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Evaluation of the anti-trypanosomal activity of *Justicia secunda* (Vahl) leaf in *Trypanosoma brucei* infected rats

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Abstract

The use of plants in traditional medicine is increasingly gaining ground in modern medicine because phytochemical components of most secondary metabolites can be used to treat a wide range of diseases. The anti-trypanosomal effects of ethanolic extracts of *Justicia secunda* leaf were investigated in albino rats. Thirty albino rats were used for the study and they were divided into six groups of five rats each. Group I was uninfected untreated (control), group II was infected untreated, group III was infected and treated with diminazene aceturate (DA), while groups IV, V and VI were infected and treated with *J. secunda* at 100mg/kg, 200mg/kg and 400mg/kg body weights, respectively. The parasite clearance time did not show any significant ($p>0.05$) difference in the extract-treated groups. However, relapse of infection occurred on day 28 post-treatment (PT) in 100mg/kg treated group and day 63 PT in 200mg/kg and 400mg/kg treated groups. The red cell parameters (PCV, Hb and RBC counts) were significantly ($p<0.05$) decreased in the infected groups, but improved in the groups treated with 200mg/kg and 400mg/kg to a level comparable to the uninfected untreated (control). The mean total white cell counts (TWBC) count was significantly ($p<0.05$) higher in the infected untreated group. The extract-treated groups did not show any variation ($p>0.05$) and were comparable with the uninfected untreated control. In conclusion, the ethanolic extract of *J. secunda* leaf exhibited dose-dependent anti-trypanosomal activity in *T. brucei*-infected rats and was able to ameliorate and conserve anaemia as shown in the improved haematological parameters and reduced risk of relapse.

Keywords: Haematology, *Justicia secunda*, Parasitaemia; Relapse, Rat, *Trypanosoma brucei*

Introduction

African animal trypanosomiasis (AAT) is a complex of disease caused by a haemoprotozoan flagellated parasite with a devastating impact on livestock productivity (Chanie *et al.,* 2013). It is also known as Nagana, which occurs throughout the tropical regions of Sub-Sahara Africa and large areas of Asia and South America (Batista *et al.,* 2011). AAT is a significant livestock disease in tsetse-infested regions of Africa, resulting in morbidity and mortality-related losses. Morbidity-related losses are characterized by low milk production, increased risk of infection by other diseases, low live weight gain, and reduced fertility (Shaw, 2004). Mortality usually occurs if treatment is

not instituted early enough in infected animals (Shaw, 2004).

The causative agents of the disease are protozoan parasites of the genus *Trypanosoma* that live and multiply extracellularly in the blood and tissue fluids of their mammalian hosts and are transmitted cyclically by the bite of infected tsetse flies of the *Glossina* species (Steverding, 2008; Brown, 2008). The disease can also be transmitted mechanically by the biting flies Tabanids and stomoxys (Desquesnes *et al*., 2009). Trypanosomosis affects cattle, sheep, goats, pigs, dogs, horses and man. The species of veterinary importance is *Trypanosoma brucei, T. congolense, T. vivax, T. simiae,* while subspecies of *T. brucei, T. brucei gambiense* and *T. brucei rhodesiense* are known to affect man causing sleeping sickness (Baker, 1995). The cardinal sign of African trypanosomiasis is anaemia with the pallor of the mucus membrane (Holmes *et al*., 2000), associated with a decline in haematological parameters (Stephen, 1986). Other clinical manifestations of trypanosomiasis are fever, listlessness, emaciation, hair loss, weight loss, ocular discharges, enlarged lymph nodes, abortion, oedema, paralysis and loss of condition, which may eventually lead to death. The disease may be acute or chronic and is affected by poor nutrition, concurrent diseases, and other stress factors (Fineile *et al*. 1983). Chemotherapy against trypanosomosis has remained a burden due to problems of toxicity, resistance and relapse of infections (Mamoudou *et al*., 2008).

