{c}	1.102 ± 0.14^{c}
T+DZ	1.543 ± 0.49^{c}	2.641 ± 0.07^{c}	$1.301 \pm 0.92^{c,d}$

^{a,b,c,d} = Different or unshared superscript in a row indicates significant difference between the group mean at ($P < 0.05$).

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

4.6.3 Effect of treatments on tissue glutathione peroxidase activity in heart, lungs and liver

The activity of GPx was higher ($P < 0.05$) in the heart of rats in T group than in PreA+T group. Theactivities of GPx in the heart of rats in groups T and T+A were not different. Similarly, those of groups PreA+T and PreA+T+DZ did not differ. The GPx activity was lower ($P < 0.05$) in the heart of rats in PreA+T groupthan in T+A group. There were no differences in GPx activities in the heart of rats in PreA+T+DZ, T+A+DZ and T+DZ groups.

In the lungs, the GPx activity in T group was higher ($P < 0.05$) compared to that of PreA+T group. The GPx activities in the lungs of rats in T and T+A groups did not differ significantly. Similarly, the activities of GPx in the lungs of rats in PreA+T and PreA+T+DZ groups were not significantly different. The GPx activitywas significantly ($P < 0.05$) lower in the lungs of rats in PreA+T group thanthat of T+A group.In the lungs, GPx activity in PreA+T+DZ, T+A+DZ and T+DZ groups did not differ.

There was a significant ($P < 0.05$) increase in GPx activity in the liver of T rats in comparison to that of PreA+T rats. The GPx activity in the liver of T rats was not different from the activities obtained in T+A rats. The liver of PreA+T rats had significant ($P < 0.05$) increase in GPx activity, when compared to that of PreA+T+DZ rats. There was a significant ($P < 0.05$) decrease in GPx activity in the liver of PreA+T group compared to that obtained in T+A group. The GPx activities in PreA+T+DZ and T+A+DZ rats were not significantly different. The GPx activity in T+A+DZ rats was lower ($P < 0.05$) in comparison to the activity recorded in T+DZ rats (Table 4.4).

Table 4.4:Mean ± SEM of tissue glutathione peroxidase(IU/L) activity in the heart, lungs, and liver of rats infected with *Trypanosoma brucei brucei* and treated with astaxanthin and diminazene aceturate

Heart		Lungs	Liver
Groups			
DW	15.73 ± 2.31[a]	18.46 ± 1.27[a]	16.18 ± 1.44[a]
S/OIL	13.84 ± 1.60[a]	18.76 ± 2.17[a]	14.19 ± 1.28[a]
T	44.07 ± 3.40[b]	50.25 ± 6.07[b]	36.89 ± 2.24[b]
PreA+T	30.60 ± 1.61[c]	34.11 ± 3.97[c]	30.48 ± 1.39[c]
PreA+T+DZ	27.14 ± 5.21[c]	32.35 ± 2.48[c]	27.66 ± 1.91[d]
T+A	43.84 ± 5.92[b]	49.91 ± 1.95[b]	35.08 ± 2.11[b]
T+A+DZ	31.64 ± 3.94[c]	35.93 ± 3.76[c]	28.52 ± 3.03[d]
T+DZ	35.51 ± 2.91[b,c]	36.83 ± 5.96[c]	33.79 ± 1.45[c]

[a,b,c,d] = Differentor unshared superscript in a column indicates significant difference between the group mean at ($P < 0.05$).

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

4.6.4 Effect of treatments on catalase activity

The activity of CAT in the heart of rats in T group was not different when compared respectively to that ofPreA+Tor T+A group. There was a significant ($P < 0.05$) decrease in CAT activity in the heart of rats in PreA+T+DZ group in comparison to PreA+T or T+A+DZ group. The activities of CAT in PreA+T, T+A, T+A+DZ and T+DZ groups did not differ.

There was a significant ($P < 0.05$) increase in CAT activity in the lungs of rats in the T group compared to the corresponding activity recorded inPreA+Tor T+A rats. The lungs of rats in PreA+Tand PreA+T+DZ groupsshowed no difference in CAT activities. The CAT activity in the lungs of rats in PreA+T group was significantly ($P < 0.05$) lower when compared to the corresponding value in T+A group. The activity obtained in PreA+T+DZ group was lower ($P < 0.05$) than that of T+A+DZ group. There was no difference in CAT activities in the lungs of rats in T+A+DZ and T+DZ groups.

The activity of CAT in the liver of T group was significantly ($P < 0.05$) higher, when compared to that of PreA+T group. Similarly, CAT activity in PreA+T group was higher ($P < 0.05$) than that of PreA+T+DZ group. TheCAT activity in the liver of rats in T and T+A groups did not differ. There was a significant ($P <0.05$) decrease in CAT activity in the liver of rats in PreA+T group, whencompared to that of T+A group.The liver of rats in PreA+T+DZ group had lower ($P < 0.05$) CAT activity than that of T+A+DZ group. The CAT activities in the liver of rats in T+A+DZ and T+DZ groups were not significantly different(Table 4.5).

Table 4.5:Mean ± SEM of catalase(IU/L) activity in the heart, lungs, and liver of rats infected with *Trypanosoma brucei brucei* and treated with astaxanthin and diminazene aceturate

Heart		Lungs	Liver
Groups			
DW	9.762±0.89[a]	25.18±1.49[a]	9.757±0.49[a]
S/OIL	11.11±0.99[a]	24.44±1.27[a]	9.041±0.21[b]
T	30.86±3.37[b]	50.78±3.82[b]	12.09±0.95[c]
PreA+T	26.53±3.41[b]	39.21±3.45[c]	10.01±0.41[d]
PreA+T+DZ	20.83±1.62[c]	39.68±2.02[c]	8.637±0.79[a,b]
T+A	30.97±3.13[b]	44.29±1.74[d]	12.91±1.08[c]
T+A+DZ	26.46±3.21[b]	47.52±5.04[b,d]	10.02±0.75[d]
T+DZ	26.39±3.32[b]	47.50±1.30[b,d]	10.37±0.84[d]

[a,b,c,d] = Different or unshared superscript in a column indicates significant difference between the group mean at ($P < 0.05$).

Keys: DW = Distilled water, S/OIL = Soya oil, T = *Trypanosoma brucei brucei*, PreA+T = Pretreated with astaxanthin and infected with *Trypanosoma brucei brucei*, PreA+T+DZ = Pretreated with astaxanthin, infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate, T+A = Infected with *Trypanosoma brucei brucei* and treated with astaxanthin, T+A+DZ = infected with *Trypanosoma brucei brucei*, treated with astaxanthin and diminazene aceturate, T+DZ = Infected with *Trypanosoma brucei brucei* and treated with diminazene aceturate.

CHAPTER FIVE

5.0 DISCUSSION

Anorexia, pale ocular mucous membrane, weakness, rough hair coat, and weight loss were the clinical signs of trypanosomosis observed in rats in this study, which were most obvious in the T and T+A groups. The clinical signs observed in this study were similar to those reported in mice, dogs and rabbits infected with *T. brucei brucei* (Anene *et al*., 1999; Abenga *et al*.,2005; Chekwube *et al*., 2014), and in cattle infected with *Trypanosoma congolense* (Mbaya *et al*., 2007).Although variable disorders occur sequel to trypanosome infection in animals (Adamu *et al*., 2009a), depending on the virulence of the infecting trypanosome, the infective dose and the immune status of the host, the symptoms usually associated with trypanosomosis include: pallor of the mucous membranes, enlargement of lymph nodes, anorexia and emaciation (Shimelis *et al*., 2015). Following treatment, the clinical signs gradually disappeared,demonstrating that diminazene aceturate and the combination treatments were effective in reversing the signs.

