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In vivo Anti-trypanosomal Evaluation and Phytochemical Analysis of Methanol Extract of *Nauclea latifolia*

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Authors' contributions

This work was carried out in collaboration between all authors. Author CEU wrote the protocol and the first draft of the manuscript. Author IOE designed the study. Authors CEU and IOE also managed the experimental process. Authors COA, NCO and SAC were in charge of literature searches and statistical analysis. The final gallery copy is approved by all authors.

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ABSTRACT

Aim: Trypanosomiasis is a pathological condition that requires serious attention. The dried root of *Nauclea latifolia* was investigated to determine the anti-trypanosomal activity on groups of Wistar rats.
Methods: A 1000 mg/kg body weight of methanol extract was administered for 7 consecutive days by intraperitoneal route (ip) to Group II while Group I received a 3.5 mg/kg of Samorinil® through the same route. The phytochemical constituents were also determined. Parasitaemia level, body weight, temperature and packed cell volume (PCV) were monitored and determined before and after the commencement of the experiment.
Results: The packed cell volume (PCV) increased significantly (P = .05) in Group I and II post

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infection from an average of 35.0 ± 0.68 to 38.05 ± 0.32% when compared to the infected - untreated group (Group III) with a PCV value of 30.0 ± 1.11%. The methanol extract at a concentration of 1000 mg/kg of the plant reduced the parasitaemia level from 7.8 to 2.53 trypanosomes per ml at the end of the experiment. In Group I, the positive group, that received commercial drug (diminazene diaceturate (Samorinil®) was aparasitic before the end of the experiment. There was significant weight improvement (p =.05) in the entire Group (I and II) from – 22 ± 0.01 to 4.4 ± 0.30 g and – 2.4 ± 0.20 to 4.1 ± 0.04 g respectively except the Group III that was untreated with – 2.4 ± 0.34 to – 6.6 ± 0.18 g that kept depreciating postinfection. First death was recorded in Group III on 12-day post-infection. Temperature of G III continued to increase slowly as the parasitaemia level increased from 35.22 to 42 .36℃ which significantly varied (p =.05) from the rectal temperature of GI and II from 35.85℃ and 35.17 to 35.53℃ and 34.40℃.
Conclusion: The experiment showed that a 1000 mg/kg methanol extract of *Nauclea latifolia* exhibited promising trypanocidal activity against *T. brucei*.

Keywords: Wistar rats; T. brucei; Nauclea latifolia; parasitaemia.

1. INTRODUCTION

Trypanosoma brucei is a flagellated unicellular pathogen with desolating health and economic value. A typical trypanosome is a colourless serpentine spindle-shape body with a round posterior and shape pointed anterior end. It is being transmitted by tse-tse fly. It is a vector of animal African trypanosomiasis (AAT) and to a little degree vector of human African trypanosomiasis (HAT). Many strains of the parasite are in existence ranging from the highly virulent to those of low virulence and each has a fixed virulence within the specie host. Man and animals are being affected by two subdivisions of trypanosomes which include the haematic groups (*T. congolense* and *T. vivax*). This group of parasites always remains in the plasma of the host's blood. The second subdivision is called tissue invading group (*T. brucei, T. evansi, T. rhodesiense, T. gambiense and T. equiperdum*). This group is found extravascularly and intravascularly [1]. The *T. vivax, T. congolense* and *T. brucei* are species in livestock.

Trypanosomiasis is an ailment caused by blood parasite called trypanosome species. It is one of the major health problems to man and animal especially in Africa. There are two stages of the disease despite the host; an early haemo-lymphatic trypanosome proliferation, and a late central nervous system infection. It is difficult to treat and deadly when untreated. Insect vector especially *Glossina* and *Stomoxy* species (tse-tse fly) transmits it to man and domestic animal causing sleeping sickness. It is a wide spread disease of livestock in Africa. It has a serious negative effect on milk production, weight gain, growth rate, reproduction and the use of draught animal in crop production [2-5]. The ailment is transmitted cyclically by *Glossinia* and *Stomoxy* species [6]. There are two approaches used in controlling trypanosomiasis such as elimination and or reduction in the number of tse-tse flies, by the use of insecticides, and trypanostatic or trypanocide drugs in prophylaxis treatment or cure of infection in man. Clinical manifestations of trypanosomiasis include fever, anaemia, emaciation, cardiovascular disorders, endocrinological disorders (oedema, impotence, libido, etc), dermatological effect (skin rashes), psychological disorder and chancre.