Justicia secunda Vahl, commonly referred to as blood root, belongs to the family *Acanthaceae*. The genus originated from South America but has been fully domesticated in tropical regions of Africa including Nigeria and has been used in African traditional medicine (Koffi *et al.,* 2013; Kitadi *et al*., 2019). It is an evergreen, perennial plant with stems that sometimes become more or less wood. It can grow from 90-200cm with a purplish green stem and pink flower. It is cultivated as a medicinal plant that is used conventionally as a blood tonic (haematinics) and also as an ornamental plant in most countries (Nigeria, India, Congo and Cameroon). The fresh leaf of the plant are boiled until a deep purple colour and drank as a tonic or beverage (Houghton, 1995). Traditionally the leaf extracts are used in the management of diabetes, hypertension and sickle cell anaemia (Theiler *et al*., 2017). Recent studies have also demonstrated that the methanol extract of *Justicia secunda* leaf exhibits antioxidant, anti-inflammatory and antinociceptive activities (Onoja *et al.,* 2017). Several studies (Carrington *et al.,* 2012; Kone *et al.,* 2012) have reported the haematinic activities of *J. secunda,* with the presence of some phytochemical components such as alkaloids, tannins, glycosides, flavonoids, saponins, coumarins and sterols (Yamoah *et al.,* 2020). Despite these previous studies, no work has been carried out on the effects of *J. secunda* in trypanosome infection. This study was therefore designed, to evaluate the possible anti-trypanosomal activity of *J. secunda* in *T. brucei*-infected rats.

Materials and Methods

Plant collection, identification and extraction

Fresh leaf of *Justicia secunda* Vahl were collected from the National Corps Research Institute, Umudike (NCRI). The leaf were identified and authenticated at the Department of Plant Science and Biotechnology, Michael Okpara University of Agriculture, Umudike. The fresh leaf were washed with water and dried under a shade at room temperature for a week. The dried plant materials were coarsely powdered using a milling machine. Four hundred and ninety-nine grams (499g) of the fine powder of *J. secunda* was weighed, using a weighing balance. The plant material was soaked with 90% of methanol in a Winchester bottle. This was shaken every 3 hours intervals during the day time and allowed to stand for 72 hours at room temperature and thereafter sieved and filtered through a Whatman number one filter paper. The filter was later concentrated at a temperature of 60^{o}C with the use of an electric oven and the extract was stored in a refrigerator at 4^{o}C for future use.

The percentage yield was calculated using the formula below:

% yield = (weight of extracted material/weight of plant material) x 100/1

Acute toxicity test

This study was carried out using the up-and-down method of acute toxicity test as described by Rispin *et al.* (2002). Six albino rats were selected for the acute toxicity test and they were randomly divided into two groups of three rats each. One group was treated with the plant extract at 200mg/kg while the second group was given an equal volume of distilled water, orally by gastric gavage. Thereafter, the rats were observed for 48 hours for signs of toxicity and mortality.

Experimental animals

Thirty apparently healthy female adult albino rats weighing between 100-110 grams were used for this study. They were sourced from the Department of Physiology and Pharmacology, Michael Okpara University of Agriculture, Umudike. The animals were housed in a well-ventilated fly proof animal house

and allowed to acclimatize for two weeks before the commencement of the study. The animals were humanely cared for in compliance with the principles of Laboratory Animal Care. They were fed commercial pelleted grower feed (Chikun®) and water was given *ad libitum.*

Parasites inoculation

The *Trypanosoma brucei* parasite used in this study was obtained from the Department of Parasitology and Entomology, University of Nigeria, Nsukka. The trypanosomes were passaged in donor rats before infection of the experimental animals. The rats were infected intraperitoneally (ip) with 0.1 ml of saline diluted blood containing 1.5×10^6 trypanosomes. The number of infective trypanosomes was determined using the rapid matching method of Herbert & Lumsden (1976).

