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Anti-trypanosomal Activity of Leaf Extracts of *Andrographis paniculata* in *Trypanosoma brucei brucei*-infected Mice

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ABSTRACT

African Animal Trypanosomiasis is a disease of major economic and public health importance, especially with the current setback in the treatment of cases using synthetic anti-trypanosomal agents. As a result, there is an urgent need to identify alternative compounds for chemotherapy. Therefore, the present study investigated the phytochemical composition, acute oral toxicity, and *in vivo* anti-trypanosomal efficacy of crude methanol, n-hexane fraction, and ethyl acetate fraction of *Andrographis paniculata* (king of bitters) leaf in *Trypanosoma brucei brucei*-infected mice. The methanol extract was screened for the presence of secondary metabolites by using standard methods. The crude plant extract and fractions were administered orally to the parasite-infected mice at doses of 300 and 600 mgkg-1 body weight (b. wt) following standard procedures. The result was compared to Diminazine aceturate which was given at the recommended dose of 3.5 mgkg-1 b. wt subcutaneously. Phytochemical screening has revealed the presence of alkaloids, flavonoids, saponins, phenols, glycosides, steroids, tannins, and anthraquinones. Diminazine aceturate gave a 100% parasite clearance, and the mice survived throughout the study period (60.00±0.00) days. *A. paniculata* extract at 600 mgkg-1 b. wt was found to be effective in reducing the parasite multiplication by 43.58 % and extending the life span of the treated mice to (10.50±0.50) days compared to the untreated control, (7.45±0.55) days. Ethyl acetate fraction on the other hand was found to be more effective in reducing the parasitaemia level (61.33 % and 75.63 %) and extending the life span of the mice to (12.50±0.34) and (15.76±0.23) days for both 300 and 600 mgkg-1 b. wt doses respectively, whereas the n-hexane gave a limited trypanocidal effect of 9.89 % and 21.15 %. The results obtained in the present study suggest the ethno-pharmacological usefulness of the plant and necessitate further studies on isolated active substances from this plant.

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Introduction

Trypanosomiasis is a chronic incapacitating haemoparasitic disease in humans and domestic animals caused by protozoans of the genus *Trypanosoma* [1]. This parasite is transmitted through the bite of the tsetse fly. Infection results in acute or chronic disease characterised by irregular fever, anaemia, occasional diarrhoea, and rapid loss of condition which often leads to death [2]. African Animal Trypanosomiasis (AAT, "Nagana") is a neglected tropical disease caused by *Trypanosoma brucei brucei, Trypanosoma vivax*, and *Trypanosoma congolense* while Human Africa Trypanosomiasis (HAT, 'sleeping sickness') is caused by two subspecies of *Trypanosoma brucei; Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* [3].

Sleeping sickness and Nagana have a noteworthy financial effect in Africa since they influence the settlement trend of people, including land use and farming. Chemotherapy, the main means of controlling the disease, is under threat because of drug resistance caused by parasites. The current chemotherapy for HAT relies on only six drugs (Suramin, Pentamidine, Melarsoprol, Eflornithine, Arsobal and Mel B), five of which were developed more than 30 years ago [4]. Other drugs, such as Homidium, Isometamidium, and Diminazene aceturate (Berenil), are used in animal infections. Each of these drugs has one or more of the following challenges: they are expensive, highly toxic, and require parenteral administration. The continued use of the same trypanocides favoured drug resistance which has been responsible for current chemotherapeutic failures [4]. The development of new, cost-effective drugs for the treatment of sleeping sickness is urgently required to manage the disease. *Andrographis paniculata* (Burm.f.) is called King of Bitters in English [5]. In Nigeria, Yorubas referred to it as 'Ewe Jogbojogbo' [6]. Traditionally, *A. paniculata* has been used as an immune system booster, blood cleanser, and herbal medicine to treat and cure several diseases, such as fever, loss of appetite, irregular stools, diarrhoea, cancer, and viral infections, owing to its powerful antimicrobial and immune strengthening capacity [7]. However, there is a dearth of information regarding its anti-trypanosomal potency. Its anti-trypanosomal potency should be evaluated in the wake of continuing parasite resistance.

