ANTIOXIDANT POTENTIAL OF *WALTHERIA INDICA* METHANOL LEAF EXTRACT IN *TRYPANOSOMA BRUCEI BRUCEI* INFECTED MICE

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Abstract
African Trypanosomiasis is parasitic disease transmitted by tsetse flies, which is an important neglected tropical disease endemic in remote regions of sub-Saharan Africa. The search for alternative antitrypanosomal drugs has become imperative due to limitations associated with commonly used conventional drugs. In the present study, antitrypanosomal and antioxidant activities of Waltheria indica Lin (W. indica) were evaluated in T. brucei infected mice. The DPPH radical scavenging activity and reducing power activity of the extract were analyzed. Serum and liver superoxide dismutase (SOD), catalase and glutathione (GSH) activities were evaluated after treatment. Thirty mice were intraperitoneally infected with Trypanosoma brucei and were randomly selected into five groups of 6 mice each. Group A -C were treated with 200, 400 and 600 mg/kg bw methanol extract of W. indica respectively, while groups D - E served as normal (untreated) control and standard control (3.5mg/kg diaminazene aceturate) respectively. Daily parasitaemia counts were carried out. The packed cell volume (PCV) and the weight changes of the mice were also monitored. The results revealed that W. indica was found to increase DPPH radical scavenging activity with IC_{50} 39.41 while it reducing power activity was lower than the standard ascorbic acid. The serum and liver SOD activities, serum catalase and liver GSH activities were significantly ($p<0.05$) lower in infected untreated mice when compared with untreated control. Treatment with the extract significantly ($p<0.05$) increases the activities of these enzymes when compared with the untreated control. There was a progressive increase in parasitaemia count (2.50±0.05 to 160.34±12.78) with decrease body weight and PCV of infected untreated mice. The mice treated with methanol extract of Waltheria indica produced dose dependent decrease in parasite multiplication for 200 mg/g kg (3.84±0.55 to 79.45±6.32), 400 mg/g (3.10±0.55 to 29.56±6.54 and 600mg/kg bw (2.94±0.32 to 18.35±4.94) respectively when compared with untreated control. While the mice treated with 600mg/kg bw showed improvement in body weight and PCV when compared with untreated control. In conclusion the methanol extract of Waltheria indica inhibited T.brucei replication and ameliorated the parasites induced anemia and oxidative stress in mice

Keywords: Trypanosomiasis, Antioxidants, *Waltheria indica, Trypanosoma brucei brucei*

Introduction
Trypanosomiasis is a parasitic disease of flagellated protozoan belonging to the genus *Trypanosoma* (Abebe, 2005; Barrett, 2003). Trypanosomiasis is a disease of both humans and animals which are referred to as Human African Trypanosomiasis (HAT) and Animal African Trypansomiasis (AAT) respectively. The disease is transmitted through the bite of tsetse fly (Glossina species) which introduces the causative agent, trypanosome, into the blood (WHO 2017; D'Archivio *et al*., 2011). The symptoms associated with the acute form of the disease is noticed with intermittent fever, anemia, emaciation, production losses, dullness with recumbency or staggering gait, labored breathing, lachrymation, bellowing, profuse salivation, twitching of muscles often terminating in convulsions and death (WHO,2014; Kennedy,2013). The chronic form last for years and is noticed with dullness, anemia, emaciation, recurrent fever, edema in dependent part of body, conjunctivitis,

lacrymation, enlargement of the superficial lymph nodes, abortions, infertility, reduced milk yield, progressive emaciation and lowered work out-put (WHO,2014; WHO, 2017; Kennedy, 2013; Isaac *et al*., 2016).

The early stage of infection involves a profound pro-inflammatory type 1 activation of the mononuclear phagocyte system (MPS) in trypano-susceptible animals causing immunopathology with anemia as the most prominent pathological feature (Naessens *et al*., 2006). The cause of anemia is considered to be as a result of dyshemopoiesis and erythrophagocytosis (Benoit *et al*., 2018; Mbaya *et al.,* 2012; Naessens *et al*., 2006). The infection with trypanosomes results in the production of large amount of reactive oxygen species (ROS) and free radicals which act as cytotoxic agent damaging vital components of the cell, including proteins and lipids. Oxidative stress occurs when there is an imbalance between radical-generating and radical-scavenging activity; a deleterious process that can damage cell structures, including lipids, proteins, and DNA (Pham-Huy *et al*., 2008). Several reports have shown an important role of free radical induced oxidative stress in the pathogenesis of Trypanosomiasis (Benoit *et al*., 2018). Antioxidants inhibit oxidative species generation and are scavengers of free radicals that provide protection to humans and animals against infections and degenerative diseases with oxidative stress etiology. Nature has endowed us with protective antioxidant mechanisms such as superoxide dismutase (SOD), catalase, glutathione peroxidases and reductase apart from many dietary components (Ahmed 2005).