Experimental procedure

The albino rats were divided into six groups of five rats each. Group I uninfected untreated (control), group II infected untreated, group III infected and treated with diminazene aceturate (DA), while groups IV, V and VI were infected and treated with *J. secunda* orally at doses of 100 mg/kg, 200 mg/kg and 400 mg/kg body weight respectively, and concentration of 100mg/ml. At the peak parasitemia (one hundred million) (10^8) trypanosomes (day 7 post-infection), group III was treated with a single dose of diminazene aceturate (Babazene ®) intramuscularly at the dose of 7 mg/kg body weight, and concentration of 70mg/ml while the groups IV, V and VI were treated with *J. secunda* for seven days.

Parasitaemia

Two methods were used to monitor the parasitaemia, the wet mount method and micro haematocrit buffy coat microscopy (MBC) as described by Murry e*t al.* (1977). The parasitaemia was monitored daily to determine the pre-patent period, parasite clearance time and weekly 60 days post-treatment.

Post-infection, parasitaemia was monitored daily to determine the onset of infection. Following treatment thereafter, mortality, parasite clearance time, relapse of infection and haematological parameters (packed cell volume (PCV), haemoglobin concentration (Hb), red blood cell count (RBC count), total white blood cell (WBC) and differential cell counts) was determined.

Blood collection and haematological parameters

Blood sample for haematology was collected weekly via the medial cantus of the rats. Two ml of blood was collected from the medial cantus of the eye into vacutainer tubes containing ethylene diamine tetra acetic acid (EDTA) as anticoagulant. The sample bottles were rocked gently to mix the blood with the EDTA to prevent clotting. Packed cell volume (PCV), haemoglobin concentration (Hb), red blood cell count (RBC count), total white blood cell (WBC) and differential cell counts (lymphocytes, neutrophils, monocytes and eosinophils) were analyzed using an Automated Haematology Analyser (model 2800 BC produced by Mindray Company, India) following standard procedures outlined by the producer.

Statistical analysis

Data obtained from the study were expressed as means ± standard deviation. Statistical significance was analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple range test with SPSS version 20 software package. The level of significance was accepted at $p<0.05$.

Results

After oral administration of *J. secunda* leaf extract at the dose of 200 mg/kg and an equal volume of distilled water, no death or any sign of toxicity was observed after 48 hours.

The pre-patent period of infection (PP) was between 5-7 days post-infection (PI) in the infected rats (Table 1). Following treatment from day 7 PI, the parasite cleared from the peripheral blood streams of DA treated group within 24-72 (48.00 ± 13.85^b) hours, 100mg/kg within 72-120 (96.00 ± 13.85^a) hours, 200mg/kg 96-120 (112.00 ± 8.00^a) hours and 400mg/kg 96-120 (104.00 ± 8.00^a) hours, which did not differ significantly ($p>0.05$) in extract treated groups, but were significantly ($p>0.05$) lower in DA treated group compared to extract treated groups.

No relapse was observed in the group that was infected and treated with DA, whereas, relapse of infection occurred in the group that was treated with 100mg/kg on day 28 post-treatment (PT) (day 35 PI). In the groups infected and treated with 200mg/kg and 400mg/kg the parasites relapsed on day 63 PT (day 70 PI) (Table 1). Deaths of the animals started on day 8 PI in the infected untreated and occurred progressively until all the animals in the group were dead. In the infected group treated with 100mg/kg, deaths of two animals occurred on days 10 and 12 PI respectively, and then following the relapse of infection. No deaths

Table 1: Parasitemia, parasite clearance time, survivability and relapse of infection in *T. brucei* infected rats treated with either diminazene aceturate or *J. secunda*