Therefore, trypanosomiasis has prevailed in sub-saharan Africa, mainly in Nigeria. The ailment has caused about 55, 000 human and 3 million livestock deaths per annual and put more than 60 million people and 45-55 million livestock at risk of the disease [7-9]. This prompted the search for more available antitrypanosomal herbal medicines with reduced cost in order to circulate to the poor developing world.

Currently, herbal medicines have received much attention as sources of useful compounds since they are less toxic, relatively safe for human consumption, environment-friendly, cheap, easily available and affordable [10]. They are considered to be an origin of new chemical substances with potential therapeutic effects [11]. World Health Organization (WHO) accredited the implicit role of herbal medicine in the Alma-Ata declaration of Health for all by the Year 2000 A.D. and in 1978, and thus approved the consumption of the natural products [12]. Therefore, medicinal plants can be applied as alternatives to orthodox medicine. *Nauclea latifolia* (*N. latifolia*) Smith (Rubiaceae) is a shrub or small spreading tree that is widely distributed

across Savannah, north Cameroon and other African countries such as Nigeria [13]. It is discovered in the forest and fringe tropical forests. In Nigeria language, *Nauclea latifolia* is known as the following '*Uvuruilu* or *ubuluinu*' in Igbo, '*Tafashiya igiva*' in Hausa and '*Egbesi* or *Ebeyesi*' in Yoruba [14]. It comprises several indole-quinolizidine alkaloids [15] and alkaloids [16]. The major alkaloids constituents include angustine, angustoline, angustifoline, nauclefine and naucletine [17]. Different parts of the plant could be used in treating ailments. *Nauclea latifolia* roots decoction is one of such herbal preparations that have been used traditionally for treating different disease conditions such as fever, pain, dental caries, septic mouth, malaria, trypanosomiasis, dysentery, diarrhea, and diseases of the central nervous system such as epilepsy, hypertension etc [18-20]. *Nauclea latifolia* decreased the level of parasitaemia in a dose-dependent manner in mice experimentally infected with *Trypanosoma brucei* [21].

This research supplied phytochemically screened *Nauclea latifolia* to determine its constituents and then, evaluated the anti-trypanosomal activity using the methanolic extract of the plant.

2. MATERIALS AND METHODS

2.1 Plant Material

The root bark of *N. latifolia* Sm, [(*Sarcocephalus latifolius* (J. E Smith.) EA Bruce family Rubiaceae] (voucher No. 137), used was collected in the dry season (March) from Item, Abia State. The collected plant materials were authenticated by a taxonomist at Bioresource Development and Conservation Program (BDCP) Centre, Nsukka.

2.2 Sources of Trypanosome

Trypanosoma brucei was obtained from the Nigerian Institute for Trypanosomiasis and Onchocerciasis Research (NITOR) Vom Plateau State, Nigeria and supplied by Dr. Ikenna Eze at Department of Veterinary Parasitology, University of Nigeria Nsukka (UNN). The parasites were inoculated into three uninfected Wistar rats that weighed between 200 – 270 g before they were used for the experiment.

2.3 Preparation of the Extract

The bark of dried roots of *N. latifolia* was cut, dried and milled. The powder (250 g) was extracted with 750 ml of methanol (90%) by cold maceration for 24 h. The methanolic extract was evaporated under ambient temperature and then, allowed to dry. The solid extract was further fractionated with chloroform and water thereby obtaining three different extracts of methanolic, water and chloroform. These were obtained by purifying the methanol extract further using column chromatographic method. Briefly, about twenty grams (20 g) of the dried extract was fractionated in a glass column (150 cm x 1.5 cm) packed with 200 g of a slurry of silica gel (70-230 mesh, 200 g). The column was eluted in succession with chloroform (volume), methanol (volume) and water (volume) to obtain chloroform (CF), methanol (MF) and aqueous (AF) fractions, respectively. This was repeated until the mother extract finished. The *N. latifolia* extracts were stored in the refrigerator till use.