Medicinal plants confer numerous health benefits as they combat oxidative stress in the body by maintaining a balance between oxidants and antioxidants (Willcox *et al*., 2004). *Waltheria indica* L. (Malvaceae) is a short-lived shrub that can reach up to 2 m in height and is widespread in subtropical and tropical regions of the world (Jansen *et al*., 2010). In traditional medicine, the roots, aerial parts, and whole plant either are taken as a decoction, infusion, or macerate or are chewed or directly applied on wounds. Many ailments such as skin ulcers, rheumatism, diarrhea, hemorrhoids, asthma, or tooth infections are treated with this plant. In Niger Republic and Nigeria, traditional healers give the whole plant to cattle as a tonic, suggesting a possible activity against "Nagana'' (trypanosomiasis in cattle) (Bala *et al*., 2009). *Waltheria indica* possess some chemical constituents capable of ameliorating trypanosomiasis and oxidative stress. The phytochemicals of the plant include alkaloids, phenols and phenolic glycosides, flavonoids, phlobatannin, steroids, tannins and terpenes (Madaki *et al*., 2016; Zango *et al*., 2013; Bala *et al*., 2011; Banso, 2009). The ant-oxidant effect (Saidu et al., 2012); trypanocidal effect (Bala *et al*., 2011), ant-bacterial effect (Olujuyigbe et al., 2011) of *Waltheria indica* have been reported. Therefore, the evaluation of oxidative stress markers and clinical studies of the herbal medicine for the safety and toxicity are required to ascertain the degree of damage to hosts tissues caused by the infection and the health status of the infected animals.

Materials and Methods

Plant Collections and Identification

The leaves of *W. indica* was collected from Bosso in Minna, Niger State, Nigeria, between the months of July, 2018. It was taken to the Department of Biological Sciences, Federal University of Technology, Minna, for the authentication.

Chemicals and Reagents

All the reagents used were of analytical grade. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), acetic acid, dimethyl sulfoxide (DMSO) was purchase from Sigma (St Louis, USA).

Sample Preparation and Extraction

The plants were destalked; the leaves were washed under running tap water and dried under laboratory condition. The dried plant sample was milled into powdered using mortar and pestle. Exactly 100 g of the plant samples was weighed and extracted with 600 ml of 70% methanol using the cold maceration method. The extracts was dried in hot water bath at 35ºC and stored in sample bottles in the refrigerator at 4ºC until required.

Calculation of extract yield

The % yield of crude extract was calculated as follows:

Weight of sample used for extraction = a (g)

Weight of extract obtained =b (g)

% yield of crude extract $\frac{b}{-}$ 10

Phytochemical analysis

Phytochemical analysis of crude extract of plant material was carried out using standard procedure to identify the constituents as described by Sofowora (1993), Trease and Evans (1989) and Harbone (2000).

Experimental Animals

A total of twenty (30) mice weighing 27-30 g were obtained from Animal Holding Unit, Department of Biochemistry, Federal University of Technology Minna, Nigeria. The animals handling and experimentation was in accordance with the guidelines for laboratory animal use and care as contained in the European Convention on Animal Care Guidelines and Protocol. Test Organism

T. brucei brucei was obtained from stabilates maintained at the Nigerian Institute of Trypanosomiasis Research (NITR), Kaduna, Nigeria; in July, 2018. The parasites were maintained in the Animal laboratory by serial passage in mice until it was required for use. Passage was carried out when the parasitaemia was in between the range of 16 – 32 parasites per field (usually 3 - 5 days post infection). 1×10^3 parasites in 0.1ml blood/PBS were injected intraperitoneally into normal mice.

In vivo Antitrypanosomal Study

Acute toxicity studies (LD_{50})

Acute toxicity study was carried out using the Lorke's method (Lorke ,1983).

Experimental design

Animals were divided into six groups of mice labeled A-F. Animals in groups A-E were infected with 10^3 *T. brucei brucei* intraperitoneally (Kabiru *et al.,* 2013). Mice in groups A-C were treated with *Waltheria indica* methanol extract 200, 400, and 600mg/kg/body weight respectively, mice in group D were infected not treated which served as negative control (NC) while mice in groups E were treated with standard drug (3.5 mg/kg bw diaminazene aceturate). Treatment was carried out orally twenty four hours after infection with parasite for eleven (11) consecutive days. Mice in group F were given orally physiological saline and served as uninfected and not treated (normal group). The Parasitaemia count was monitored daily using a wet blood film viewed under light microscope at x40 magnification according to the method of Herbert and Lumsden, (1976).While packed cell volume (using Hematocrit method) and weight (using analytical balance) were determined at the first day before parasite inoculation and seventh day before mice were sacrificed for antioxidant enzymes assay.