The significant body weight gain observed in the groups treated with astaxanthin and/or diminazene aceturate may be due to the less severity of the clinical signs observed in those groups, which could be attributed to the important role played by astaxanthin in regulating immunity and disease aetiology (Park *et al*., 2010; Kuan-Hung*et al*., 2016), and the trypanocidal action of the diminazene aceturate. However, rats in T and T+A groups showed no difference in body weight at week 4 when compared to week 1, and this finding may be attributed to the acute nature of the infection. Reduced feed intake and impaired efficiency of feed conversion, fever (in association with increased heat production), increased metabolisation energy and reduction in the proportion of protein used for growth and the increased synthesis of protein at the expense of

muscle protein catabolism have all been suggested as the cause of weight loss in trypanosome - infected animals (Eghianruwa, 2012).

In this study, pre-treatment with astaxanthin and in combination with diminazene aceturate led to higher survivabilityof the rats compared to those that were infected and not treated, or infected before treatment with astaxanthin. This observation agrees with the results of Eze *et al*. (2008), who reported a higher survival rate inrats treated with a diminazene/selenium combination than those groups that had selenium and vitamin E supplements alone. The pathogenesis of trypanosomosis is associated with severe inflammation and production of radicals such as nitric oxide, which affect adversely the survival of the host (Mbuthia *et al*., 2011). This production of nitric oxide is a disease-exacerbating factor; and in murine trypanosomosis, it causes damage to lymphocyte function of the host (MacLean *et al*., 2001; Tedeschi *et al*., 2004). The prolonged survivability of the rats pre-treated with astaxanthin and/or diminaze aceturate may be due to the antioxidant activity of the astaxanthin, which ameliorated the inflammatory reactions and the damages caused by the generation of ROS during the course of the infection. The decrease in survival rate of rats infected and not treated may be attributed to the enormous increase in the number of circulating trypanosomes and their by-products, causing haemolysis of the red blood cells (Yakubu *et al*., 2014). The survivability of rats in the astaxanthin post-treated group was not different from that of the infected untreated,indicating that astaxanthin was better asa prophylactic than curative agent.

Experimental infection of rats with *Trypanosoma brucei brucei* was successful. Parasites were observed in the blood of infected rats from day 2 post-infection. By day 4 post-infection, all the rats in the groups infected with *Trypanosoma brucei brucei*showed parasitaemia. The prepatent

period of 2-4 days disagrees with the findings of Chekwube *et al*. (2014) in rats and Kobo *et al*. (2015) in mice, who reported a prepatent period of 5 and 4 days, respectively. The pre-patent period of 2-4 days observed in this study may be related to the level of virulence and the number of parasites inoculated. It has been reported that the number of parasites inoculated can influence the pre-patent period (Egbe-Nwiyi *et al*., 2017). Pre-treatment with astaxanthin did not affect the onset of parasitaemiaand was unable to clear the parasites from blood, but led to a decrease in the level of parasitaemia. This finding agrees with the earlier reportsof Kobo *et al*. (2015) in rats infected with *Trypanosoma brucei brucei* and treated with Daflon® 500 mg (an antioxidant) and Eze *et al*. (2015a) in *Trypanosoma brucei*-infected rats supplemented with dietary zinc that exhibits antioxidant activity. Astaxanthin has been reported to influence host immune system and disease aetiology (Park *et al*., 1998, 2010; Kuan-Hung*et al*., 2016), thus altering the susceptibility of the host to infectious diseases. Parasitaemia in susceptible animals may be influenced by the number of parasites inoculated, nutritional stress, intercurrent infections, host immune competence and pathogenicity of the strain of *T. brucei brucei*.

All the groups treated with diminazene aceturate alone or in combination with astaxanthin became aparasitaemic 48 hours post- treatment;an exception was the group post-treated with astaxanthin and diminazene aceturate, where there was resurgence of parasitaemia in 10% (that is one out of three) of the rats at day 7 post-treatment and the rat died a day after the resurgence of parasitaemia was observed. This corroborates the report of Ezeh *et al*. (2009, 2016), who observed relapse infection after treatment with diminazene aceturate. Reports of relapse of *T. b. brucei* infection have been attributed to the presence of *T. b. brucei* in drug inaccessible sites such as the brain; and this often occurs when there is a prolonged period between infection and

treatment (Eke *et al*., 2017) such that the parasite migrates to brain and after treatment recede from the brain to the blood stream (Eze *et al*., 2019). However, in the present study, the treatment was started on day 4 post-infection, when parasites were 3 or 4 per microscopic field, suggesting that the parasites may not have invaded the brain as treatment was started immediately. Therefore, the resurgence of parasitaemia may not be as a result of the parasites resurfacing into the blood stream from the brain but may be attributed to other factors such as mutation, amplification or deletion, altered drug uptake, drug metabolism, drug target interaction or efflux (Eze *et al*., 2019). The preponderance of fake and adulterated drugs may also lead to emergence of drug-resistant trypanosomes (Ezeokonkwo *et al*., 2007).

Measurement of anaemia gives an indication of severity of the disease (Adeyemi *et al*., 2010). The significant decrease in the levels of RBC, PCV and Hb concentration observed in the infected untreated rats agrees with the findings of Chekwube *et al*. (2014), Kobo *et al*. (2015), Karaye *et al*. (2017) and Oparah *et al*. (2017),which may be attributed to the trypanosome-induced disruption of RBC membrane (Akanji *et al*., 2009). This could have resulted in subsequent haemolysis as reflected in the low RBC count. Acute haemolysis has been demonstrated as a cardinal feature in African trypanosomosis (Adamu *et al*., 2009a; Chekwube *et al*., 2014; Kobo *et al*., 2015). The decrease in PCV may also be attributed to the infection inducing low concentration of reduced glutathione on the membrane surface of the RBC, thus rendering the membrane liable to oxidative lysis, secondary to the metabolic activities of the proliferating trypanosomes. Low level of reduced glutathione has been reported to predispose RBCs to oxidative damage (Akanji *et al*., 2009). Oxidative cell damage is a prominent feature in *T. brucei brucei* infections (Omer *et al*., 2007; Saleh *et al*., 2009; Kobo *et al*., 2015). Other

factors have also been reported to be responsible for the reductions in RBC, PCV and Hb concentration in trypanosome-infected animals. They include depression of erythropoiesis, immunological mechanisms and erythrophagocytosis, increased plasma volume, haemodilution, and disorders of coagulation (Chekwube *et al*., 2014).

The RBC, PCV and Hb concentration of astaxanthin-pretreated group alone and astaxanthin pretreated and administered with diminazene aceturate group remained significantly higher than the infected untreated group. This result is consistent with the report of Kobo *et al*. (2015) who observed that the pretreatment of rats with a flavonoid mixture (Daflon® 500 mg) kept the RBC, PCV and Hb concentration higher than in the infected untreated rats. This finding could be attributed to the beneficial effect of astaxanthin as an antioxidant and immunostimulant that resulted in the protection of the integrity of RBCs, thereby preventing the cells from oxidative haemolysis. The group pretreated with astaxanthin and administered with diminazene aceturate had the highest RBC, PCV and Hb concentration, when compared to other treatment groups. This result may be attributed to the antioxidant effect of astaxanthin and the trypanocidal effect of diminazene aceturate that eliminated the parasites from peripheral circulation, thereby protecting the RBCs from destruction by the lashing action of the trypanosome flagella (Oparah *et al*., 2017). The RBC, PCV and Hb values in the astaxanthin post-treated group alone were not different from that of the infected untreated group, indicating that astaxanthin was better as a prophylactic than curative agent.