2.4 Experimental Design

The animals used in this experiment were cared for and all treatment protocols were performed in accordance with guidelines on animal ethics in Nigeria and University of Nigeria, Nsukka which complied with European community directive for animal experiment [22]. Twenty healthy Wistar rats of either sex weighing between 200 - 280 g were sourced from Faculty of Veterinary Medicine, UNN. They were screened and confirmed free of trypanosomiasis [23]. They were randomly grouped into four groups (I–IV) of five Wistar rats each and used for determination of anti-trypanosomiasis activity of methanol extract of *N. latifola*. Group I: Comprises 5 Wistar rats that were treated with diminazene diaceturate (Samorinil®) as positive control by intraperitoneal route. Group II: Comprises 5 Wistar rats that were treated with a 1000 mg/kg of methanolic extract of *N. latifolia* by intraperitoneal route. Group III: Comprises 5 Wistar rats that were untreated as negative control. Group IV: Comprises 5 Wistar rats that were not infected as the second positive control. The animals were housed in clean metallic cages and fed with chicken growers mash; containing 16% of protein and water was given *ad libitum*. The Wistar rats were allowed to acclimatize for one week. Heavily infected blood sample from donor Wistar rat was collected through ocular vein puncture and immediately diluted with physiological saline to give $1x10^6$ parasites per ml to obtain inoculums. Healthy Wistar rats (G I - III) were then inoculated by intraperitoneal route with 0.2 ml of the diluted blood sample containing $1x10^6$ trypanosome cells. The parasitaemia level

was monitored daily to determine the time for the establishment of trypanosome in the blood and also post- infection. The packed cell volume (PCV) was also determined. The physical parameters such as weight and rectal temperature of the Wistar rats were also monitored. The experiment terminated on the 28th -day post infection.

2.5 Phytochemical Tests

The phytochemical analysis was carried out using the method of Trease and Evans [24].

2.6 Haematological Examination

The parasitaemia levels were estimated by using the rapid matching technique as described by Herbert and Lumsden [25]. A drop of blood was collected by cleaning the tail with cotton wool soaked with methylated spirit before pricking with a sterile lancet and massaging the tail and then examined under X40 magnification of a table microscope and the number of trypanosome per field were counted. Each counting per field was matched with log figures obtained from the reference table [25]. The log figures were converted to antilog and subsequently converted to absolute numbers which reflected the number of trypanosome per ml.

2.7 Packed Cell Volume (PVC)

This was determined using the microhaematocrit method [26]. Blood was collected from the eyes of the rats using heparinized haematocrit capillary tubes. The filled end of the tube was sealed with plasticizer and the heparinized capillary tube was centrifuged in a microhaematocrit centrifuge at 10, 000 rpm for 5 min. The PCV was estimated in percentage using spiral microhaematocrit reader by measuring the height of red blood cell column and compared to the total height of the column of the whole blood. This was done to all the groups starting from day zero prior to infection till the end of the experiment at four days interval.

2.8 Survivability and Clinical Signs

The rats were observed throughout the period of the experiment. The disease symptoms include depression, anorexia, dullness, anaemia, facial edema and pyrexia. The deaths were noted.

2.9 Weight Variation

The increase and decrease in the weights of the Wistar rats during infection (0-8) and post-treatment (9-15) were determined with a weighing balance (Avery, UK).

2.10 Temperature

Fever was one of the symptoms that occurred during post-infection. Pyrexia occurred as the body homoeostasis tried to maintain the system. The rectal temperature was taken on a daily basis using a digital clinical thermometer.

2.11 *In vivo* Anti-trypanosomiasis Study

The presence of the parasites in the blood was examined starting on the third-day post infection. It was observed to be high on 9 day post infection. The duration of treatment occurred by ip for a period of one week as follow:

G 1(positive control): Received single dose treatment of 3.5 mg/kg diminazene diaceturate (Samoniril®). G II: Received 1000 mg/kg methanol extract of *Nauclea latifolia* that was reconstituted with distilled water. G III (negative control): Received no treatment. G IV: This was another positive control. They were uninfected and therefore, untreated.

2.12 Statistical Analysis

The statistical analysis of the relationship between the haematological parameters, weight- and rectal temperature was carried out using GraphPad InStat Demo (USA). Values were expressed as mean ± SD (standard deviation). Data were calculated and analyzed with one-way analysis of variance (ANOVA). Differences between means were assessed by a two-tailed student's T-test and p =.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

The yield of 42.67% was obtained as the mother methanol extract. The fractionated parts of chloroform, aqueous and methanol yielded 7.17, 15.20, and 18.0% respectively.

