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Effect of Kaempferol, Diminazene Aceturate and their Combination on Hematological Parameters in *Trypanosoma brucei brucei* Experimentally Infected Mice

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Abstract

Kaempferol is a polyphenolic compound and are widely distributed in plants. It is used in the treatment of different disease conditions. With the endemic resistant parasites against most antitrypanosomal agents and the toxicity associated with diminazene aceturate, the search for safer and more effective alternative therapy of trypanosomosis becomes paramount. In this study the effect of treatment with kaempferol and diminazene aceturate on Hematological parameters in mice with experimental *Trypanosoma brucei brucei* infection was evaluated. Thirty six adult swiss albino mice of either sex were randomly divided into six groups of six mice each. Mice in group I were untreated uninfected. Mice in group II were pre-treated with kaempferol (1 mg/kg) for 14 days. Mice in groups II to VI each were inoculated with blood containing *Trypanosoma brucei brucei* (10⁶ trypanosomes/ml of blood/animal) intraperitoneally. Following establishment of the infection (four days post-inoculation), mice in group III were treated once with diminazene aceturate (3.5 mg/kg) I.P. Mice in group IV were treated with diminazene aceturate (3.5 mg/kg) once I.P. and then continued with kaempferol (1 mg/kg) for nine days.

Keywords: Hematology, Kaempferol, *Trypanosoma brucei brucei*

Introduction

In Africa, trypanosomosis is one of the neglected parasitic diseases of animal and human, which accounts for the low livestock productivity (Welburn et al., 2006). About 70 million people distributed over 1.55million km² (Simarro et al., 2012), on the other hand, about 250 million animals distributed over approximately 25 million km² in Africa are at risk of the disease (WHO, 2006). The pathogenesis of African trypanosomosis is partly due to the generation of reactive oxygen species (ROS) due to stress induced by the parasite, which causes degenerative changes in cells, tissues and organs of the infected animals (Kobo et al., 2014). The ROS attack both the membrane polyunsaturated fatty acids and proteins of RBCs, leading to oxidative hemolysis and consequently, anemia as well as the depletion of endogenous antioxidant reserved in the blood and other tissues of trypanosome-infected animals (Kobo et al., 2014). The interplay of several factors acting either individually or synergistically also contributes to the development of hemolytic anemia in human and animal

trypanosomosis, most common among these factors are erythrocyte injury caused by lashing action of trypanosome flagella, platelet aggregation, toxins and metabolites produced by trypanosomes, lipid peroxidation and malnutrition (Murray and Morrison, 1978; Morrison et al., 1981; Saror, 1982; Igbokwe, 1994). Chemotherapy and chemoprophylaxis by trypanocides using isometamidium, homidium and diminazene, formed the most important aspect of control and eradication of trypanosomosis in animals (Leach and Roberts, 1981; Kinabo, 1993; Anene, et. al. 2001). Reports from East, South and West Africa (Ndung'u et al. 1999; McDermott et al., 2000; Maikai et al., 2007) have shown that the prevalence of trypanocidal drug resistance to be very high. Antioxidants are substances that protect cells from the damage caused by unstable molecules known as free radicals (Kobo et al., 2014). Antioxidants scavenge free radicals and prevent tissues and organs from damage caused by free radicals (Shimelis et al., 2015). In the present study, the effects of treatment with kaempferol and/or diminazene aceturate on hematological parameters of mice

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with experimental *Trypanosoma brucei brucei* infection was investigated using established procedures.

Materials and Methods

Location of the Research

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

Experimental Animals

Thirty six adult Swiss albino mice of either sex weighing between 18 and 22 grams were used in this study. The mice were reared in the animal house, Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria. These animals were housed in locally fabricated mice cages at room temperature, 25°C. Wood shavings were used as beddings and changed once every week. The experimental mice were allowed free access to rat chow and water ad-libitum. All animal experiments were carried out according to international guidelines as approved by the postgraduate (ethical) committee of the Faculty of Veterinary Medicine, Ahmadu Bello University Zaria.