Materials and Methods

Plant Collection and Authentication

Andrographis paniculata was obtained from the Biological Garden of the Federal University of Technology Minna. The plant was identified in the Department of Plant Biology, Federal University of Technology, Minna. The voucher specimen with authentication number FUT/PLB/ACA/027 was kept in the herbarium of the Department of Plant Biology, FUT Minna.

Plant Preparation and Extraction

Fresh leaves were harvested, washed, and air dried on a laboratory bench for two weeks. Dried plants were pulverised using a mortar and pestle. Soxhlet extraction of plant materials was carried out by weighing 50 g of the powdered samples in 200 ml of methanol for each cycle. The resulting extract was then placed in a water bath. The extract was stored on a cupboard at room temperature until use. Ethyl acetate and n-hexane were used as solvents to obtain fractions. This was performed using the method described in [8].

Percentage Yield

Fresh *A. paniculata* leaves were ground using an electronic blending machine. Pulverised fresh plant samples (100 g) were dissolved in 600 ml of n-hexane and ethyl acetated fractions and methanol for 72 h. The mixture was filtered through Whatman filter paper (No. 1), and the solvent was removed under reduced pressure in a rotary evaporator. A green-coloured paste was obtained, weighed, and stored in a refrigerator at 4 °C until required. The residue was weighed, and the percentage yield was calculated using the formula described by [9]

$$\%\,\text{yield} = \frac{\text{weight of the extract residue (g)} \times 100}{\text{weight of the powdered plant (g)}}$$

Qualitative Phytochemical Screening

The phytochemical screening of some essential phytochemicals (alkaloids, saponins, glycosides, tannins, anthraquinone, steroids, flavonoids) in the plant leaves was done according to the methods of [10,11]

Experimental Mice

Albino Mice were obtained from the Nigeria Institute for Trypanosomiasis Research (NITR), Kaduna. The mice were housed in standard cages with wood shavings as bedding which were changed daily. The mice were fed with a grower's mash and allowed access to clean water ad libitum. The experiment was conducted in compliance with international guidelines for biochemical research involving animals [12].

Test Organism

Trypanosoma brucei brucei was obtained from the parasitology division of the Nigeria Institute for Trypanosomiasis Research (NITR), Kaduna, Kaduna State, Nigeria. Three healthy rats were inoculated with *T. brucei brucei* and transported to the Animal Biology Laboratory of the Federal University of Technology, Minna. The parasites were maintained in the laboratory by continuous passage through trypanosome-free mice throughout the study period. Passage was considered necessary when parasitaemia was in the range of 16 – 32 parasites per field (usually 3–4 days post-infection in mice) [13].

Determination of Acute Toxicity

The Acute toxicity profile was determined according to a previously described method [14]. In the initial phase, 9 mice of both sexes were randomly divided into three groups of 3 mice each. Mice in groups 1, 2, and 3 were treated with the crude methanol extract at 10, 100, and 1000 mg/kg, respectively.

In the second phase, higher doses (1600, 2900, and 5000 mg/kg) of each extract were administered to nine mice in Groups 1, 2, and 3. All doses (low and high) were administered to monitor toxicity and estimate the lethal dose value. All animals were kept under strict observation for behavioural, neurological, autonomic, and physical changes, such as alertness, motor activity, restlessness, convulsions, coma, diarrhoea, and lacrimation, for 24 h. These observations were continued for an additional 14 days for signs of overt toxicity. The LD_{50} values were then extrapolated.

Inoculation of Experimental Mice

Blood was obtained intraperitoneally from heavily infected donor mice using a syringe. Blood (1 ml) was diluted immediately in 2 ml normal saline to serve as the inoculum containing 1×10^6 parasites/ml. Diluted blood was injected into trypanosome-free mice.