3.1 Phytochemical Results

The phytochemical analysis result of the fractionated extract of *N. latifolia* is shown in Table 1. The result showed a range of secondary metabolites that could be responsible for the trypanocidal effect of the plant extract. So many plants with antitrypanosomal activities have been

found to contain the following active constituents such as the presence of alkaloids [25,27,28], saponins [29] and flavonoids [28,30] were more in the methanol and aqueous extracts.

3.2 Parasitaemia

The result of parasitaemia determination in the three groups of Wistar rats is recorded as shown in Fig. 1. On the day 3 post-infections, there was presence of the trypanosomes in the blood of few rats but they appeared almost on all the Wistar rats on day five post-infection. However, the level of parasitaemia was high enough up to 7.8 x 10^6 trypanosome/ ml, on day 9 post infection which made the commencement of treatment in G I and II with 3.5 mg/ kg of diminiazine diaceturate and 1000 mg/kg methanolic extract solution respectively. The level of parasitaemia persistently increased in G III till death. On the day-12 post-infection the parasitaemia level of G III increased up to 8.0 x 10^6 trypanosome/ ml which resulted in the death of some Wistar rats of that group. The level of parasitaemia in G I started to decrease from 7.68 - 0 x 10^6 trypanosome/ ml after treatment until day 12 post infection when the whole Wistar rats became aparasitic as observed from the microscope. This showed that 3.5 mg/kg Samorinil® cleared the trypanosomes completely. The level of parasitaemia also decreased from 7.62 to 2.53 x 10^6 trypanosome/ ml in the Wistar rats of G II after commencement of treatment with 1000 mg/kg followed by disappearance of the pathological signs. There was a significant variation ($p = .05$) in the parasitaemia levels of the infected groups (G I-II). A report has shown that *Nauclea latifolia* decreased the level of parasitaemia in a dose-dependent manner in mice experimentally infected with *T. brucei* [22]. The trypanosomes decreased to almost minimum level in G II at the given dose of methanolic extract of *N. latifolia* indicating that if the extract is continued for a longer time, it will lead to aparasitaemia. This might be stipulated as a result of the secondary metabolites of the methanolic extracts. Some research reports have been reported on secondary metabolites of some plants to determine the trypanosomiasis activities of the extracts. Alkaloids have been characterized to inhibit protein biosynthesis and microtubule formation, intercalate DNA, disturb membrane fluidity and induce programmed cell death in blood stream of trypanosomes [31]. Flavonoids have also been reported to exhibit both *in vivo* (significantly decreases parasitaemia level) and *in vitro* antitrypanosomal activity [27,32,33].

3.3 Packed Cell Volume (%)

The PCV result is shown in Table 2. PCV helps to forecast the effectiveness of the test samples by inhibiting haemolysis due to rise in parasitaemia level. The level of anemia depicts a disease status and productive performance of parasite infected livestock and is the main cause of death in them [23,34]. PCV level detected by anemia level depreciates with a rise in parasitaemia [35]. This might be to the fact that trypanosomes generate reactive oxygen species that attack RBC membranes, and induce oxidation leading to haemolysis [36,37]. The average PCV of all the groups falls within 46.4 ± 0.12 before inoculation of the parasites. The result showed that there was a decrease in PCV in G I to III within the period of the post infection. This might be acute haemolysis as a result of the increase in parasitaemia level. This has also been reported by Tesfaye et al. [33] and Ekanem et al. [37]. The PCV of G I and II gradually increased after the commencement of treatment but the level differed due to the difference in their recovery though not significant. While in G III their PCV continued to decrease as there was no treatment till death which varied significantly ($p =.05$) from PCV of G I and IV. In the G IV which was another positive control, their PCV continually increased throughout the course of the experiment because they were uninfected.