Increased MCV of RBCs (macrocytosis) in the infected untreated group corroborates the findings of Abenga *et al*. (2005) in *T. congolense* infection of Nigerian puppies, butcontrastswith the report of Kobo *et al*. (2015) in rats infected with *T. brucei brucei*. Increase in MCV and

MCH are typically seen in cases of haemolytic anaemias. Macrocytosis in African trypanosomosis usually arises from increased erythropoiesis in the bone marrow, with resultant release of macrocytic immature red cells into circulation, known to occur during the acute phase of the anaemia (Abenga *et al*., 2005; Oparah *et al*., 2017). The increase in the value of MCH recorded in the infected untreated group was probably due to free circulating haemoglobin arising from the haemolysis (Igbokwe and Anosa, 1989). The MCHC is a more reliable predictor in the classification of anaemia. The increase in MCHC suggests a qualitative bone marrow output during the acute stage of infection. The significant decreases in the MCV and MCH recorded in the group pre-treated with astaxanthin alone and its combination with diminazene aceturate suggests that astaxanthin reduced the anaemic effect of *T. brucei brucei* in the infected rats.

The decrease observed in platelet count in the infected untreated group corroborates the findings of Ezebuiro *et al*. (2012) in rabbits, Kobo *et al*. (2015) in rats and Oparah *et al*. (2017) in donkeys. Thrombocytopaenia could be the result of damage to platelets due to haemorrhage, vasoconstriction or tissue damage observed in trypanosomosis (Davis, 1982). Low platelet count may also be due to increased splenic sequestration of platelets, bone marrow suppression, resulting in a decrease in platelet production, or an increased destruction of the platelets due to disseminated intravascular coagulation reaction (Kobo *et al*., 2015; Oparah *et al*., 2017). In addition, the thrombocytopaenia recorded in the infected untreated group may also be due to oxidative damage to the platelet membranes, provoked by the trypanosome parasite. This may result in the formation of lipid peroxides within the platelet membranes, thereby causing cellular lysis (Kobo *et al*., 2015). The result of post-treatment with astaxanthin was not different from that of the infected untreated group. This finding shows that, post-treatment with astaxanthin

alone did not prevent the platelet membrane from oxidative damage caused by the parasite. Pre-treatment with astaxanthin and diminazene aceturate significantly increased the platelet count better than pre-treatment with astaxanthin alone, post-treatment with astaxanthin and diminazene aceturate, and diminazene aceturate alone. This result may be due to the immune-stimulatory and antioxidant effects of astaxanthin that protected the platelet membrane from oxidative damage via the scavenging of ROS evoked by the parasite.

The significant increase in total leucocyte count observed in this study agrees with the reports of Ukpai and Nwabuko (2014) and Abenga *et al*. (2017) in *T. brucei brucei*-infected rats and pigs, respectively, but disagrees with the findings of Kobo *et al*. (2015) in *T. brucei brucei*-infected rats, and El-Ashmawy *et al*. (2016) in *T. evansi* infected rats. The increase in WBC count in the infected untreated rats was an indication of infection. It may be attributed to the ability of the body to employ its immune arsenals to combat the invading parasites, and in the process of immune response to enhance more WBC production (Ukpai and Nwabuko, 2014). Leucocytosis which may be due to increase in lymphocyte, neutrophil, monocytes or eosinophil counts, has been implicated in trypanosomosis (Ezebuiro *et al*., 2012). It is believed to be part of an immunological response influenced by the ever-changing variable surface glycoprotein of the infecting trypanosomes (Abenga *et al*., 2017; Oparah *et al*., 2017). Lymphocytosis could also result from the host's inflammatory response due to the presence of the infecting organisms (Satue *et al*., 2014). Thisreport supports the findings in this study where there was an increase in lymphocyte and neutrophil counts in the infected groups, when compared to the uninfected control groups. Treatment of infected rats with astaxanthin and/or diminazene aceturate caused a decrease in WBC, lymphocyte and neutrophil counts, when compared to the infected untreated group. This may be due to the finding that astaxanthin,being a potent antioxidant with anti-

inflammatory activity (Liu and Lee, 2003; Ambati *et al*., 2010),was able to counter the host's inflammatory response induced by the parasite, thereby maintaining the WBC, lymphocyte and neutrophil counts at lower levels. The group pre-treated with astaxanthin and administered with diminazene aceturate had lower WBC, lymphocyte, and neutrophil counts when compared to the group that was pre-treated with astaxanthin alone before the infection. This result may be due to the combined anti-inflammatory and trypanocidal effects of astaxanthin and diminazene aceturate. In addition, the group that was post-treated with astaxanthin alone had WBC, lymphocyte and neutrophil counts that were not different from those of the infected untreated group, demonstrating that pre-treatment with astaxanthin was better at stimulating the immune system than the post-treatment.

Erythrocyte osmotic fragility which measures the extent of resistance of erythrocyte to intracellular pressure has been used as an indicator of oxidative stress (Kobo *et al*., 2014; Pati *et al*, 2017; Yusuf *et al*., 2018). The extent of osmotic stress is dependent upon volume, surface area, and functional integrity of membrane of the erythrocytes (Islah *et al*., 2016). Therefore, erythrocyte osmotic fragility is frequently employed for the diagnosis of haemolytic anaemia and oxidative damage due to large scale destruction of RBCs (Hanzawa and Watanabe, 2000). Haemolytic anaemia has been reported to be a consistent finding in different blood protozoan diseases including trypanosomosis. In this study, infection of rats with *T. brucei brucei* induced a significant increase in erythrocyte osmotic fragility in the infected untreated group. The result is consistent with earlier reports in *T. brucei brucei* infected rats (Mijares *et al*., 2009; Kobo *et al*., 2014) that showed increase in osmotic fragility. The increase may be ascribed to the increase in the generation of ROSresulting in oxidative damage, caused by *T. brucei brucei* infection. Umar *et al*. (2007) reported that infection with *T. brucei brucei* may alter the host's antioxidant defence

against ROS, resulting in over-production of ROS or depletion of antioxidants. The ROS capture electrons from the phospholipids in the erythrocyte membrane and, thus, damage the cell. Erythrocyte membrane damage has been postulated to be associated with adhesion of erythrocytes and reticulocytes to the trypanosome surface via sialic acid receptors (Esievo *et al*., 1982; Dagnachew *et al*., 2015). The parasite utilises sialoglycoprotein of RBC membrane for its multiplication and differentiation (Briones *et al*., 1994).

According to the above mentioned-mechanism, the erythrocyte becomes extremely vulnerable to oxidative stress resulting in loss of its membrane integrity and subsequent lysis. This study revealed that the erythrocytes of the infected untreated group were more susceptible to haemolysis, suggesting that the erythrocytes had undergone some deleterious changes in their surface membrane, which increased susceptibility to haemolysis. As observed in this study, pre-treatment with astaxanthin protected erythrocyte membrane from oxidative haemolysis. Astaxanthin provides cell membranes withpotent protection against ROS or otheroxidative attack.The carotenoids have been reported tooffer the best protection against ROS in rats (Ambati *et al*., 2010, 2013a). Astaxanthin shows the strongest singlet oxygen ($^1O_2$) quenching activity (Nishida *et al*., 2007), and more potent than α-tocopherol as hydroxyl radical scavenger (Hama *et al*., 2012). The result of the study suggests that astaxanthin exerted antioxidant effect by scavenging the ROS produced by the trypanosomes, preserving the integrity of the erythrocyte membrane, thus, protecting RBCs against haemolysis. The group pretreated with astaxanthin before the administration of diminazene aceturate had significantly lower percentage of haemolysis, compared to the group pretreated with astaxanthin alone.This finding suggeststhat

pre-treatment with astaxanthin and diminazene aceturate exhibited better protecting activity of the erythrocyte membrane against haemolysis.