Experimental Set-up

The forty-five (45) Swiss albino mice were divided into nine groups (n = 9) five (5) mice each. It comprised three (3) control and six (6) experimental groups. The grouping and dosage administrations were as follows:

Group 1: Uninfected and treated (Normal control)
Group 2: Infected and treated with 0.5ml distilled water (Negative control)
Group 3: Infected and treated with 5mg/kg of Diminazine Aceturate (Berenil) (Positive control)
Group 4: Infected and treated with 300mg/kg crude methanol extract of *A. paniculata* extract
Group 5: Infected and treated with 600mg/kg crude methanol extract of *A. paniculata* extract
Group 6: Infected and treated with 300mg/kg ethyl acetate fraction of *A. paniculata* extract
Group 7: Infected and treated with 600mg/kg of ethyl acetate fraction *A. paniculata* extract
Group 8: Infected and treated with 300mg/kg n-hexane fraction of *A. paniculata* extract
Group 9: Infected and treated with 600mg/kg n-hexane fraction of *A. paniculata* extract

Treatment of Infected Mice

The plant extract, standard drug, and normal saline were administered to each group, as described above. The extract was dissolved in a normal saline solution. The mice were administered the extracts, drugs, and normal saline at doses corresponding to their body weight. The treatment began three days post-inoculation and was routinely repeated daily for 14 consecutive days. The extracts were administered through the oral cavity using a cannula.

Determination of Parasitaemia

Parasitaemia was monitored daily by microscopic examination of the blood obtained from the tail of each mouse. The tail was cut to extrude the blood. A drop of blood was placed on a clean grease-free microscope slide, and physiological buffered saline was added and covered with a cover slide. Blood samples were examined microscopically at ×40 magnification. The level of parasitaemia was determined using the ''rapid matching'' method [15]. The reduction in parasite motility after treatment was attributed to the anti-trypanosomal activity of the extract. The percentage parasite clearance was calculated using the following formula:

$$\% \text{ parasite clearance} = \frac{(\text{Initial parasitaemia} - \text{Final parasitaemia}) \times 100}{\text{Initial parasitaemia}}$$

Determination of Body Weight

The body weight of each mouse in all groups was measured before infection, on the day the treatment commenced, and every other day until Day 14.

Determination of Mean Survival Time (MST)

Mortality was monitored daily and the number of days from inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the experimental period. The mean survival time was calculated using the formula described by [16].

$$\text{Mean Survival Time (MST)} = \frac{\text{Sum of survival of all mice in a group (days)}}{\text{Total number of mice in the group}}$$

Data Analysis

Values were analysed using the statistical package for social science (SPSS) version 16 and are presented as mean ± SE of the mean. Comparisons between separate groups were performed using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT). The level of significance was set at $P < 0.05$.

Results

Plant Extract Yields

The crude methanol extraction and the sequential solvent extractions gave rise to crude extracts and fractions whose yields are summarised in Table 1. The crude methanol extract of *A. paniculata* had the lowest yield (13.16 %). In contrast, the ethyl acetate fraction had the highest yield (39.80 %), whereas the hexane fraction had a yield of 21.05 %.

Table 1: Yield of crude extract, hexane, and ethyl acetate fractions of *A. paniculata*

Extract/fractions	Yield (%)
Methanol crude extract	13.16
Hexane fraction	21.05
Ethyl acetate fraction	39.89

Phytochemical Study

Various secondary metabolites were detected in the crude methanol extract of *A. paniculata*. The *A. paniculata* extract showed positive results for the presence of alkaloids, flavonoids, saponins, phenols, glycosides, steroids, tannins, and anthraquinones (Table 2).

Table 2: Phytochemical composition of crude methanol extract of *A. paniculata*

Phytochemical	Inference
Alkaloids	+
Flavonoids	++
Saponins	++
Phenols	+
Glycosides	+
Steroids	+
Tannins	+
Anthraquinones	+

Key: + = present - = absent

Acute Toxicity

No toxic symptoms were observed at a dose of 1600 mg/kg BW. No mortality was observed in any of the experimental animals that lived up to 14 days after administration (Table 3). The behavioural patterns of animals were observed for the first 1 h, followed by 6 h and 24 h after the administration of the extract. All animals in the control and extract-treated groups administered 10 – 1,600 mg/kg BW of the extracts were normal and did not display observable effects on skin, breathing, food intake, water consumption, postural patterns, or hair loss. The LD_{50} value after oral administration was found to be more than 5,000 mg/kg body weight.