Table 1. The phytochemical analysis of *N. latifolia* in chloroform, methanol, and water extract

Test	Aqueous extract	Methanol extract	Chloroform extract
Alkaloids	+++	+++	–
Glycosides	++	++	+
Saponins	++	++	–
Flavonoids	+++	++	–
Tannins	+++	++	+
Carbohydrate	+	+	–
Reducing sugar	+	+	–

+++ shows highly present, ++ shows moderately present, + slightly present and – shows absent

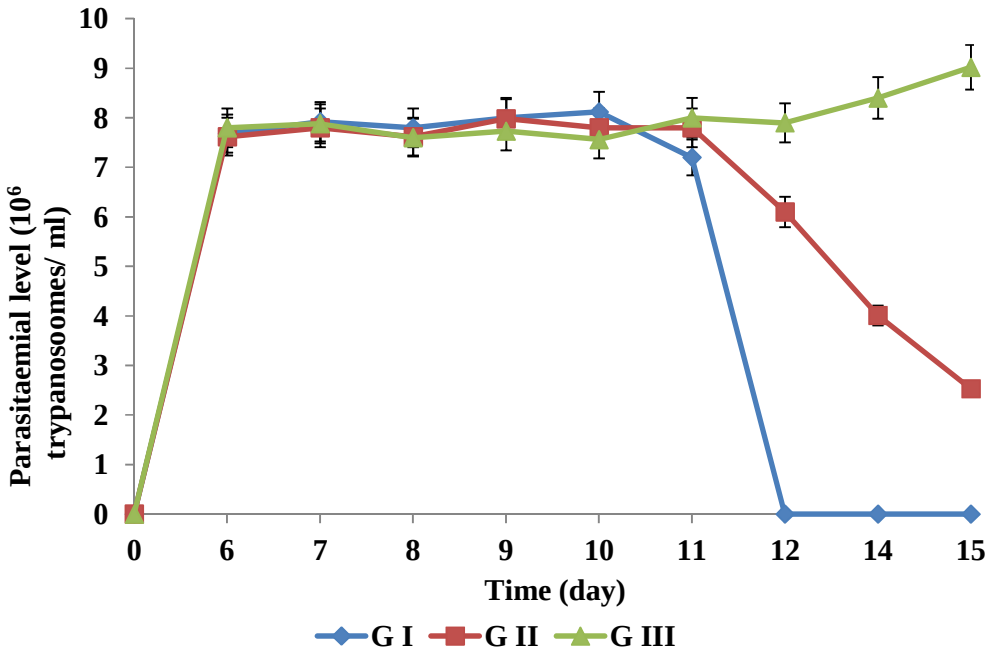

Fig. 1. Mean Parasitaemia level (10^6 trypanosomes / ml) of G I, G II and G III (G stands for group I, group II and group III)

Table 2. The result of packed cell volume of G I – IV

PI (day)	G I ± SD (%)	G II ± SD (%)	G III ± SD (%)	G IV ± SD (%)
0	48.0 ± 0.93	47.6 ± 1.80	48.0 ± 0.17	42.0 ± 0.16
6	45.0 ± 1.01	43.6 ± 0.51	43.4 ± 0.44	42.7 ± 0.11
10	35.6 ± 0.02	34.4 ± 1.33	33.6 ± 0.13	43.0 ± 0.21
14	39.8 ± 0.41	36.3 ± 0.23	30.0 ± 1.11	43.3 ± 1.22

PI= post-infection; SD= standard deviation; G I to GIV = Group I to IV

Table 3. The survival rate of the groups (GI - GIV)

DOD (day)	GI	GII	GIII	GIV
0 - 10	0	0	0	0
11 -20	0	0	5	0
21 -28	5	5	-	0

DOD= day of death; G I – G IV = Group I to IV

3.4 Survival Rate

The death of G III Wistar rats started on day 12 post infection as shown in Table 3. This might have occurred due to pancytopenia and other clinical-pathological signs during the acute crises and sub-acute stages [38,39]. In G I and II, they were sacrificed at the end of the experiment. The result showed that methanol extract of *N. latifolia* at the dose of 1000 mg/ ml prolonged the survival time of G II wistar rats infected *T. brucei*.