The Parasite

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

Drug, Sources and Preparation

Kaempferol was sourced from whitehead scientific (pty) limited, South Africa, it came along with the following details; CAS number (520-18-3), Catalog number (3603), EC number (208-287-6) and batch number (3). Diminazene aceturate was purchased from pharmacy unit of the Veterinary Teaching Hospital (VTH), Faculty of Veterinary Medicine, Ahmadu Bello University (A.B.U), Zaria, Kaduna State, Nigeria.

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

Experimental Infection of the Mice

Trypanosomes infected blood was obtained from the tail of the infected donor mice at peak of Parasitaemia (109) and used to maintain parasite suspension in physiological saline. The mice were inoculated (1 mL/mice) intraperitoneally with a suspension, containing 3 or 4 trypanosomes per view at $\times 100$ magnification (approximately 106 trypanosomes per mL) as described by Ekanem and Yusuf (2008). The Thirty six adult mice were randomly divided into six groups of six mice each and were treated as follows:

Group I- Mice in group I were neither infected nor treated.

Group II- Mice in group II were pre-treated individually with kaempferol (1 mg/kg per os) for 14 days and then infected with *Trypanosoma brucei brucei* (106 trypanosomes/ml of blood).

Group III- Mice in group III were infected with *Trypanosoma brucei brucei* (106 trypanosomes/ml of blood), after detection of Parasitaemia, each mouse was treated once with diminazene aceturate (3.5 mg/kg i.p).

Group IV- Mice in group IV were infected with *Trypanosoma brucei brucei* (106 trypanosomes/ml of blood), after detection of Parasitaemia, each mouse was treated once with diminazene aceturate (3.5 mg/kg) intraperitoneally, and then continued with kaempferol (1 mg/kg per os) for 9 consecutive days.

Group V- Mice in group V were infected with *Trypanosoma brucei brucei* (106 trypanosomes/ml of blood i.p), after detection of Parasitaemia, they were treated with kaempferol (1 mg/kg per os) for 9 consecutive days.

Group VI- Mice in group VI were infected with *Trypanosoma brucei brucei* (106 trypanosomes/ml of blood i.p) and then administered normal saline at (5 ml/kg per os) for 9 consecutive days.

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

Hematological Analysis

At the end of the experiment, mice from each group were sacrificed by severing the jugular vein. About 1.5 ml of blood was collected from each mouse in a 20 ml vial containing EDTA as an anti-coagulant and used to determine the Hematological parameters; packed cell volume (PCV), total erythrocyte count, hemoglobin concentration, and total and differential leucocyte counts were determined using methods described by Dacie and Lewis (1991). The method described by Schalm et al (1975) was also used to calculate erythrocytic indices from PCV, hemoglobin concentration and erythrocyte count.

Statistical Analysis

Data obtained was expressed as mean \pm standard error of mean (S.E.M) and then subjected to one-way analysis of variance (ANOVA) and compared with Turkey post-hoc test, using Graph Pad Prism version 5.0 for windows (Graph Pad Software, San Diego, California, USA). The level of significance was set at $p < 0.05$.

Results

Mean survival rate

Figure 1 shows the effect of treatments with kaempferol and/or diminazene aceturate on mean survival rate in mice with experimental *Trypanosoma brucei brucei* infection. Mean survival rate significantly ($P < 0.001$) decreased in mice pretreated with kaempferol (group II) and those that were infected and administered normal saline (group VI) when compared to mice infected and treated with diminazene aceturate (group III), diminazene aceturate and kaempferol (group IV) and kaempferol only (group V).