In vitro antioxidant study

DPPH Radical scavenging activity

This test was conducted as described by Blois, (1958) and reported by Nisha *et al.,* (2012). The ability to scavenge the DPPH radical was calculated using the following equation:
DPPH scavenging activity (%) = $(A_0 - A_1) / A_0 \times 100$ Nisha *et al.,* (2012).
Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Reducing power assay

Reducing power was determined by the method described by Oyaizu *et al*., 1986 and reported by Nisha *et al.,* 2012. The absorbance was measured at 700 nm. A stronger absorbance indicates increased reducing power

In vivo antioxidant study

Collection and preparation of Serum and liver

The blood sample collection was according to the method of Shittu *et al.,* (2014).

Estimation of Glutathione Peroxidase

The activity of Glutathione peroxidase (GPx) in tissues was determined by using the method of Rotruck *et al*., (1973). The glutathione peroxidase activity was expressed as Units per milligram protein (Units/mg protein)

Calculations:

$$\text{GPx activity} = \frac{\Delta OD/min \times GSH\ Std. \times Total\ reaction\ volume}{OD\ of\ standard \times 307.32 \times volume\ of\ enzyme\ source \times protein\ conc}$$

Where: 307.32 = molecular weight of GSH

Estimation of Superoxide Dismutase (SOD)

Superoxide dimutase (SOD) is an enzyme responsible for the removal of superoxide formed from oxygen in tissues. This was done by the method of Yusuf, (2010). The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds.

Calculation:

$$\text{Increase in absorbance per minute} = \frac{A_3 - A_0}{2.5}$$

Where A_o = absorbance after 30 seconds
A_3 = absorbance after 150 seconds

Estimation of Catalase Activity

The catalase activity in serum was determined using the modified method as described by Atawodi *et al*., (2011).

Determination of Protein

The total protein content of the serum was assayed using commercially available total protein kit (Randox Laboratories, UK), employing direct Biuret method as described by (Onoja *et al*., 2014).

Statistical analysis

All values were expressed as the Mean ± SEM of three replicate experiments. The analysis was performed using SPSS statistical package for WINDOWS (version 16.0; SPSS Inc,

Chicago). Results were subjected to one-way ANOVA followed by Duncan Multiple range test and $p<0.05$ were considered to be statistically significant.

Results

Percentage Yield of *Waltheria indica*

The yield obtained from methanol extract of *Waltheria indica* was 28%

Phytochemical composition of *Waltheria indica*

Table1 shows the quantitative phytochemical composition of crude methanol extract of *Waltheria indica*. The results revealed that flavonoids were the highest followed by phenols, saponins, alkaloids and tannins was the lowest phytochemical.

Table 1. Quantitative phytochemical composition of methanol extract of *waltheria indica*

Phytochemicals	mg/100g
Flavonoids	988.92±5.49
Phenols	270.38±4.10
Tannins	12.54±1.21
Saponins	237.50±30.00
Alkaloids	29.32±0.33

Values are expressed as Mean ±SEM

In vitro antioxidant study

Figure1 shows the result of the DPPH Radical Scavenging Activity of *Waltheria indica* methanol extract. *Waltheria indica* had higher antioxidant activity (IC_{50}= 39.41) when compared to ascorbic acid (IC_{50}= 64.84*),*

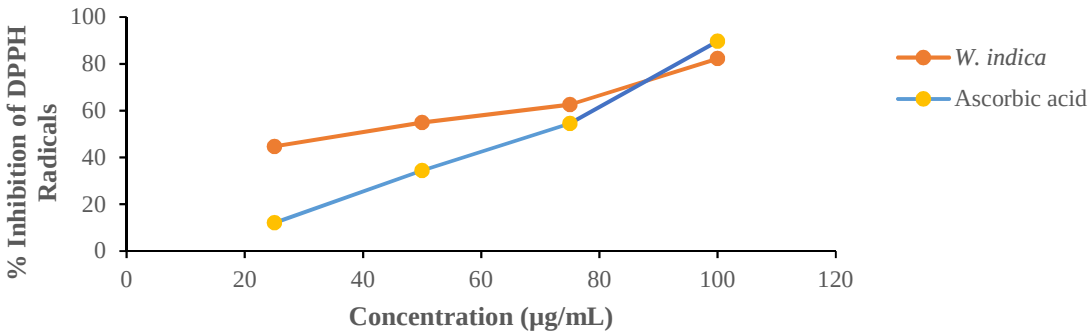


Figure 1: DPPH Radical Scavenging Activity of methanol extract of *Waltheria indica*

Figure 2 shows the result of the Ferric oxide reducing power of *Waltheria indica* methanol extract. The reducing power activity of *Waltheria indica* is lower than the standard ascorbic acid.