Days post-infection	Uninfected untreated (control)	Infected untreated	Infected treated with DA (7mg/kg)	Infected treated with *J. secunda* (100mg/kg)	Infected treated with *J. secunda* (200mg/kg)	Infected treated with *J. secunda* (400mg/kg)
0	A5/5	A5/5	A5/5	A5/5	A5/5	A5/5
7	A5/5	P5/5	P5/5*	P5/5*	P5/5*	P5/5*
14	A5/5	P2/2	A5/5	A3/3	A5/5	A5/5
21	A5/5	P1/1	A5/5	A3/3	A5/5	A5/5
28	A5/5	M5/5	A5/5	A3/3	A5/5	A5/5
35	A5/5	M5/5	A5/5	R2/2	A5/5	A5/5
42	A5/5	M5/5	A5/5	R1/1	A5/5	A5/5
49	A5/5	M5/5	A5/5	M5/5	A5/5	A5/5
56	A5/5	M5/5	A5/5	M5/5	A5/5	A5/5
63	A5/5	M5/5	A5/5	M5/5	A5/5	A5/5
70	A5/5	M5/5	A5/5	M5/5	R1/5	R1/5
77	A5/5	M5/5	A5/5	M5/5	R1/4	R2/5
84	A5/5	M5/5	A5/5	M5/5	A4/4	A3/3

P = Parasitaemic
A = Aparasitaemic
*= Day of treatment
R= Relapse
M= Mortality
Numerator = Number either aparasitaemic or parasitaemic
Denominator =Number of infected animals per group

were recorded in the groups treated with 200mg/kg and 400mg/kg until after relapse of infection. The survivability and time relapse of infection did not show any significant ($p>0.05$) difference in the groups treated with 200mg/kg and 400mg/kg, but were significantly ($p<0.05$) higher from the infected treated with 100mg/kg.

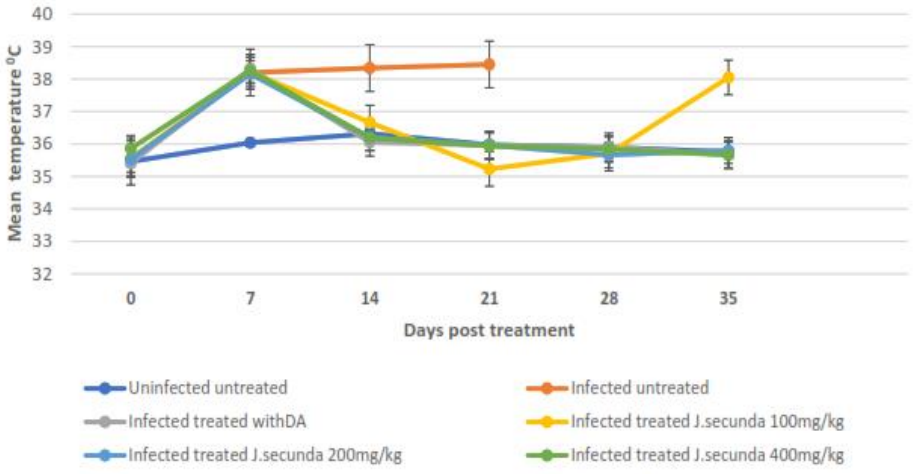

Figure 1: The mean rectal temperature (°C) of *T. brucei* infected rats treated with either diminazene aceturate or *J. secunda*

The mean temperature was significantly ($p<0.05$) higher on day 7 PI in all the infected groups. From day 14 PI (day 7 PT) the mean rectal temperature of both the DA and extract treated groups were significantly ($p<0.05$) lower than the infected untreated and comparable with the uninfected untreated control. However, on day 35 PI (day 28 PT) the mean temperature of the group treated with 100mg/kg became significantly ($p<0.05$) higher than all other infected treated groups and the control (Figure 1). The mean packed cell volume (PCV), haemoglobin concentration (Hb) and red blood cell counts were significantly ($p<0.05$) lower in all the infected groups on day 7 PI. Following treatment, the parameters significantly ($p<0.05$) increased on days 14 and 21 PI (day 7 and 14 PT) in DA and extract treated groups compared to the infected untreated group, which declined progressively until the deaths of all the animals. While on days 28 and 35 PI (day 21 and 28 PT) the parameters became significantly ($p<0.05$) lower in 100mg/kg treated group compared to