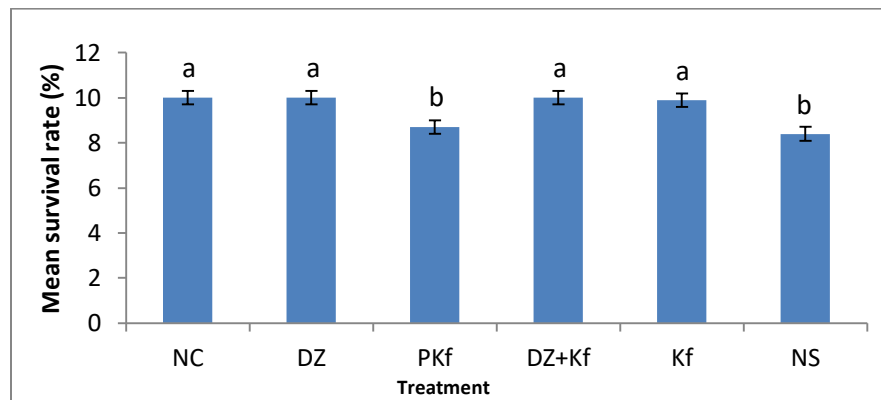


Figure 1: Effect of treatments with kaempferol and/ or diminazene aceturate on mean survival rate in mice experimentally infected with *Trypanosoma brucei brucei*. Mean values with different alphabets are statistically different ($P < 0.001$), **Key:** NC= Neutral control, DZ=Diminazene aceturate, PKf=pre-treated with kaempferol, DZ+Kf=Diminazene aceturate and kaempferol, Kf= kaempferol and NS= Normal saline.

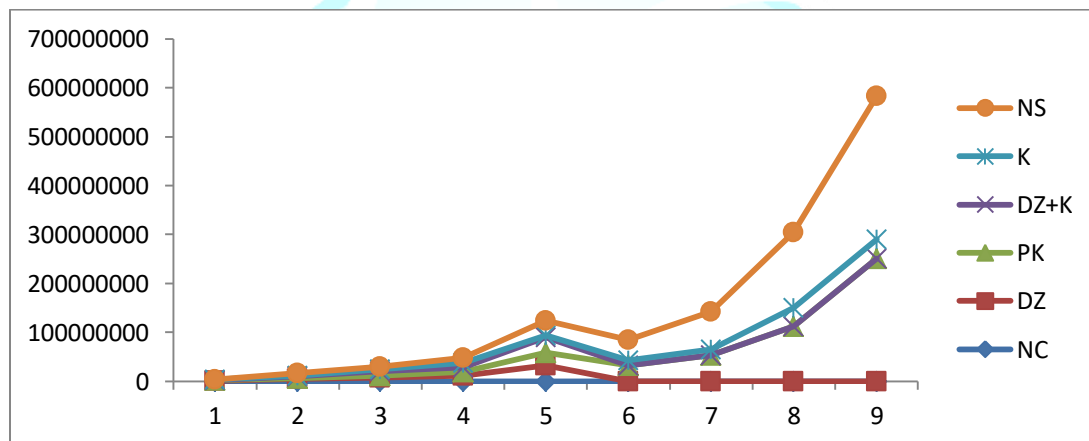


Figure 2: Effect of the treatments with kaempferol and/ or diminazene aceturate on the level of Parasitaemia in mice experimentally infected with *T. brucei brucei*.

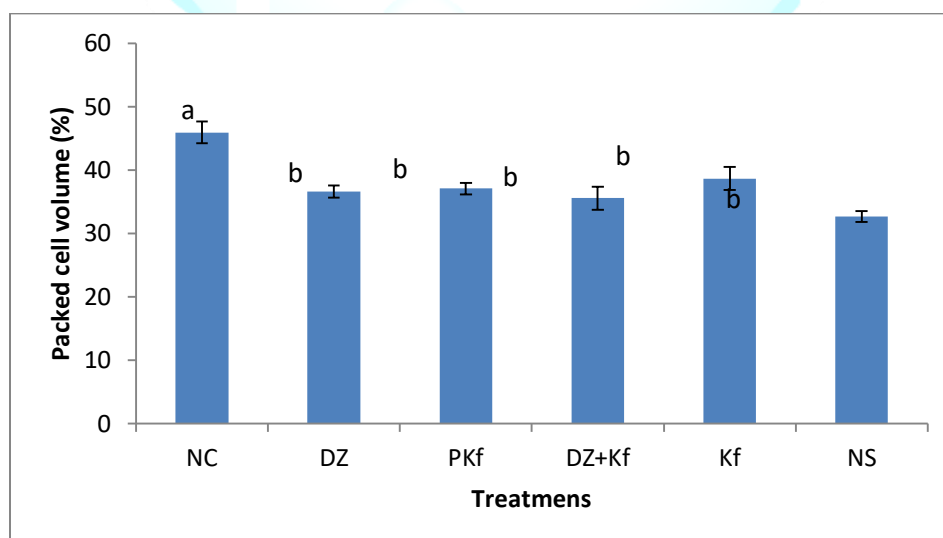


Figure 3: Effect of treatments with kaempferol and/ or diminazene aceturate on Packed Cell Volume in mice experimentally infected with *Trypanosoma brucei brucei*. Mean values with different alphabets are statistically different ($P < 0.001$).