Biochemical evaluation of body fluids gives an indication of the functional stateof various body organs, and biochemical changes in body fluids that result from infections depend on the species of the parasite and its virulence (Awobode, 2006; Abuessailla *et al*., 2017). The AST and ALT are biomarkers of hepatic integrity and to a certain level may be used to assess the extent of hepatocellular damage. The ALT activity, however, gives more valuable information relevant to the integrity of the hepatocytes than AST (Bashir *et al*., 2015; Uraku, 2017). Raised ALP activity occurs in inflammatory conditions of the gut and liver (Wurochekke *et al*., 2008; Oyewole and Malomo, 2009). The study showed that infection with *T. brucei brucei* significantly increased AST, ALT and ALP activities in the infected untreated and astaxanthin post-treated and administered with diminazene aceturate groups, when compared to other treatment groups. This finding agrees with the report of Do Carmo *et al*. (2015) and Shittu *et al*. (2017) in *T. brucei brucei*-infected rats. Increases in serum ALT and AST activities, urea and creatinine concentrations have been reported in experimental trypanosomosis (Kalu *et al*., 1989; Adah *et al*., 1992) and are indications of damage to hepatic and renal tissues (Kaplan *et al*., 1988). Therefore, the increases observed in the present study may be due to the damage to tissues caused by trypanosome infections. The parasites may also release the enzymes as metabolites into the blood circulation. Homogenates and suspensions of trypanosomes have been reported to show activity for these enzymes (Nwoha *et al*., 2013).

In the group pretreated with astaxanthin and administered with diminazene aceturate, there was considerable prevention of the disease-induced increases in serum AST, ALT and ALP, compared to other treatment groups. This could be due to the combined effects of astaxanthin and diminazene aceturate. The antioxidant property of astaxanthin, apparently, protected the liver from the ROS generated by trypanosome infection, thereby providing greater protection to the cells against oxidative damage. The trypanocidal property of diminazene aceturate may have cleared the parasites fromcirculation and consequently, reducedthe damaging effect of the parasite on the tissues. The study has shown, for the first time, that pretreatment with astaxanthin was better at ameliorating the organ damage caused by the trypanosome parasite than post-treatment, indicating that pretreatment offers better antioxidant protection against ROS generated during trypanosome infection.

The findings of this study indicate that *T. brucei brucei* infection of experimental rats resulted in increased serum cholesterol, triglycerides and HDL, thus agreeing with earlier findings by Ngure *et al*. (2008) and Waema *et al*. (2013) in Rhodesian monkey model of HAT, but contradicts the report of Adamu *et al*. (2008, 2009b) in *T. congolense* infected sheep and *T. brucei* infected pigs, respectively. It has been observed that protozoan parasites, including trypanosomes depend on their hosts for energy and nutrients required for growth, motility and reproduction (Bala *et al*., 2011). The role of lipids in the pathogenesis of trypanosomosis has been reported (Tizard *et al*., 1978). There is evidence suggesting that parasites can take up the lipids and cholesterol they need from lipoproteins present in the host body, which may lead to alterations in serum concentrations of such metabolites (Gillet and Owen, 1992). Several studies have also indicated that changes in the intracellular redox balance may modify lipid metabolism. Indeed, oxidative

stress has been associated with accumulation in adipose tissue (Fujita *et al*., 2006) and it affects the regulation of hepatic lipid synthesis (Napolitano *et al*., 2001). Abnormalities of lipid metabolism have been identified in several laboratory and domestic animals infected with various species of trypanosomes (Eze *et al*., 2015b). Inconsistent changes in cholesterol levels following trypanosome infection have been observed as a rise in rabbits inoculated with *T. brucei* (Ngure *et al*., 2008), a slight decrease in rats infected with *T. b. rhodesiense* (Dixon, 1967), and a decrease in ruminants infected with *Trypanosoma congolense* and *Trypanosoma vivax* (Roberts, 1975). Changes in cholesterol, therefore, appear to depend on the infecting trypanosome species and the host. The alterations in triglycerides and HDL could be due to the acute phase responses during early infection (Gaithuma *et al*., 2011). The TNF produced during acute infection has been shown to inhibit adipose tissue enzyme lipoprotein lipase that is responsible for clearing lipids from plasma (Khovidhunkit *et al*., 2004; Waema *et al*., 2013). Marked increase in pro-inflammatory cytokines including TNF obtained in this study may be responsible for the observed changes.

The increase in serum triglycerides concentrations may also be due to reduced serum concentration of albumin. Albumin (reduced plasma concentration of which leads to reduced total serum proteins) is required to bind to neutral fats (triglycerides and cholesterol), for these lipids to be transported in the plasma. This is because lipids are hydrophobic in nature and, therefore, require some form of hydrophilic adaptation in the form of lipoproteins. However, infection leads to a fall in serum albumin due to decreased synthesis of albumin and/or increase catabolism of albumin, consequently causing reduction in the binding capacity and leading to increased plasma free concentration of these analytes (Bala *et al*., 2011). The groups treated with

astaxanthin alone and/or diminazene aceturate had lower values of cholesterol, triglyceride and HDL when compared to the infected untreated group. An exception was the group post-treated with astaxanthin alone, where the levels of cholesterol, triglyceride and HDL were not different from the infected untreated group. The decreases observed in those groups may be attributed to the combined effects of astaxanthin and diminazene aceturate. The anti-oxidative, anti-inflammatory and dyslipidaemic effects of astaxanthin have been reported (Hussein *et al*., 2007; Ikeuchi *et al*., 2007), this may be responsible for the decreases observed in this study. Increased levels of LDL receptor, 3-hydroxy-3-methylglutaryl CoA reductase and sterol regulatory element-binding protein 2 in the liver may be involved in the hypocholesterolaemic effect of astaxanthin (Yang *et al*., 2011). Astaxanthin increases the expressions of carnitine palmitoyl transferase 1, acetyl-CoA carboxylase β and acyl-CoA oxidase mRNA, suggesting that the triglyceride-lowering effect of astaxanthin may be due to increased fatty acid β-oxidation in the liver (Yang *et al*., 2011). The results show that the group pre-treated with astaxanthin and administered with diminazene aceturate maintained the concentrations of cholesterol, triglyceride and HDL at lower level, when compared to other groups, showing that astaxanthin may be better as a prophylactic than therapeutic agent.

In this study, a significant increase in IgG level was recorded in all the infected groups compared to DW and S/oil groups. The finding agrees with the reports of Lejon *et al*. (2003) in calves experimentally infected with *Trypanosoma congolense* and Isaac *et al*. (2010) in humans naturally infected with *Trypanosoma brucei gambiense* who reported increases in serum IgG level. Increase in IgG and IgM levels during trypanosome infection have been implicated in the disease pathogenesis (Kobayashi *et al*., 1976). Pre-treatment with astaxanthin significantly

increased IgG level when compared to other treatment groups. The current result may be attributed to the immune-modulating effect of astaxanthin that led to the increase in IgG recorded in the groups. Treatment with diminazene aceturate decreased the level of IgG, which may be attributed to the trypanocidal effect of diminazene aceturate, and this is in agreement with the findings of Luckins (1976) in cattle infected with *Trypanosoma congolense* and Whittle *et al*. (1977) in humans infected with *Trypanosoma brucei gambiense*, who recorded decreases in IgG levels after treatment with diminazene aceturate.