200mg/kg, 400mg/kg and DA treated group. No significant (p>0.05) variation was observed between 200mg/kg and 400mg/kg extract treated groups (Figures 2, 3 and 4). The mean TWBC count was significantly (P<0.05) higher in all the infected groups on day 7 PI compared with the uninfected untreated control. On day 14 PI (day 7 PT) the TWBC counts of the infected treated groups did not show any significant (P>0.05) difference, but were significantly (P<0.05) lower than the infected untreated group.

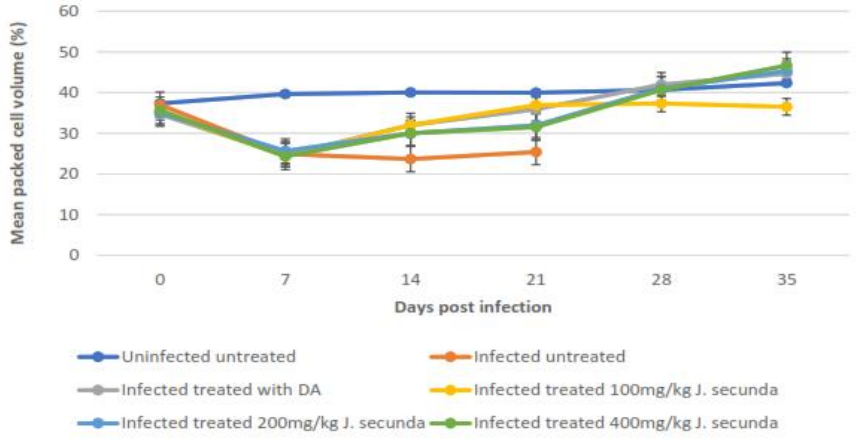

Figure 2: The mean packed cell volume (%) of *T. brucei* infected rats treated with either diminazene aceturate or *J. secunda* leaf extract

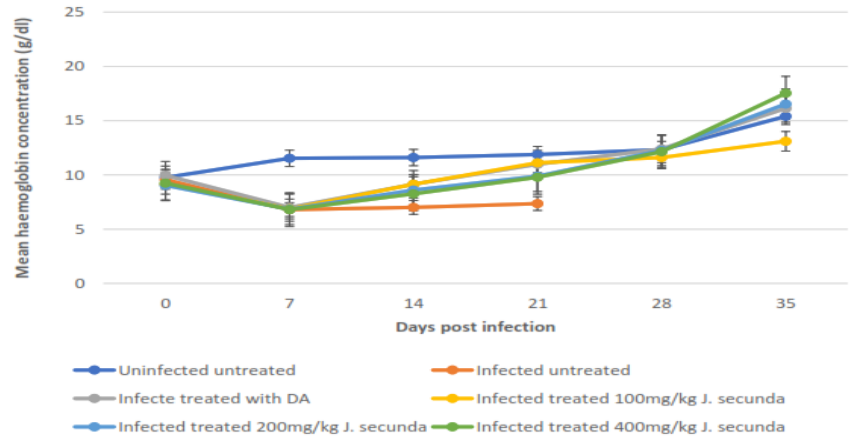

Figure 3: The mean haemoglobin concentration (g/dl) of *T. brucei* infected rats treated with either diminazene aceturate or *J. secunda* leaf extract