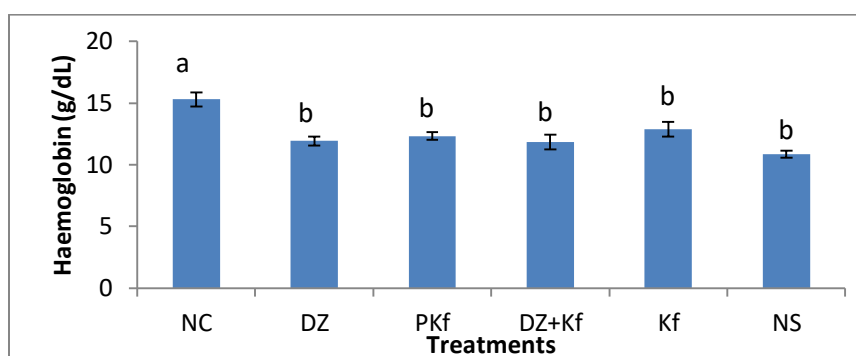


Figure 4: Effect of treatments with kaempferol and/ or diminazene aceturate on hemoglobin concentration in mice experimentally infected with *Trypanosoma brucei brucei*. Mean values with different alphabets are statistically different ($P<0.001$).

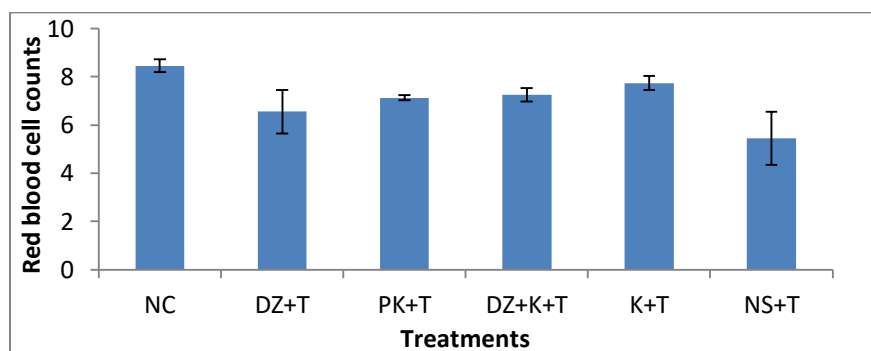


Figure 5: Effect of treatments with kaempferol and/ or diminazene aceturate on red blood cell count of mice experimentally infected with *Trypanosoma brucei brucei*. Mean values with different alphabets are statistically different ($P<0.001$).

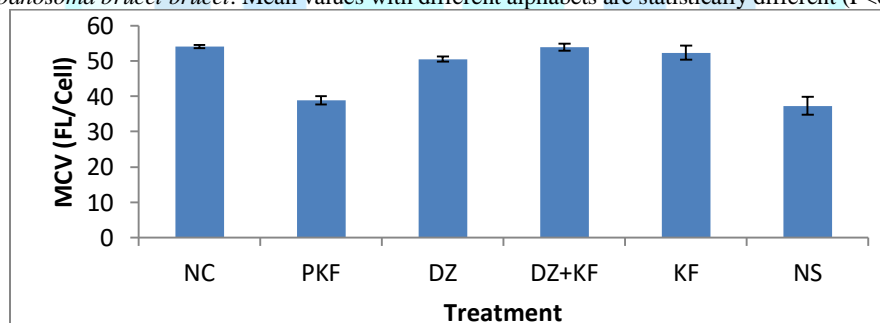


Figure 6: Effect of treatments with kaempferol and/ or diminazene aceturate on MCV of mice experimentally infected with *Trypanosoma brucei brucei*. Mean values with different alphabets are statistically different ($P<0.001$).