In this study, the increase in the cytokine (IL-1α, IL-6 and TNF-α) levels was concomitant and directly correlated with parasitaemia and development of anaemia as observed in the groups infected with *Trypanosoma brucei brucei*which corroborates with the report of Paim *et al*. (2011). Trypanosome-derived products have been shown to activate the generation of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α, playing an important role in the replication process of the parasite as well as in the host immune response (Magez *et al*., 2007; Paim *et al*., 2011). Cytokines exhibit suppressor activity on erythropoiesis (Paim *et al*., 2011) and are probably central players in anaemia associated with inflammation (Noyes *et al*., 2001; Musaya *et al.*, 2015). The cytokines are reported to inhibit haematopoiesis and may be associated with the chronic disease-related anaemia. An increase in the levels of these cytokines may lead to haematopoiesis suppression and erythrocyte degradation inducing a reduction in PCV (Taniguchi *et al*., 1997). Faquin *et al*. (1992) reported an inhibition of hypoxia-induced erythropoietin secretion in the presence of high levels of TNF-α, IL-1α and β. The increase in the level of cytokines may be related to the inflammatory response and the parasitaemia control, occurring in the infected rats (Sileghem *et al*., 1989; Gao and Pereira, 2002).

Titus *et al*. (1991) reported that the first host response to protozoan infection is the secretion of several cytokines, including TNF-α, IL-1 and IL-6. The combined action of these cytokines leads to leukocytosis, fever and acute phase protein production. These initial responses provide an important contribution to the course of infection by regulation of the immune response to the parasite. The finding of increase in serum TNF-α concentration supports the report of Okomo-Assoumon*et al*. (1995) that the levels of serum TNF-α of *T. b. gambiense*-infected patients correlates with disease severity. *In vitro* study suggests that components of glycosyl phosphatidyl inositol (GPI)-anchored trypanosome variant surface glycoprotein (VSG) triggers macrophages (Magez *et al*., 1998; Paulnock and Coller, 2001) to produce TNF-α (Daulouede *et al*., 2001). The TNF-α has been suggested to be involved in *T.b. gambiense* growth control in the face of increase in the trypanosome number and lifespan, when anti-TNF-α was introduced into cultures of macrophages and trypanosome parasite (Daulouede *et al*., 2001). It is therefore, hypothesised that the greater the severity of HAT infection, the more GPI-anchored trypanosome VSG in blood circulation and, hence, greater production of TNF-α. This suggests that the elevated serum TNF-α level in trypanosome infection may implicate the cytokine in the immunopathogenesis of the disease.

The IL-1 is a potent mediator cytokine in the pathogenesis of trypanosomosis. An increase in the level of IL-Iα was obtained in the infected untreated group and this corroborates the findings of Sileghem *et al*. (1989) who detected high level of IL-1during the acute phase of the disease in rats experimentally infected with *T. brucei*. The IL-1 induces the production of acute phase proteins in the liver (Paim *et al*., 2011) and stimulates the lymphocyte-mediated immune response (Reed *et al*., 1989),which may also be responsible for the lymphocytosis observed in

the present study. The increase in serum level of IL-6 observed in this study could represent the host immune response to infection. Increase in IL-6 has been reported in *T. evansi* (Paim *et al*., 2011) and *T. brucei* (Sternberg *et al*., 2005) infection of rats. In mice infected with *T. cruzi*, those deficient in IL-6 were more susceptible to infection and presented high parasitaemia with increased mortality rates (Gao and Pereira, 2002).

Pretreatment with astaxanthin and/or diminazene aceturate was able to keep the levels of thecytokines lower than in the infected untreated groups and in the group post-treated with astaxanthin and administereddiminazene aceturate. This finding may be because the pretreatment with astaxanthin and/or diminazene aceturate reduced the severity of infection in these groups as evident by the lower level of parasitaemia, lymphocytosis and higher PCV. The concentrations of the cytokines in astaxanthin pretreated groups were relatively lower than those of astaxanthin post-treated group and administered diminazene aceturate, or those administered diminazene aceturate alone. This could be due to the antioxidant and immune- modulating effects of astaxanthin in reducing the disease severity. The trypanocidal effect of diminazene aceturate also eliminated the parasite from circulation, thus further reducing the severity of the disease.

Lipid peroxidation is one of the most common deleterious effects associated with oxidative stress, and the measurement of lipid peroxidative products has been used to evaluate oxidative stress in *in vivo* conditions (Halliwell and Grootveld, 1987). Kobo *et al*. (2014) showed that infection with *Trypanosoma brucei brucei* in rats induced significant alteration in an oxidative stress biomarker (malondialdehyde concentration), which was ameliorated by the administration of a flavonoid fraction (Daflon® 500 mg). In the present study, oxidative stress was evident as

indicated by higher levels of 8-isoprostane, a lipid peroxidation biomarker in the liver, kidney and heart of rats infected with *Trypanosoma brucei brucei* without treatment. Recently, isoprostane has been regarded as the most valuable, accurate and reliable marker of oxidative stress *in vivo* and their quantification is recommended for assessing oxidant injuries in humans and animals (Pilacik *et al*., 2002). Numerous studies have shown that quantitative measurement of isoprostane formed during oxidative stress may be used as significant marker of pro-oxidative status during pathogenesis of different diseases (Pilacik *et al*., 2002). The increase in the level of 8-isoprostane recorded in the liver, kidneys and heart of infected untreated rats may be as a result of increased production and accumulation of ROS in these organs caused by the infection. The production of ROS induces lipid peroxidation of polyunsaturated fatty acids and produces highly reactive substances such as isoprostane, which have long-term adverse effects on tissues and organs (Eweda *et al*., 2018). The levels of 8-isoprostane in the liver, kidneys and heart of astaxanthin pretreated rats were lower compared to other treatment groups. This finding agrees with the report of Kobo *et al*. (2014) in rats infected with *Trypanosoma brucei brucei* and treated with a flavonoid mixture and/or diminazene aceturate. The decrease may be attributed to the antioxidant effect of astaxanthin that protected the organs against oxidative stress and lipid peroxidation induced by increased production of ROS caused by trypanosome infection. Pretreatment with astaxanthin and diminazene aceturate administration exerted protecting effects on the organs against the damaging effect of ROS compared to post-treatment with astaxanthin and diminazene aceturate administration. This finding further confirms that pretreatment with astaxanthin was better at protecting tissues and organs against the damaging effect of ROS. The group pretreated with astaxanthin alone had lower level of 8-isoprostane than those post-treated with astaxanthin alone and/or with diminazene aceturate. This could be attributed to the unique

molecular structure of astaxanthin, which enables the molecule to stay both in and outside the cell membrane, thus, making it better at quenching singlet oxygen, scavenging radicals and inhibiting lipid peroxidation than other antioxidants (Liu and Osawa, 2007; Ambati *et al*., 2013b).