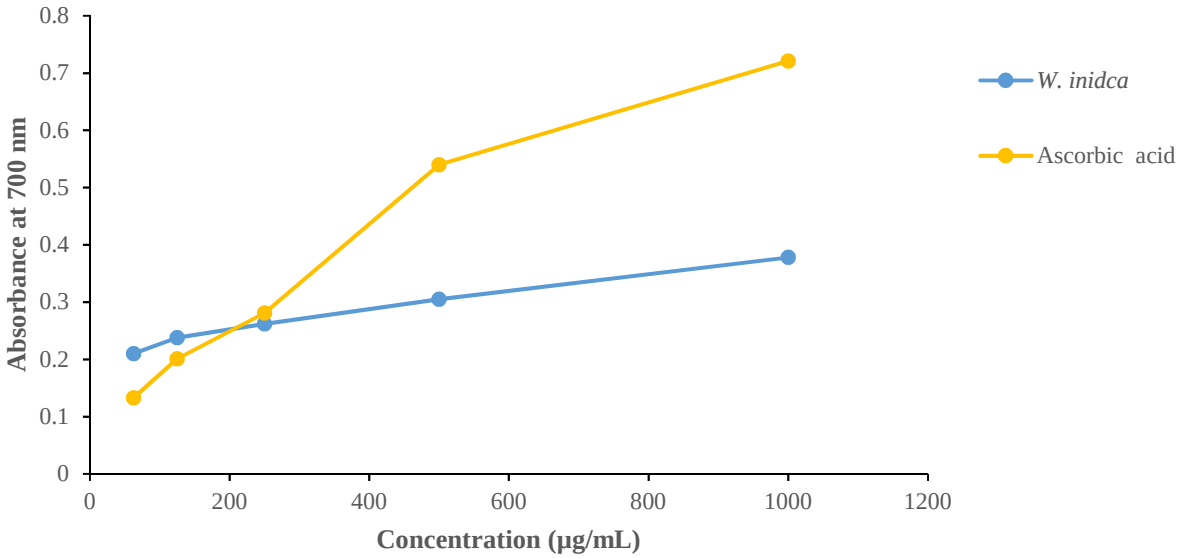


Figure 2: Reducing Power Activity of methanol extract of *Waltheria indica*

In vivo Antitrypanosomal Study

Acute toxicity

Acute toxicity study of the methanol extract of *Waltheria indica* L. administered in mice by intraperitoneally showed no mortality at the highest dose of 5000 mg / kg weight of the animal.

Parasitaemia count

The results of average parasitaemia count of *T. brucei* infected mice treated with methanol extract of *Waltheria indica* is presented in Figure 3. Infected untreated mice produce significant and progressive increase parasite replication. The mice treated with 200, 400 and 600 mg/kg bodyweight of methanol extract of *Waltheria indica* produced dose dependent decrease in parasite multiplication when compared with untreated control. The infected mice treated with the diaminazene aceturate shows complete parasite clearance on day 3 and the animals survived throughout the experiment.

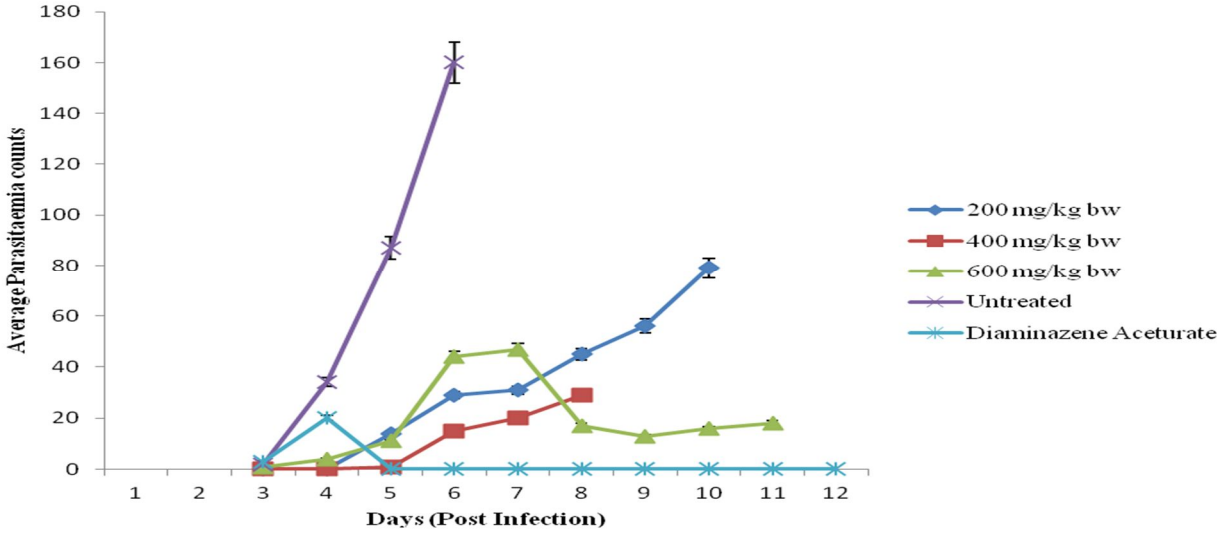


Figure 3: The average (n=3) course of parasitaemia of *T. brucei* infected mice treated with *Waltheria indica* methanol leaf extract