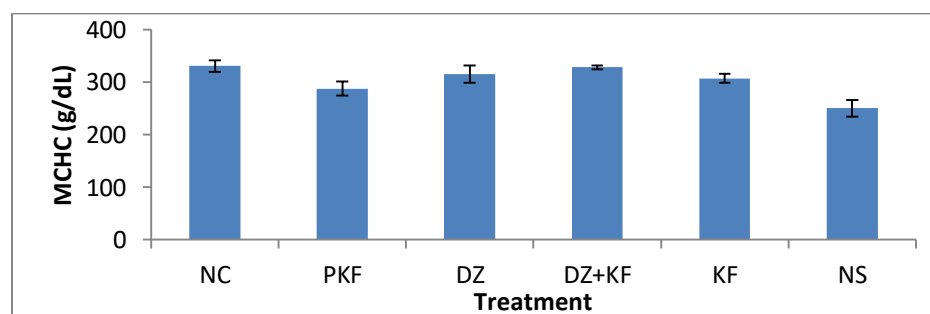


Figure 7: Effect of treatments with kaempferol and/ or diminazene aceturate on MCHC of mice experimentally infected with *Trypanosoma brucei brucei*. Mean values with different alphabets are statistically different ($P<0.001$).

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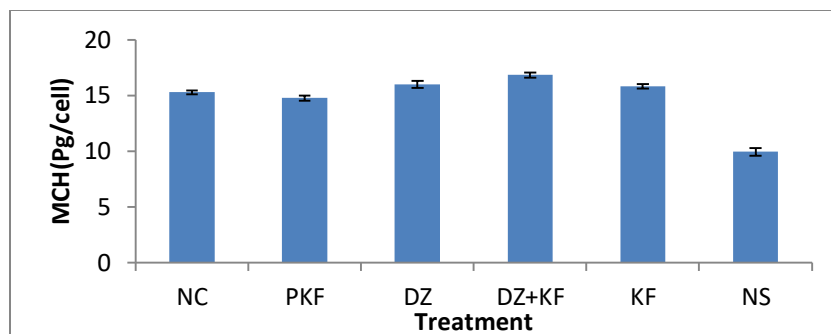


Figure 8: Effect of treatments with kaempferol and/ or diminazene aceturate on MCH of mice experimentally infected with *Trypanosoma brucei brucei*. Mean values with different alphabets are statistically different ($P < 0.001$).

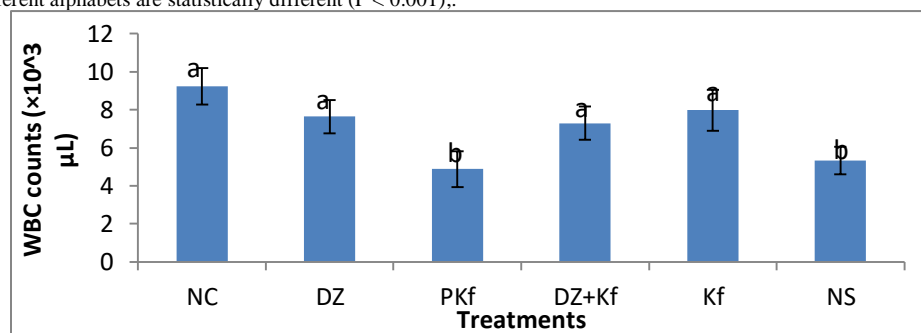


Figure 9: Effect of treatments with kaempferol and/ or diminazene aceturate on absolute white blood cell counts in mice experimentally infected with *Trypanosoma brucei brucei*. Means with different alphabets differ significantly ($P < 0.001$).