In this study, increases in antioxidant enzyme activities were observed in the heart, lungs and liver of infected untreated and astaxanthin post-treated group alone, when compared to other treatment groups. This result contradicts the findings of many researchers (Omer *et al*., 2007; Saleh *et al*., 2009; Mishra *et al*., 2017) who reported a decrease in antioxidant enzyme activities in rats infected with trypanosomes; but agrees with the work of Ogunsanmi and Taiwo (2007), who reported an increase in the enzyme activities. The increase in the activities observed in the infected untreated and astaxanthin post-treated alone may be due to the mobilisation of antioxidant defence system to combat the high parasitaemia recorded in these groups, compared to other groups.The SOD, CAT and peroxidases are the main enzymes incorporated in defence mechanism against ROS (Eljalii *et al*., 2015). The antioxidant defence system of the rats was,apparently, not yet suppressed or exhausted at the very early stage (acute) of infection, which may be the reason for the increase in the antioixidant enzymes recorded in these groups. Ataley *et al*. (2000) also reported that under condition of oxidative stress, activities of antioxidant enzymes,including SOD, CAT and GPx, increase. In high oxidant environment such as occurs in trypanosomosis, the need to scavenge the oxidants is important in order to prevent oxidative stress. In the groups pretreated with astaxanthin and/or diminazene aceturate, the activities of antioxidant enzymes were lower compared to the infected untreated and astaxanthin post-treated groups, indicating that pretreatment with astaxanthin and/or diminazene aceturate

reduced the level of oxidative stress caused by trypanosome infection.This was evident by the lower level of parasitaemia recorded in these groups, thus decreasing the level of activities of SOD, CAT and GPx.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

Experimental infection of adult Wistar rats with *Trypanosoma brucei brucei* and treated with astaxanthin and/or diminazene aceturate caused:

i. A decrease in body weight gain in the positive control and the group post-treated with astaxanthin alone when compared to other treatment groups.

ii. Reduction in survival rate in the positive control and the group post-treated with astaxanthinalone.

iii. An increase in the level of parasitaemia in positive control and the group post-treated with astaxanthin alone. Also, there was resurgence of parasitaemia in 10% of rats in the group that was post-treated with astaxanthin and administered with diminazene azeturate at day 7 of post-treatment.

iv. Reduction in some haematological parameters such as RBC, PCV, Hb concentration, platelet count and increase in MCV, MCH, MCHC, WBC and neutrophil counts in the positive control and astaxanthin post-treated group alone when compared to other treatment groups.

v. An increase in erythrocyte osmotic fragility in positive control and the group post-treated with astaxanthin alone, compared to other groups.

vi. An increase intissue concentration of 8-isoprostane in liver, kidneys and heart of rats in the positive control and the group post-treated with astaxanthin alone.

vii. An increase in the serum activities of AST, ALT and ALP in the infected untreated group and the group post-treated with astaxanthin alone.

viii. An increase in the serum concentrations of IL-1α, IL-6 and TNF-α in positive control and astaxanthin post-treated group alone when compared to other treatment groups.

ix. An increase in serum cholesterol, high density lipoprotein and triglyceride levels in the infected untreated group and the group post-treated with astaxanthin alone.

x. Higher immunoglobulin G in the positive control and astaxanthin post-treated group alone when compared with other groups.

xi. Higher activities of SOD, GPx and CAT in the positive control and the group post-treated with astaxanthin alone.

xii. Reduced level of parasitaemia and prevented resurgence of parasitaemia in rats infected with *T. brucei brucei.*

xiii. Amelioration of trypanosome-induced changes in haematological,serum biochemical parameters, concentrations of cytokines and immunoglobulin G.

xiv. Ameliorationof oxidative stress caused by *T. brucei brucei* infection, which was evidenced by the decreases observed in erythrocyte osmotic fragility, concentration of 8-isoprostane and the activities of SOD, GPx and CAT.

6.2 Conclusion

In conclusion, this study has shown that:

i. Pretreatment of rats with astaxanthin alone or in combination with post-infection administration of diminazene aceturate enhanced body weight gain, increased the survival rate of rats, decreased the level of parasitaemia and prevented resurgence of parasitaemiathat occurred in astaxanthin post-treated group and administered with diminazene aceturate.

ii. Trypanosome-induced alterations in haematological and biochemical parameters of *T. b. brucei*-infected rats were ameliorated by pretreatment with astaxanthin alone or in combination with post-infection administration of diminazene aceturate.

iii. Post-treatment with astaxanthin alone had no beneficial effect on parasite-induced alterations in haematological and biochemical parameters of *T. b. brucei*-infected rats.

iv. *Trypanosoma brucei brucei* infection induced alterations in lipid profile of infected rats which were ameliorated by pretreatment with astaxanthin alone or in combination with post-treatment with diminazene aceturate.

v. Pretreatment with astaxanthin alone or in combination with post-treatment with diminazene aceturate enhanced the immune response of rats infected with *T. brucei brucei.*

vi. In the *T. b. brucei*-infected rats, the increase in cytokine (IL-1α, IL-6 and TNF-α) levels was concomitant and directly correlated with parasitaemia and development of anaemia, suggesting that cytokines may be involved in the immunopathology of animal trypanosomosis.

vii. Infection with *T. brucei brucei* increased activities of antioxidant enzymes, EOF and concentration of 8-isoprostane in Wistar rats.

viii. Pretreatment with astaxanthin alone or in combination with post-infection administration of diminazene aceturate resulted in a significant decrease in the activities of antioxidant enzymes, EOF and 8-isoprostane concentration, indicating that this treatment regimen ameliorated the oxidative damage induced by *T. brucei brucei* infection in the rats.

6.3 Recommendations

From the findings of this work, it is recommended that:

i. Pre-administration of astaxanthin be employed as an adjunct in the management of trypanosomosis.

ii. The use of such combination (pre-infection admistration of astaxanthin and post-infection treatment with diminazene aceturate) therapy in animal trypanosomosis.

iii. Ttreatment of other trypanosome species with the combination (pretreatment with astaxanthin and post-treatment with diminazene aceturate) for comparative study.

iv. Pretreatment with astaxanthin and post-treatment with diminazene aceturate should also be carried out in chronic infection.

v. Further work should be carried out to evaluate the combined effect of astaxanthin and other trypanocides in the management of trypanosomosis.

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APPENDICES

Appendix 1: Effect of treatment with astaxanthin and/or diminazene aceturate on weekly body weight (kg) changes of rats experimentally infected with *Trypanosoma brucei brucei*

Treatment	Week 1	Week 2	Week 3	Week 4
DW	117 ± 9.34	4.53 ± 0.68	3.69 ± 0.38	1.47 ± 0.27
S/OIL	136.5 ± 13.44	5.08 ± 0.27	3.97 ± 0.66	2.13 ± 0.18
T	133.4 ± 6.85	7.63 ± 0.32	8.94 ± 0.89	4.01 ± 0.16
PreA+T	128 ± 5.41	6.14 ± 0.14	6.21 ± 0.49	3.02 ± 0.31
PreA+T+DZ	123.4 ± 9.56	4.24 ± 0.14	5.69 ± 0.20	2.52 ± 0.12
T+A	128 ± 7.22	7.85 ± 0.12	8.65 ± 0.43	3.73 ± 0.05
T+A+DZ	133.5 ± 5.16	6.57 ± 0.23	6.14 ± 0.41	3.36 ± 0.24
T+DZ	126.6 ± 7.20	6.63 ± 0.25	6.38 ± 0.65	3.57 ± 0.06

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

A = Astaxanthin

DZ = Diminaazene aceturate

Appendix 2: Effect of treatment with astaxanthin and/or diminazene aceturate on the survivability (Days) of rats experimentally infected with *Trypanosoma brucei brucei*

Treatments							
DW	**S/OIL**	**T**	**PreA + T**	**PreA + T + DZ**	**T + A**	**T + A + DZ**	**T + DZ**
8.0 ± 0.0	8.0 ± 0.0	6.2 ± 0.41	7.25 ± 0.28	8.0 ± 0.0	6.0 ± 0.37	7.7 ± 0.30	8.0 ± 0.0

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

A = Astaxanthin

DZ = Diminaazene aceturate

Appendix 3: Effect of treatment with astaxanthin and/or diminazene aceturate on the level of parasitaemia of rats experimentally infected with *Trypanosoma brucei brucei*