Body Weight Changes

Effect of methanol extract of *Waltheria indica* on bodyweight of *T. brucei* infected mice are shown in figure 4. Infected untreated mice shows decrease in body weight post treatment. However, groups of mice treated with 200 and 600 mg/kg bodyweight of *Waltheria indica* causes slight improvement in body weight post treatment as compared with the infected untreated control. While 400 mg/kg bodyweight showed no significant change in body weight.

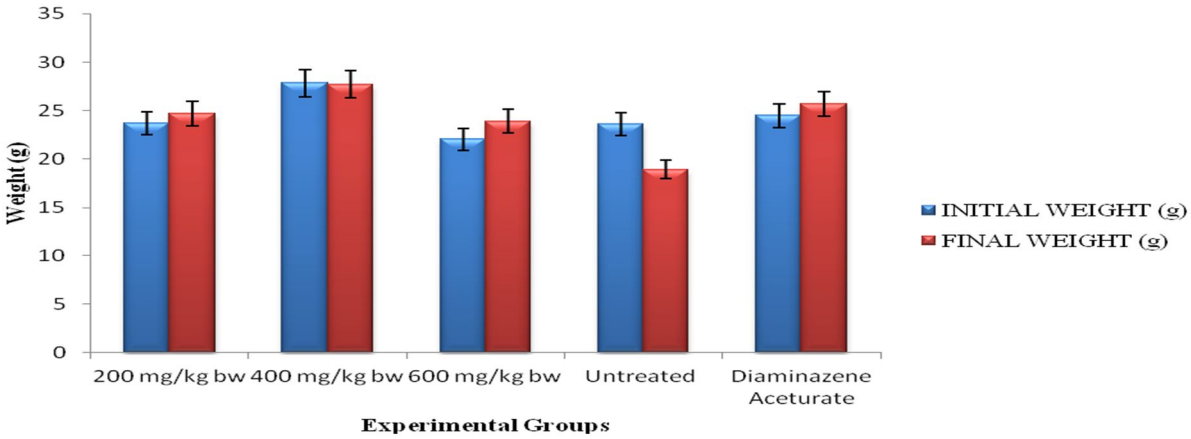


Figure 4: Effect of *Waltheria indica* methanol leaf extract on bodyweight of *T. brucei* infected mice

Packed Cell Volume (PCV)

Effect of methanol extract of *Waltheria indica* on packed cell volume of *T. brucei* infected mice are shown in figure 5. Infected untreated mice as well as those treated with 200 and 400 mg/kg bodyweight shows decreases in PCV post treatment. However, groups of mice treated with 600 mg/kg bodyweight of *Waltheria indica* and those treated with diaminazene aceturate showed an improvement in PCV post treatment when compared with the infected untreated control.

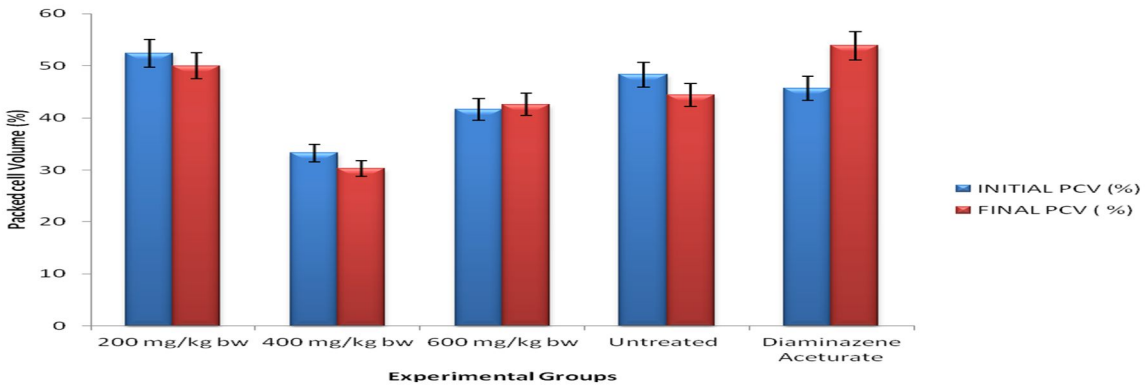


Figure 5: Effect of Methanol Extract of *Waltheria indica* on Packed Cell Volume (PCV) of *T. brucei* Infected Mice

Antioxidants Studies

Serum and Liver Total Proteins

The serum and liver total proteins concentrations were significantly ($p<0.05$) lower in infected untreated mice when compared with normal control. Treatment of infected mice with methanol extract of *Waltheria indica* significantly increased the serum and liver total

proteins concentration when compared with the untreated control. Mice treated with diaminazene aceturate showed the highest liver concentration of total proteins when compared with other experimental groups.