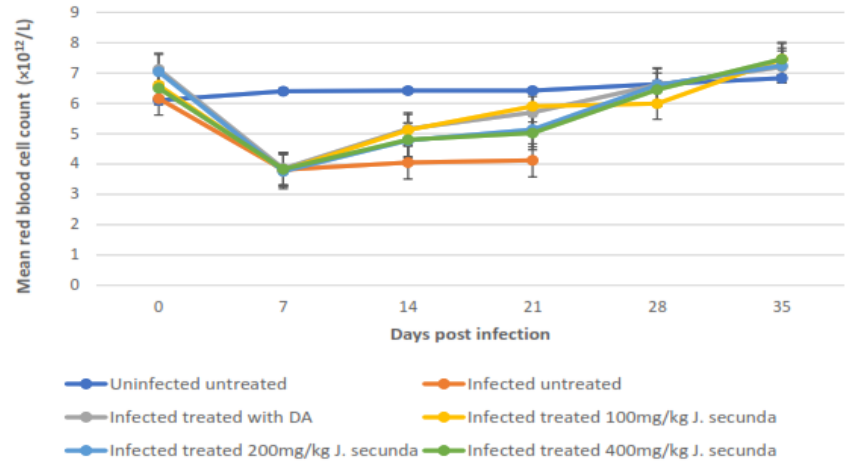

Figure 4: The red blood cell counts ($\times 10^{12}$/L) of *T. brucei* infected rats treated with either diminazene aceturate or *J. secunda* leaf extract

There was significant (P<0.05) variation in the TWBC in the groups treated with DA, 200mg/kg and 400mg/kg on day 21 PI (day 14 PT), while the group treated with 100mg/kg and uninfected untreated control did not show any significant (P>0.05) difference. From day 28 PI (day 21 PT) the TWBC count of the infected treated groups and the uninfected untreated control did not show any significant (P>0.05) difference (Figure 5). The mean lymphocyte counts of all the infected groups did not show any significant (P>0.05) difference on day 7 PI, but were significantly (P<0.05) lower than the uninfected untreated control. From day 14 PI (day 7 PT) the lymphocyte counts of all the infected groups did not show any significant (P>0.05) difference, but were significantly (P<0.05) higher than the infected untreated group and comparable with the control (Figure 6). The mean neutrophil counts of all the infected groups did not show any significant (P>0.05) difference on day 7 PI, but were significantly (P<0.05) higher than the uninfected untreated control. From day 14 PI (day 7 PT) the neutrophil counts of the infected treated groups became significantly (P<0.05) lower compared to infected untreated group and there were no significant (P>0.05) variations between the infected treated groups and the uninfected untreated control till the end of the experiment (Figure 7).

Discussion

The experimental infection of the rats with *T. brucei* was successful with a pre-patent period of 5-7 days post-infection. This result is consistent with the findings of Anene *et al.* (1999) and Ezeh *et al*. (2019) in rats, Ezeokonkwo & Agu (2004) in rabbits and Akpa *et al.* (2022) in dogs. This study also revealed a prolonged survival time and reduced the risk of relapse in the groups treated with the extract of *J. secunda* at the doses of 200mg/kg and 400mg/kg body weight. These findings may be attributable to the phytochemical components of the plant such as flavonoids, saponins and tannins and their secondary metabolites, which are largely responsible for the medicinal role of the plants (Yamoah *et al.,* 2020). *J. secunda* has also been reported to possess antinociceptive, anti-inflammatory and antioxidant activities (Onoja *et al.,* 2017). The ability of *J. secunda* leaf extract to control parasitaemia level and also extend the survival time of the rats, in groups treated with 200mg/kg and 400mg/kg shows that the plant possesses anti-trypanosomal activity, which is dose-dependent.