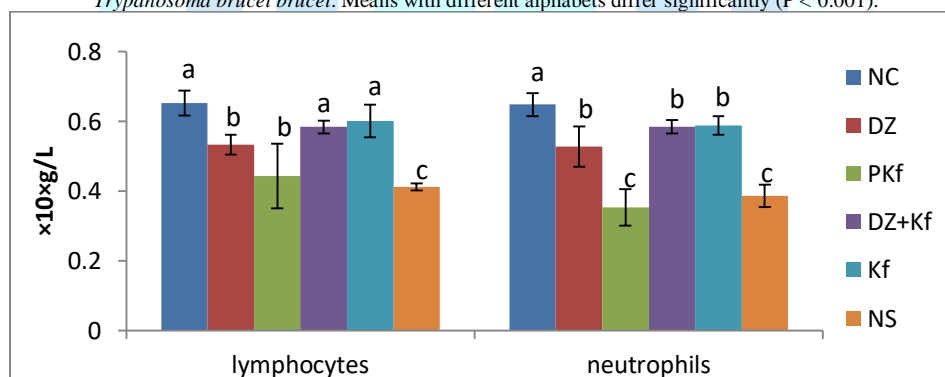


Figure 10: Effect of treatments with kaempferol and/ or diminazene aceturate on mean lymphocyte and neutrophil count in mice experimentally infected with *Trypanosoma brucei brucei*. Means with different alphabets differ significantly ($P < 0.05$).

Effects of Treatments on the Level of Parasitaemia in Infected Mice

The effects of treatments with kaempferol and/ or diminazene aceturate in mice experimentally infected with *Trypanosoma brucei brucei* on the level of Parasitaemia is shown in **Figure 2**. Parasitaemia increased progressively in mice pre-treated with kaempferol (group II), treated with kaempferol only

(group V) and those that were administered normal saline (group VI) up to day 9 post infection. The parasites were cleared completely from the blood stream of mice treated with diminazene aceturate only (group III) and those that were treated with diminazene aceturate and kaempferol (group IV) on day six post infection, hence remain aparasitaemic.

Although the onset of Parasitaemia was not different from other groups, but there was significant ($P < 0.001$) reduction in the level of Parasitaemia in mice treated kaempferol (group V) when compared to mice pre-treated with kaempferol (group II) and those that were infected and administered normal saline (group VI).

Effects of Treatments on Hematological Parameters

Packed Cell Volume: **Figure 3** shows the effects of treatments with kaempferol and/ or diminazene aceturate on packed cell volume in mice infected with *T. brucei brucei*. The mean packed cell volume increased significantly ($P < 0.001$) in mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV), kaempferol only (group V) when compared to mice pre-treated with kaempferol (group II) and those that were infected and administered normal saline (group VI).



Hemoglobin Concentration: Figure 4 shows the effects of treatments with kaempferol and/or diminazene aceturate on hemoglobin concentrations in mice infected with *T. brucei brucei*. There was significant ($P < 0.001$) decrease in the mean hemoglobin concentration in mice pre-treated with kaempferol (group II) and those that were infected and administered normal saline (group VI) when compared to mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV) and kaempferol only (group V).

Red Blood Cell Count: Figure 5 shows the effect of treatments with kaempferol and/or diminazene aceturate on red blood cell count of mice infected with *T. brucei brucei*. A significant ($P < 0.001$) increase in the mean red blood cell count was recorded in mice treated with diminazene aceturate only (groups III), kaempferol and diminazene aceturate (group IV), kaempferol only (group V) when compared to mice pre-treated with kaempferol (group II) and those that were infected and administered normal saline (group VI).

Effect of the Treatment on Erythrocytic Indices

Mean Corpuscular Volume (MCV): Figure 6 shows the effect of the treatment with kaempferol and/or diminazene aceturate on mean corpuscular volume (MCV) of mice infected with *T. brucei brucei*. There was significant ($P < 0.001$) decrease in the mean values of MCV in mice pre-treated with kaempferol (group II) and those that were infected and administered normal saline (group VI) when compared to mice treated with diminazene aceturate (III), diminazene aceturate and kaempferol (IV) and kaempferol only (V).