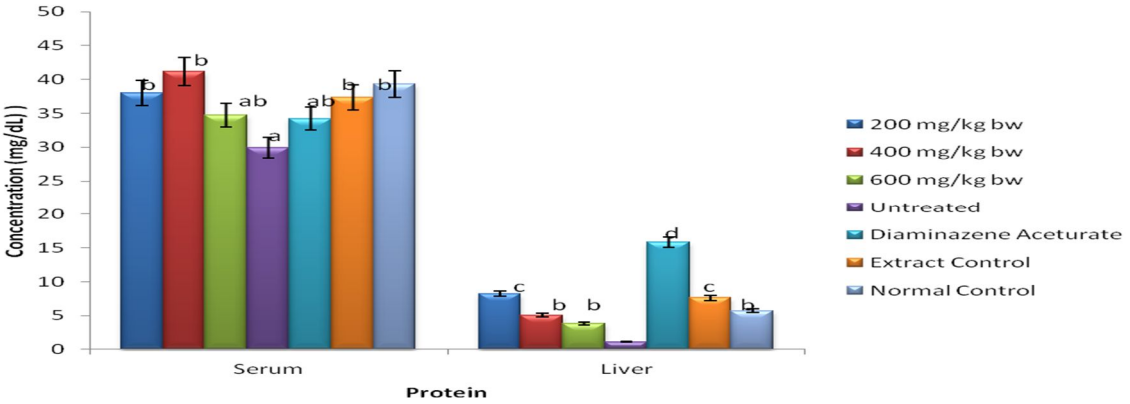


Figure 6: Effect of methanol extract of *Waltheria indica* on total protein concentration in *T. brucei* infected mice

Serum and Liver Glutathione Peroxidase

The GSH activities were significantly higher ($p<0.05$) in liver and significantly lower in serum of uninfected and treated mice when compared with normal control and infected treated groups. Mice treated with 400 mg/kg have the highest serum glutathione peroxidase activities when compared with control and other experimental groups. However, infected untreated mice recorded the least ($p<0.05$) liver GSH activities when compared with normal control and infected treated groups.

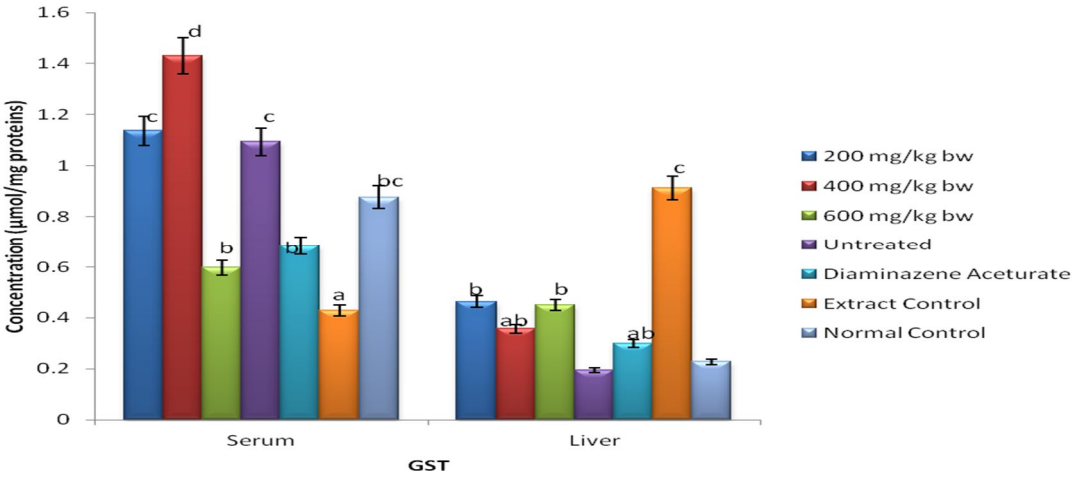
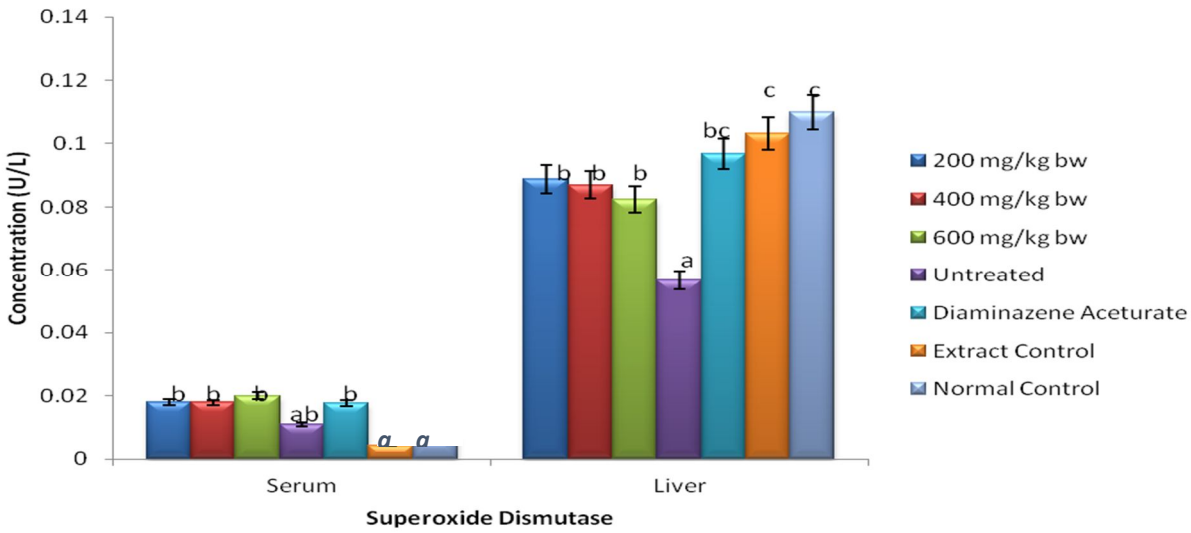