No relapse was observed in the DA treated group unlike the extract treated groups. This result contrasts the findings of Anene *et al*. (2006) who recorded relapsed infection by day 42 PI in rats treated with DA; but agrees with the findings of Rani & Suresh (2007) who recorded no relapse in Pomeranian dog treated with a single dose of DA. This result is likely due to the early treatment commenced in the group

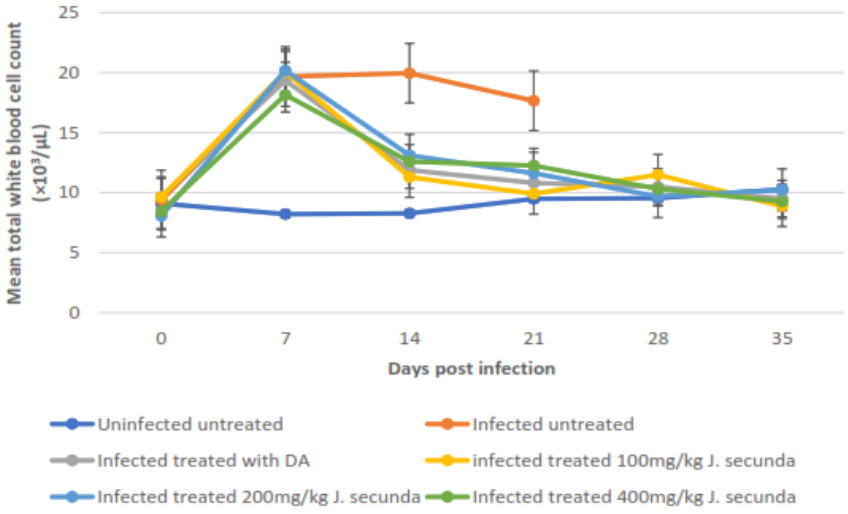

Figure 5: The mean total white blood cell counts (×10^3/µL) of *T. brucei* infected rats treated with either diminazene aceturate or *J. secunda* leaf extract

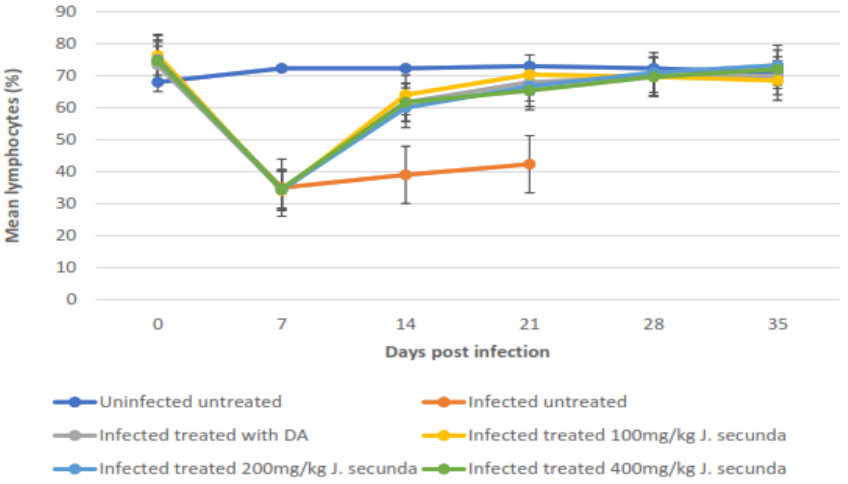

Figure 6: The mean lymphocyte counts (%) of *T. brucei* infected rats treated with either diminazene aceturate or *J. secunda* leaf extract