Day 0	Day 2		Day 4	Day 6	Day 8
Treatment					
DW	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S/OIL	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
T	0.0 ± 0.0	1.68 ± 0.88	7.27 ± 0.10	7.05 ± 0.32	8.1 ± 0.09
PreA+T	0.0 ± 0.0	2.27 ± 0.95	5.58 ± 1.08	7.05 ± 0.15	7.67 ± 0.15
PreA+T+DZ	0.0 ± 0.0	1.35 ± 1.05	5.88 ± 1.11	0.0 ± 0.0	0.0 ± 0.0
T+A	0.0 ± 0.0	2.8 ± 0.10	6.96 ± 0.70	7.63 ± 0.39	8.1 ± 0.15
T+A+DZ	0.0 ± 0.0	1.53 ± 1.01	7.23 ± 0.16	0.0 ± 0.0	0.0 ± 0.0
T+DZ	0.0 ± 0.0	3.73 ± 1.18	7.53 ± 0.14	0.0 ± 0.0	0.0 ± 0.0

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

A = Astaxanthin

DZ = Diminaazene aceturate

Appendix 4: Effect of treatment with astaxanthin and/or diminazene aceturate on RBC, PCV and Hb concentration of rats experimentally infected with *Trypanosoma brucei brucei*

RBC ($\times 10^{12}$/L)	PCV (%)	Hb (g/dL)

Treatment			
DW	8.10 ± 0.39	44.9 ± 0.39	14.93 ± 0.67
S/OIL	8.5 ± 0.14	45.08 ± 0.50	15.3 ± 0.50
T	5.80 ± 0.25	33.84 ± 0.80	10.3 ± 0.46
PreA+T	7.27 ± 0.21	39.3 ± 1.49	11.98 ± 0.28
PreA+T+DZ	7.60 ± 0.21	41.98 ± 1.03	13.88 ± 0.53
T+A	6.28 ± 0.47	33.7 ± 0.59	9.85 ± 0.50
T+A+DZ	7.06 ± 0.07	38.28 ± 1.0	12.35 ± 0.56
T+DZ	6.69 ± 0.17	37.6 ± 1.29	11.05 ± 0.78

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

A = Astaxanthin

DZ = Diminaazene aceturate

RBC = Red blood cell

PCV = Packed cell volume

Hb = Haemoglobin

Appendix 5: Effect of treatment with astaxanthin and/or diminazene aceturate on erythrocytic indices of rats experimentally infected with *Trypanosoma brucei brucei*

MCV	MCH	MCHC (g/dL)

(femtolitre/cell) Treatment		(pictogram/cell)	
DW	67.23 ± 2.67	16.85 ± 0.32	23.23 ± 0.27
S/OIL	66.3 ± 1.22	16.1 ± 0.35	22.83 ± 0.69
T	80.13 ± 0.98	19.52 ± 0.42	29.12 ± 1.47
PreA+T	73.8 ± 1.09	17.95 ± 0.44	24.2 ± 0.41
PreA+T+DZ	71.49 ± 1.19	17.64 ± 0.15	23.38 ± 0.35
T+A	80.33 ± 1.05	19.87 ± 0.32	27.24 ± 0.31
T+A+DZ	76.17 ± 1.38	18.63 ± 0.17	24.83 ± 0.31
T+DZ	74.68 ± 2.06	19.48 ± 0.42	25.7 ± 0.28

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

A = Astaxanthin

DZ = Diminaazene aceturate

MCV = Mean corpuscular volume

MCH = Mean corpuscular haemoglobin

MCHC = Mean corpuscular haemoglobin concentration

Appendix 6: Effect of treatment with astaxanthin and/or diminazene aceturate on leukocyte and platelet counts of rats experimentally infected with *Trypanosoma brucei brucei*

WBC	Lymphocyte	Neutrophil	Platelet

Treatment	(× 10^9/L)	(× 10^9/L)	(× 10^9/L)	(× 109/L)
DW	5.5 ± 0.40	4.08 ± 0.34	2.27 ± 0.22	711.3 ± 17.1
S/OIL	5.77 ± 0.55	3.88 ± 0.33	2.33 ± 0.33	724.3 ± 11.36
T	8.13 ± 0.40	6.75 ± 0.44	4.8 ± 0.12	315.1 ± 6.36
PreA+T	6.7 ± 0.26	6.13 ± 0.71	3.45 ± 0.26	541.8 ± 13.09
PreA+T+DZ	6.16 ± 0.27	5.88 ± 0.42	3.03 ± 0.03	601.8 ± 10.52
T+A	8.37 ± 0.67	6.85 ± 0.41	5.03 ± 0.58	326.2 ± 10.34
T+A+DZ	7.33 ± 0.75	6.1 ± 0.51	3.7 ± 0.21	498.5 ± 27.47
T+DZ	6.87 ± 0.41	6.27 ± 0.95	3.6 ± 0.35	482 ± 17.64

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

A = Astaxanthin

DZ = Diminaazene aceturate

WBC = White blood cell

Appendix 7: Effect of treatment with astaxanthin and/or diminazene aceturate on erythrocyte osmotic fragility of rats experimentally infected with *Trypanosoma brucei brucei*

0.9% 0.7 %			**0.5 %**	**0.3 %**	**0.1 %**	**0.0 %**
Treatment						
DW	13.69 ± 0.99	19.03 ± 1.09	32.42 ± 2.82	95.69 ± 4.31	96.68 ± 2.04	100 ± 0.0
S/OIL	11.28 ± 0.36	14.26 ± 2.17	31.44 ± 2.09	96.66 ± 2.36	99.08 ± 0.92	100 ± 0.0
T	25.05 ± 1.77	40.03 ± 4.52	74.92 ± 2.18	94.17 ± 2.41	95.62 ± 2.54	100 ± 0.0
PreA+T	17.19 ± 0.64	24.44 ± 3.09	48.92 ± 4.53	98.51 ± 1.49	94.37 ± 3.30	100 ± 0.0
PreA+T+DZ	14.68 ± 0.49	17.45 ± 1.32	36.95 ± 3.14	95.17 ± 2.45	99.27 ± 0.59	100 ± 0.0
T+A	25.69 ± 0.87	45.80 ± 5.33	69.17 ± 6.87	100 ± 0.0	95.04 ± 4.73	100 ± 0.0
T+A+DZ	19.50 ± 0.68	27.84 ± 3.03	50.52 ± 3.24	92.27 ± 7.73	100 ± 0.0	100 ± 0.0
T+DZ	18.18 ± 0.54	26.4 ± 3.24	53.11 ± 2.90	99.52 ± 0.48	99.65 ± 0.35	100 ± 0.0

Keys:
DW = Distilled water
S/OIL = Soya oil,
T = *Trypanosoma brucei brucei*
A = Astaxanthin
DZ = Diminaazene aceturate

Appendix 8:Effect of treatment with astaxanthin and/or diminazene aceturate on the activities of 8-isoprostane (Pg/mL) in the liver, kidney and heart of rats infected with *Trypanosoma brucei brucei*

Liver		**Kidney**	**Heart**
Treatments			
DW	2540±186.0	3797±105.8	2768±190.7
S/OIL	2401±301.2	3353±232.1	2630±200.5
T	5629±229.6	5854±61.07	5002±468.9
PreA+T	3240±215.3	4922±242.5	4063±432.6
PreA+T+DZ	3579±383.4	4610±98.99	3838±372.3
T+A	5074±144.9	5776±39.08	5347±416.2
T+A+DZ	4633±257.7	5290±190.5	4207±184.2
T+DZ	4553±352.1	5123±117.7	4660±197.0

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

A = Astaxanthin

DZ = Diminaazene aceturate

Appendix 9: Effect of treatment with astaxanthin and/or diminazene aceturate on liver enzymes of rats experimentally infected with *Trypanosoma brucei brucei*