Figure 7: Effect of methanol extract of *Waltheria indica* on GSH activities in *T. brucei* infected mice a a

Serum and Liver Superoxide Dismutase (SOD)The serum and liver activities of superoxide dismutase (SOD) were significantly ($p<0.05$) lower in infected untreated mice when compared with normal control. Treatment of infected mice with methanol extract of *Waltheria indica* significantly increased the serum and liver superoxide dismutase (SOD)



when compared with the untreated control.

Figure 8: Effect of methanol extract of *Waltheria indica* on superoxide dismutase activities in *T. brucei* infected mice

Serum and Liver Catalase activities

The serum activities of catalase were significantly ($p<0.05$) lower in infected untreated mice when compared with normal control and other treatment groups. Treatment of infected mice with methanol extract of *Waltheria indica* significantly increases the catalase activities when compared with the untreated control. Mice treated with diaminazene aceturate recorded the highest liver catalase activities compared with control and other experimental groups

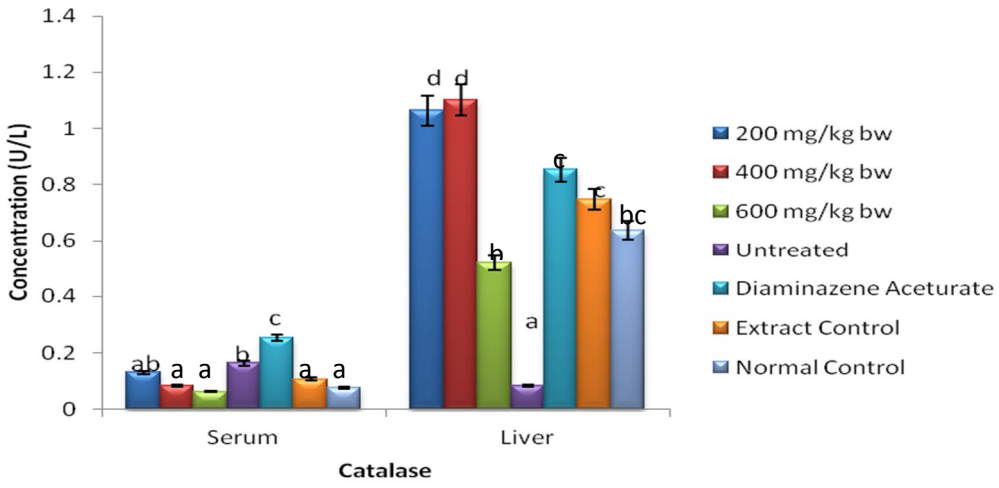


Figure 9: Effect of methanol extract of *Waltheria indica* on catalase activities in *T. brucei* infected mice

Discussion

Recently, there has been an upsurge of interest in the therapeutic potential of plants as antioxidants in reducing oxidative tissue injuries (Patel *et al*., 2010). *Waltheria indica* is a plant used in traditional medicine to treat several pathologies (Saidu *et al*., 2012; Yougbare-Ziebrou *et al.,* 2016). The study assessed the *in vivo* and *in vitro* antioxidant and antitrypanosomal properties this plant. The result of this study revealed *Waltheria indica* extract yield as 28% which is similar to extract yield (13.68%) obtained by Madaki *et al*., 2016, for extracting 50 g of *W. indica* leaf powder*. W. indica* methanol leaf extract was found to contain high amount of flavonoids, while alkaloids was the least phytochemical observed as shown in Table 1. Phytochemicals contained in medicinal plants enables them exert their therapeutic effects and serve as precursors for the synthesis of useful drugs (Abolaji *et al*., 2007).