Mean Corpuscular Hemoglobin Concentration (MCHC): Figure 7 shows the effect of the treatments with kaempferol and/or diminazene aceturate on mean corpuscular hemoglobin concentration (MCHC) of mice infected with *T. brucei brucei*. There was significant ($P < 0.001$) decrease in the mean values of MCHC in mice pre-treated with kaempferol (group II) and those that were infected and administered normal saline (group VI) when compared to mice treated with diminazene aceturate (III), diminazene aceturate and kaempferol (IV) and kaempferol only (V).

Mean Corpuscular Hemoglobin (MCH): Figure 8 shows the effect of the treatments with kaempferol and/or diminazene aceturate on mean corpuscular hemoglobin (MCH) of mice infected with *T. brucei brucei*. There was significant ($P < 0.001$) decrease in the mean values of MCH in mice pre-treated with kaempferol (group II) and those that were infected and administered normal saline (group VI) when compared to mice treated with diminazene aceturate (III), diminazene aceturate and kaempferol (IV) and kaempferol only (V).

Total White Blood Cell Count: Figure 9 shows the effects of the treatments with kaempferol and/or diminazene aceturate on total white blood cell count of mice infected with *T. brucei brucei*. There was significant ($P < 0.001$) decrease in the mean total leucocyte count in mice pre-treated with kaempferol (group II) and those that were infected and administered normal saline (group VI) when compared to mice treated with diminazene aceturate only (groups III), diminazene aceturate and kaempferol (group IV) and kaempferol only (group V).

Effect of Treatments on Differential Leucocyte Count

Figure 10 shows the effects of treatments with kaempferol and/or diminazene aceturate on lymphocyte and neutrophil count of mice infected with *T. brucei brucei*. Significant ($P < 0.05$) decrease in the mean lymphocytes and neutrophil count were observed in mice pre-treated with kaempferol (group II) and those that were infected and administered normal saline (group VI) when compared to mice treated with diminazene aceturate only (groups III), diminazene aceturate and kaempferol (group IV) and kaempferol only (group V).

Discussion

The clinical signs observed were; anorexia, loss of body weight, pale ocular mucous membrane and weakness and are more obvious in mice pre-treated with kaempferol (group II) and those that were infected and administered normal saline (group VI), this agrees with the report of Kobo et al (2014) in rats with experimental *Trypanosoma brucei brucei* infection and then treated with two mixture of flavonoids (hesperidin and Daflon®), severity of these signs depends on the strain of infecting trypanosomes, dose of the parasite during infection, immune status of the host and host susceptibility. Variable disorders occur sequel to trypanosome infection in animals (Adamu et al., 2009), depending on the virulence of the infecting trypanosome, the infective dose and the immune status of the host. The symptoms usually associated with trypanosomiasis includes; pallor of the mucous membranes, enlargement of lymph nodes, anorexia and emaciation (Shimelis et al., 2015).

Parasitaemia was detected four days post-infection, it agrees with the findings of Ibrahim et al (2016) in mice, rats and rabbits experimentally infected with *Trypanosoma brucei brucei*. So far, little has been achieved in terms of understanding the variations in pre-patent period and course of *T. b. brucei* infection in various laboratory animal species because it rapidly divide in the blood stream of their host by binary fission, resulting in the large population of the parasites within short time (Ibrahim et al., 2016). Parasitaemia increased progressively in groups II (pre-treated with kaempferol), V (treated with kaempferol only) and VI (administered normal saline) up to day nine post-infection where all mice were sacrificed by severing their jugular veins.

There was significant ($P < 0.001$) decrease in the level of Parasitaemia in mice treated with kaempferol only when compared to mice pre-treated with kaempferol and those that were administered normal saline, this agrees with the findings of Kobo et al (2014) which showed delayed proliferation of *Trypanosoma brucei brucei* in rats treated with mixtures of flavonoids (hesperidin and daflon®), the effect was attributed to scavenging ability of the flavonoids on free radicals generated during the course of infection. Kaempferol is also extensively metabolized in the liver to form glucurono-conjugated and sulfo-conjugated forms (Calderon-Montano et al., 2011), these forms of kaempferol, and kaempferol itself, can then be excreted in urine. About 2.5% of kaempferol ingested is excreted as urine; much of the ingested kaempferol is present in the plasma and tissues in nanomolar concentrations (Calderon-Montano et al., 2011). In addition, flavonoids may regenerate other antioxidants with known immune-enhancing activity, such as vitamin E (Zhu et al., 2000) and carotenoids (Pietta and Simonetti, 1998), therefore, this could be the reason why kaempferol was effective at reducing the level of parasitaemia in the infected mice treated with kaempferol. There was an absolute clearance parasite from the blood stream of mice treated with diminazene aceturates only (group III) and the combination of diminazene aceturate and kaempferol (group V) one day after treatment and that the clearance may be due to diminazene (Saba et al., 2007).