AST (IU/L)		ALT (IU/L)	ALP (IU/L)
Treatments			
DW	26.67 ± 1.86	16.67 ± 0.67	103.1 ± 3.86
S/OIL	30 ± 5.51	12.5 ± 1.26	97.43 ± 11.08
T	60.33 ± 2.96	48 ± 4.62	286.1 ± 30.63
PreA+T	46 ± 2.27	32 ± 8.0	223.3 ±19.62
PreA+T+DZ	42.67 ± 3.28	20 ± 2.31	186.7 ± 21.65
T+A	56.33 ± 1.76	44.25 ± 1.93	280 ± 19.87
T+A+DZ	50 ± 5.13	31 ± 2.52	213.4 ± 14.5
T+DZ	53.33 ± 1.20	33 ± 5.26	218.9 ± 8.76

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

A = Astaxanthin

DZ = Diminaazene aceturate

AST = Aspartate aminotransferase

ALT = Alanine aminotransferase

ALP = Alkaline phosphatase

Appendix 10: Effect of treatment with astaxanthin and/or diminazene aceturate on tumour necrosis factor alpha (Pg/mL), interleukin-1alpha (Pg/mL) and interleukin-6 (Pg/mL) of rats experimentally infected with *Trypanosoma brucei brucei*

TNF-α		**IL-1α**	**IL-6**
Treatments			
DW	116.3 ± 5.09	21.53 ± 2.04	125.3 ± 14.65
S/OIL	135.6 ± 15.39	19.04 ± 0.90	115.2 ± 11.92
T	280.9 ± 17.27	30.46 ± 2.37	366.3 ± 41.45
PreA+T	179.5 ± 25.59	25.86 ± 1.44	228 ± 24.51
PreA+T+DZ	191.5 ± 18.5	25.3 ± 2.15	191.6 ± 17.59
T+A	251.2 ± 38.75	27.93 ± 0.10	364.8 ± 51.92
T+A+DZ	206.6 ± 13.85	26.26 ± 1.03	233.2 ± 35.6
T+DZ	203.2 ± 22.52	24.99 ± 1.21	266.9 ± 8.74

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

PreA = Pretreated with astaxanthin

DZ = Diminaazene aceturate

TNF-α = Tumour necrosis factor alpha

IL-1α = Interleukin 1alpha

IL-6 = Interleukin 6

Appendix 11: Effect of treatment with astaxanthin and/or diminazene aceturate on lipid profile of rats experimentally infected with *Trypanosoma brucei brucei*

Cholesterol (mg/dL)		Triglyceride (mg/dL)	HDL (mg/dL)
Treatments			
DW	4.53 ± 0.68	3.69 ± 0.38	1.47 ± 0.27
S/OIL	5.08 ± 0.27	3.97 ± 0.66	2.13 ± 0.18
T	7.63 ± 0.32	8.94 ± 0.89	4.01 ± 0.16
PreA+T	6.14 ± 0.14	6.21 ± 0.49	3.02 ± 0.31
PreA+T+DZ	4.24 ± 0.14	5.69 ± 0.20	2.52 ± 0.12
T+A	7.85 ± 0.12	8.65 ± 0.43	3.73 ± 0.05
T+A+DZ	6.57 ± 0.23	6.14 ± 0.41	3.36 ± 0.24
T+DZ	6.63 ± 0.25	6.38 ± 0.65	3.57 ± 0.06

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

A = Astaxanthin

DZ = Diminaazene aceturate

LDL = Low density lipoprotein

Appendix 12: Effect of treatment with astaxanthin and/or diminazene aceturate on Immunoglobulin G(ng/mL) of rats experimentally infected with *Trypanosoma brucei brucei*

Treatments							
DW	**S/OIL**	**T**	**PreA + T**	**PreA + T + DZ**	**T + A**	**T + A + DZ**	**T + DZ**
4.72 ± 0.10	5.36 ± 0.33	6.40 ± 0.28	7.50 ± 0.34	7.17 ± 0.51	6.11 ± 0.23	5.10 ± 0.52	6.10 ± 0.24

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

A = Astaxanthin

DZ = Diminaazene aceturate

IgG = Immunoglobulin G

Appendix 13:Effect of treatment with astaxanthin and/or diminazene aceturate on the activity of superoxide dismutase (IU/L) in the heart, lungs, and liver of rats infected with *Trypanosoma brucei brucei*

Heart		**Lungs**	**Liver**
Treatments			
DW	0.3692 ± 0.13	2.187 ± 0.04	0.2582 ± 0.02
S/OIL	0.4492 ± 0.21	2.124 ± 0.04	0.3035 ± 0.05
T	1.711 ± 0.22	2.904 ± 0.09	1.842 ± 0.29
PreA+T	1.352 ± 0.07	2.543 ± 0.07	1.170 ± 0.04
PreA+T+DZ	1.347 ± 0.12	2.552 ± 0.03	1.056 ± 0.07
T+A	1.778 ± 0.11	2.667 ± 0.02	1.529 ± 0.08
T+A+DZ	1.486 ± 0.19	2.612 ± 0.10	1.102 ± 0.14
T+DZ	1.543 ± 0.49	2.641 ± 0.07	1.301 ± 0.92

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

A = Astaxanthin

DZ = Diminaazene aceturate

SOD = Superoxide dismutase

Appendix 14:Effect of treatment with astaxanthin and/or diminazene aceturate onglutathione peroxidase (IU/L)activity in the heart, lungs, and liver of rats infected with *Trypanosoma brucei brucei*

Heart		**Lungs**	**Liver**
Treatments			
DW	15.73 ± 2.31	18.46 ± 1.27	16.18 ± 1.44
S/OIL	13.84 ± 1.60	18.76 ± 2.17	14.19 ± 1.28
T	44.07 ± 3.40	50.25 ± 6.07	36.89 ± 2.24
PreA+T	30.60 ± 1.61	34.11 ± 3.97	30.48 ± 1.39
PreA+T+DZ	27.14 ± 5.21	32.35 ± 2.48	27.66 ± 1.91
T+A	43.84 ± 5.92	49.91 ± 1.95	35.08 ± 2.11
T+A+DZ	31.64 ± 3.94	35.93 ± 3.76	28.52 ± 3.03
T+DZ	35.51 ± 2.91	36.83 ± 5.96	33.79 ± 1.45

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

A = Astaxanthin

DZ = Diminaazene aceturate

GPx = Glutathione peroxidase

Appendix 15:Effect of treatment with astaxanthin and/or diminazene aceturate on catalase(IU/L)activity in the heart, lungs, and liver of rats infected with *Trypanosoma brucei brucei*

Heart		**Lungs**	**Liver**
Treatments			
DW	9.762 ± 0.89	25.18 ± 1.49	9.757 ± 0.49
S/OIL	11.11 ± 0.99	24.44 ± 1.27	9.041 ± 0.21
T	30.86 ± 3.37	50.78 ± 3.82	12.09 ± 0.95
PreA+T	26.53 ± 3.41	39.21 ± 3.45	10.01 ± 0.41
PreA+T+DZ	20.83 ± 1.62	39.68 ± 2.02	8.637 ± 0.79
T+A	30.97 ± 3.13	44.29 ± 1.74	12.91 ± 1.08
T+A+DZ	26.46 ± 3.21	47.52 ± 5.04	10.02 ± 0.75
T+DZ	26.39 ± 3.32	47.50 ± 1.30	10.37 ± 0.84

Keys:

DW = Distilled water

S/OIL = Soya oil,

T = *Trypanosoma brucei brucei*

A = Astaxanthin

DZ = Diminaazene aceturate

GPx = Glutathione peroxidase