3.5 Weight Change of the Experimental Animals

Body weight also determines the activity of the drugs on the animals. During the period of infection, there was a significant weight change (p =.05) within the infected groups before and after the commencement of treatment on day 9 post infection as shown in Table 4. At the end of the experiments, there was also a significant difference in weights of the infected treated groups (GI and II) and infected-untreated group (GIII). In G I, the group depreciated to–2.2 g post infection days (0-8) and increase to 4.4 g post treatment (8-15) before the end of treatment. The group II lost– 2.4 g post infection (0-8) and recovered 4.1 g after treatment (8-15) days. But in group III recovery stage was not observed as there was progressive decrease in body weight as the group was not treated. Loss in weight occurred due to anorexic nature of the

Table 4. The weight change in the experimental animals post infection days

PI (Day)	G I ± SD	G II ± SD	G III ± SD	G IV ± SD
0-8	–2.2±0.01	–2.4 ± 0.20	–2.4 ± 0.34	15.7± 0.11
8-15	4.4±0.30	4.1± 0.04	–6.6±0.18	26 ± 0.22

PI= post infection; SD= standard deviation; G I to GIV = Group I to IV

Table 5. Mean temperature (℃) of the various group s

PI (day)	G I (°C) ± SD	G II (°C) ± SD	G III (°C) ± SD	GIV (°C) ± SD
0	35.85 ± 0.03	35.17 ± 0.21	35.22 ± 0.18	35.81 ± 0.11
1	34.99 ± 0.31	36.24 ± 0.01	35.76 ± 0.05	35.88 ± 0. 13
2	35.84 ± 0.12	36.11 ± 0.33	35.64 ± 0.02	35.79 ± 0. 21
3	36.08 ± 0. 22	36.75 ± 0. 08	36.00 ± 0.04	35.5 ± 0 . 17
5	37.43 ± 0.01	37.12 ± 0.06	37.87 ± 0.04	35.11 ± 0. 20
7	38.66 ± 0.05	38.40 ± 0.09	38.87 ± 0.01	35.54 ± 0. 11
9	39.70 ± 0.03	39.38 ± 0.11	39.80± 0.32	35.51 ± 0.0 7
11	36.06 ± 0.06	36.60 ± 0.02	39.89 ± 0.11	35.11± 0.01
13	35.81± 0.16	35.55 ± 0.05	40.99 ± 0.02	35.17 ± 0. 02
14	35.53± 0.12	34.40 ± 0.44	42.36± 0.09	35.34 ± 0.1 1

PI= post infection; SD= standard deviation. G I to GIV = Group I to IV

disease as a result of energy deficit and loss of tissue associated with catabolism of body fat, deficiencies of vitamin C, B and essential amino acids [38]. Group IV had a continuous increase in weight as there was no infection. While the recovery or weight increase in other infected groups might be they feed better with decrease loss of protein from tissues as a result of the administered drug or extract and perhaps by reduction of proliferating parasites [23,35].

3.6 Variations in Body Temperature

Increase in body temperature of the animals was observed during the infection and treatment periods. Their body temperatures were normal before infection but subsequently became pyrexic during the infection period especially on day 9 post-infection before the commencement of treatment. During treatment, temperature of G III continued to increase slowly as the parasitaemia level increased which significantly varied (p =.05) from rectal temperature of GI and II. While the rectal temperature of G IV remained fairly constant. The result was recorded and shown in Table 5.

4. CONCLUSIONS

The current work evaluated the *in vivo* anti-trypanosomal activity of methanol extract of *Nauclea latifolia* using Wistar rats and phytochemical screening. Some parameters like parasitaemia, PCV, weight and temperature were used in the monitoring the efficacy of the *N. latifolia* extract. The standard drug diaceturate a derivative of diminazene aceturate, an aromatic diamidine was used which caused a rapid reduction in the parasitaemia of G I. This was observed by the reduction in the rectal temperature, weight gain, increases in PCV and survival rate of the rats. The parasites were eventually cleared by this drug in the blood of treated G I. Methanol extract of *Nauclea latifolia* extract (1000 mg/kg) administered for one week in Group II was observed at this concentration to have trypanocidal effect on *T. brucei*. This was observed as a result of significant decrease effect in the parasitaemia level, accompanied by a moderate increase in PCV, weight gain and normal rectal temperature. The prolongation of lives of treated animals may therefore, be associated with the ability of this extract to improve the PCV possibly by reducing the parasite load or neutralizing the toxic metabolites produced by trypanosomes. Therefore, this research has shown evidence that methanolic extract of *N. latifolia* might have a trypanocidal activity, improve PCV level, promote weight and quality of life of Wistar rats due to the presence of some secondary metabolites of the extract and should be supportive of its use in ethno-medicine by the public.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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