The result of the parasitaemia counts (Fig.3) shows that methanol extract of *W. indica* has trypanocidal properties by the ability to extend the life span of T.brucei infected mice as well as lower the replication of trypanosome of treated mice in a dose dependent pattern. Different phytochemicals including flavonoid alkaloid and polyphenols have been reported for antitrypanosomal activities (Hoet *et al*., 2004). Therefore, the antitrypanosomal activities of *W. indica* demonstrated in this study could be attributed to the presence of these phytochemicals as shown in Table 1 which may be acting singly or synergistically to bring about the trypanocidal effect observed.

However, the activities demonstrated by *W. indica* are lower than that of diaminazene aceturate. This observation is expected because, it, been suggested that crude plant extracts tend to have lower antitrypanosomal effects because unpurified bioactive compounds may require initial conversions which time lag allows for parasite proliferation (Noedl *et al*., 2003).

Hepatomegaly that occurs in *T. brucei* infection has been reported to be directly related to the severity of anaemia and levels of parasitaemia (Anosa, 1988). Anaemia is a fairly common problem encountered in trypanosome infection. The haemolysis may be due to the parasite consumption and degradation of the intracellular proteins which are mainly hemoglobin (Gavigan *et al*., 2001). Infected untreated mice as well as those treated with 200 and 400 mg/kg bodyweight of *W. indica* shows decrease PCV post infection. These decreases however were considerably reversed in the infected mice treated with 600 mg/kg bodyweight and standard group.

Oxidative stress plays important etiologic role in the pathogenesis of African trypanosomiasis (Ogunsami & Taiwo, 2007). Removal of the parasite from the system and simultaneously boosting the host immune and antioxidant system could be very relevant in the control of African trypanosomiasis (Hoet *et al*, 2004).

The *in vitro* antioxidant activity exhibited by *W. indica* as shown in the result of reducing power activity (Figure 2) was lower than standard ascorbic which has a stronger absorbance and hence higher reducing power while the result of DPPH radical scavenging activity (Figure 1), showed that *Waltheria indica* had higher antioxidant activity (IC_{50}= 39.41 µg/ mL) when compared to ascorbic acid (IC_{50}= 64.84 µg/ mL). This result opposes the result of Yougbare-Ziebrou *et al.,* (2016) which shows a low DPPH radical activity with an IC50 of 79.5 µg/ mL when compared to that of quercetin whose inhibitory concentration is 0.69 µg /mL. The disparity in result could be as a result of different solvent and standard used. Therefore, this activity could be attributed also to the presence of phytochemicals. It is well documented that many plants with high contents of phenolic compounds (polyphenols,

flavonoids and tannins) exhibit a strong antioxidant activity, although, *W. indica* was more effective than standard (ascorbic acid) and various factors like stereo-selectivity, solubility of the extract, polarity of the solvent, functional groups present in phytochemical compounds could be the possible reason for increase in the reaction with DPPH radical (Herrera-Calderon *et al*., 2016).

The decrease activities of catalase, SOD and GSH observed in infected untreated mice could be due to the severity of trypanosome infection which led to the mobilization of antioxidant enzymes to fight the presence of the parasites and were overwhelmed or exhausted by the high level of free radicals towards the late stage of infection. Alteration in antioxidant enzyme level may result from the effect of the trypanosome lyses resulting from effect of the host defense mechanism (Pentreath & Kennedy, 2004). Also reports (Opara *et al*., 2017) has shown that under late condition of oxidative stress, activities of antioxidant enzymes such as SOD, catalase and GSH decreases. Importantly, the extract was able to increase the SOD, GSH and the catalase levels as compared to infected negative control mice. Perhaps the extract was able to provide some antioxidants components and spared the use of endogenous catalase, to fight trypanosomes-generated free radicals (Yusuf *et al*., 2012). Antioxidant system are normally put in place in living aerobic organism to counter the effect of oxidative stress (Shittu *et al*., 2014). According to the studies of Sepulveda – Boza and Cassels (1996) suggested that many natural products exhibited their trypanocidal activity by virtue of their interference with the redox balance of the parasites acting either on the respiratory chain or on the cellular defenses against oxidative stress.

Conclusion

Based on the results obtained from this study it is concluded that methanol extract *Waltheria indica* has potential in the management of African trypanosomiasis due to its ability to reduce the parasitaemia level and extension of the life span of the infected treated mice. It also has ameliorative effect on *T. brucei* induced oxidative stress in mice and thus could be considered as a source for alternative antitrypanosomal agent.

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