The mean PCV, Hb concentration and RBC count reduced significantly ($P < 0.001$) in groups II (pre-treated with kaempferol), V (treated with kaempferol only) and VI (administered normal saline) indicating anemia, this agrees with the work of Ukpai and Nwabuko (2014) who reported the effects of *Trypanosoma brucei brucei* on Hematological parameters and pathology of internal organs in albino rats, Anemia observed was attributed to mechanical injury to RBC, chemicals produced by live and dead trypanosomes and also lipid peroxidation. Anemia which is regarded as the most consistent finding in trypanosomiasis of man and domesticated animal has also been reported in *T. vivax* infected cattle and goats (Saror, 1980), *T. congolense* infected sheep (Bisalla, 2007), *T. congolense* infected dogs (Gow et al., 2007), *T. brucei brucei* infected goats, sheep and rabbits (Taiwo et al.,

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2003; Seed, 1969). The pathophysiology of *Anemia* in trypanosomiasis is complex and multi factorial in origin (Naessens et al., 2005). It initiates a cascade of events leading to hemolytic *Anemia* and cardiovascular collapse (Anosa, 1988). Kaempferol used in this study showed significant level of protection of the red blood cells in the infected mice treated with kaempferol only (group V), this may be due to its antioxidant effect and ability to scavenge the free radicals generated by the parasite during the course of infection, thereby reducing the free radical loads hence prevent erythrocyte membrane from oxidative damage (Procházková et al., 2011). Umar et al (2007; 2001) have reported protective effect of vitamin E and C in trypanosomes induced *Anemia*. Murray and Dexter (1988) showed that the severity of *Anemia* during trypanosome infection could be related to differences in virulence among trypanosome strains.

Erythrocytic indices were determined so as to classify *Anemia* morphologically (Adamu et al., 2009). There was a significant ($p < 0.001$) reduction in the mean values of MCV and MCHC in mice pre-treated with kaempferol (II) and those that were administered normal saline (group VI), resulting in microcytic hypochromic *Anemia*, therefore the results obtained in this study agrees with that reported by Kobo et al., (2014) where microcytic hypochromic *Anemia* was observed only in untreated rats infected with *T. brucei brucei* and disagrees with the finding of Ibrahim et al (2016) where macrocytic hypochromic *Anemia* was observed in mice, and rats with experimental *Trypanosoma brucei brucei* infection. Microcytic hypochromic *Anemia* occur due to erythropoiesis that takes place after the onset of infection trypanosoma, at which time immature erythrocytes are released into the systemic circulation (Igbokwe et al., 1994). Microcytic hypochromic anemia is a blood disorder characterized by small red blood cells (erythrocytes) which have insufficient hemoglobin and hence have a reduced ability to carry oxygen through the body (Ford, 2013). The increased MCV and MCHC in kaempferol treated (group V) may be due to kaempferol ability to protect red blood cells from oxidative damage by scavenging free radicals that are detrimental to biomolecules. Mean WBC, lymphocytes and neutrophils significantly ($P < 0.05$) decreased in mice pre-treated with kaempferol (groups II) and those that were administered normal saline (group VI) agrees with the work of Takeet and Fagbemi (2009) in rabbits with trypanosomiasis, these researchers attributed this reduction to immunosuppressive actions of trypanosomes. Leucopenia after an initial period of leucocytosis is a common finding in *T. b. brucei* infection in laboratory animal (Ibrahim et al., 2016). Leucopenia in animal with trypanosomiasis has been reported to be due largely to ineffective or depressed granulopoiesis in the bone marrow (Anosa et al., 1997a).

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