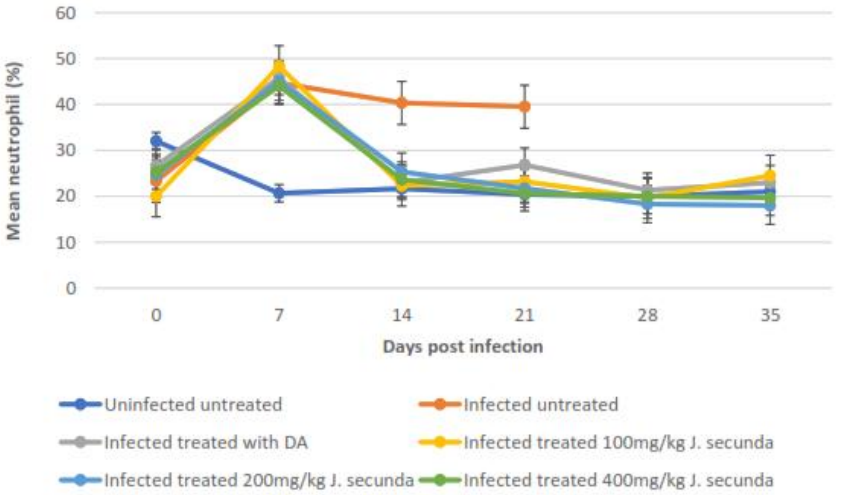

Figure 7: The mean neutrophil counts (%) of *T. brucei* infected rats treated with either diminazene aceturate or *J. secunda* leaf extract

treated with DA (day 7 PI). Early treatment after infection usually leads to permanent cure unlike late treatment (from day 14 PI) which normally leads to a relapse of infection (Adieme *et al*., 2013). Death occurred progressively in the infected untreated group and the group treated with 100mg/kg due to anaemia associated with immunosuppression commonly seen in African trypanosomosis (Anosa *et al*., 1997). The increased parasitaemia may have overwhelmed the immune response of the infected rats, thereby not allowing the rats enough time to produce sufficient antibodies to fight the invading parasites. The trypanosome infection of the rats also induced pyrexia, which is a common feature of African trypanosomosis (Taylor & Authie, 2004). The treatment with *J. secunda* extracts ameliorated the pyrexia, which may also be due to its hepatoprotective, anti-inflammatory and antioxidant activities (Aimofumeh *et al.,* 2020). The presence of structural hydroxyl functional groups has also been thought to be responsible for the beneficial biological effects of *J. secunda* in the management of various health conditions (Araujo *et al.,* 2015).

The red cell parameters (PCV, Hb and RBC counts) were decreased in the infected groups but improved in the groups treated with 200mg/kg and 400mg/kg to a level comparable with DA treated group and uninfected untreated (control). Anaemia in African animal trypanosomosis is due to increased erythrocyte fragility and susceptibility to oxidative damage as previously reported by other researchers (Taiwo *et al.,* 2003; Sivajothi *et al.,* 2015). The marked improvement in the haematological parameters and eventual reversal of anaemia in the extract treated groups as seen in this study is attributable to the decrease in oxidative stress markers caused by the administration of the extracts. This result corroborates the findings of Abdullahi *et al*. (2023), who reported improved haematological parameters in *T. evansi* infected rats treated with *Balanties aegyptiaca.* The presence of some phytochemicals and iron reach content of *J. secunda* has been reported to be responsible for the observed haematinic activity of the plant extract (Yamoah *et al.,* 2020).

Then infection of the rats with *T. brucei* showed leucocytosis, which was maintained in the infected untreated group throughout the study. This result could be attributed to the immune response associated with African trypanosomosis (Anosa *et al*., 1997; Ndoutamia *et al.,* 2002), which agrees with other workers of Ukwueze *et al.* (2022) who reported leucocytosis in dogs infected with *T. brucei,* but disagrees with Kobo *et al*. (2014) who observed decreased total leucocyte counts in infected untreated rats. Following treatment with *J. secunda* leaf extract the total leucocyte counts returned to pre-infection values, which is an indication that the administration of the extract was able to clear the parasite from the blood stream and stabilize the immune system. Leucocytic response is important marker for assessing the level of immune response under stressful and diseased conditions as they are essential in protecting the body against infectious agents (Hardie *et al.,* 1991; Ufele *et al.,* 2007).

In conclusion, the ethanolic extract of *J. secunda* leaf exhibited dose-dependent anti-trypanosomal activity in *T. brucei* infected rats. The extract was also able to ameliorate and conserve anaemia in *T. brucei* infected rats, as seen in the improved haematological parameters, increased survival time and reduced risk of relapse.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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