Table 3: Acute toxicity profile of methanol extract of *A. paniculata* in mice

Dose (mg/kg BW)	Observations	Mortality
10	Animals showed no apparent changes in appearance and activity.	0/3
100	Animals were calm and devoid of unusual reactions.	0/3
1000	Animals showed no apparent changes in appearance and activity.	0/3
1600	Animals showed no apparent changes in appearance and activity.	0/3
2900	Sustained agitation, increased heart rate, decreased feed intake, and Animals had intense ethrema.	0/3
5000	Animals had intense ethrema and disorientation.	0/3

In vivo Anti-trypanosomal Efficacy of the Crude Extract of Andrographis Paniculata

The average parasitaemia count of *T. brucei brucei* infected mice treated with methanol leaf extract of *A. paniculata* is presented in Figure 1. The results showed progressive increases in the parasite load of the infected untreated mice before death. The methanol extract of *A. paniculata* leaves showed some degree of dose-dependent anti-trypanosomal activity against *T. b. brucei* (Figure 1). The extract showed 15.04 % and 43.58 % parasite clearance at 300 and 600 mg/kg BW, respectively. The groups treated with Diminazene aceturate showed total parasite clearance on day 4 post-infection (100.00% cure) and survived throughout the period of the experiment. Groups of mice treated with 300 and 600 mg/kg BW survived for 8.05±0.55 and 10.50±0.50 days respectively, while the untreated control survived for 7.45±0.55 days (Table 4).

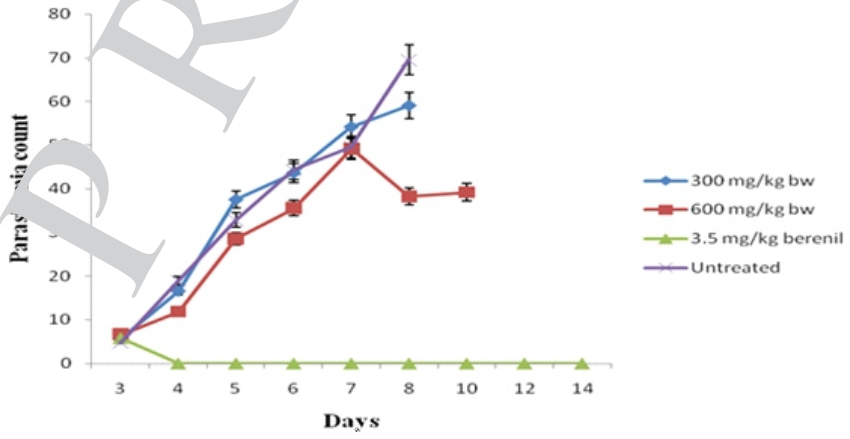

Figure 1: Effect of Crude Methanol Extract of *A. paniculata* on parasitaemia count in *T. b. brucei*-infected mice

In-vivo Anti-trypanosomal Efficacy of the Hexane and Ethyl Acetate Fraction of Andrographis paniculata

The average parasitaemia count of *T. brucei brucei* infected mice treated with hexane and ethyl acetate fractions of *A. paniculata* is presented in Figure 2, which shows a progressive increase in the parasite load of infected untreated mice before death. The hexane and ethyl acetate fractions of *A. paniculata* showed dose-dependent anti-trypanosomal activity against *T. b. brucei* (Figure 2). The hexane fraction had the lowest parasite clearance rates of 9.894 % and 25.15 % at 300 and 600 mg/kg BW, respectively. The ethyl acetate fraction showed more promising anti-trypanosomal activities, with parasite clearances of 61.33 % and 75.33 % at 300 and 600 mg/kg BW, respectively (Table 4). The group treated with Diminazine aceturate showed total parasite clearance on day 4 post-infection (100.00% parasite clearance) and survived throughout the experimental period (60.00±0.00) days. Groups of mice treated with 300 and 600 mg/kg BW of hexane fraction survived only for 8.94±0.23 and 9.53±0.63 days, respectively. Groups of mice treated with 300 and 600 mg/kg BW of ethyl acetate fraction survived for 12.50±0.34 and 15.76±0.23 days respectively while the untreated control survived for 7.45±0.55 (Table 4).

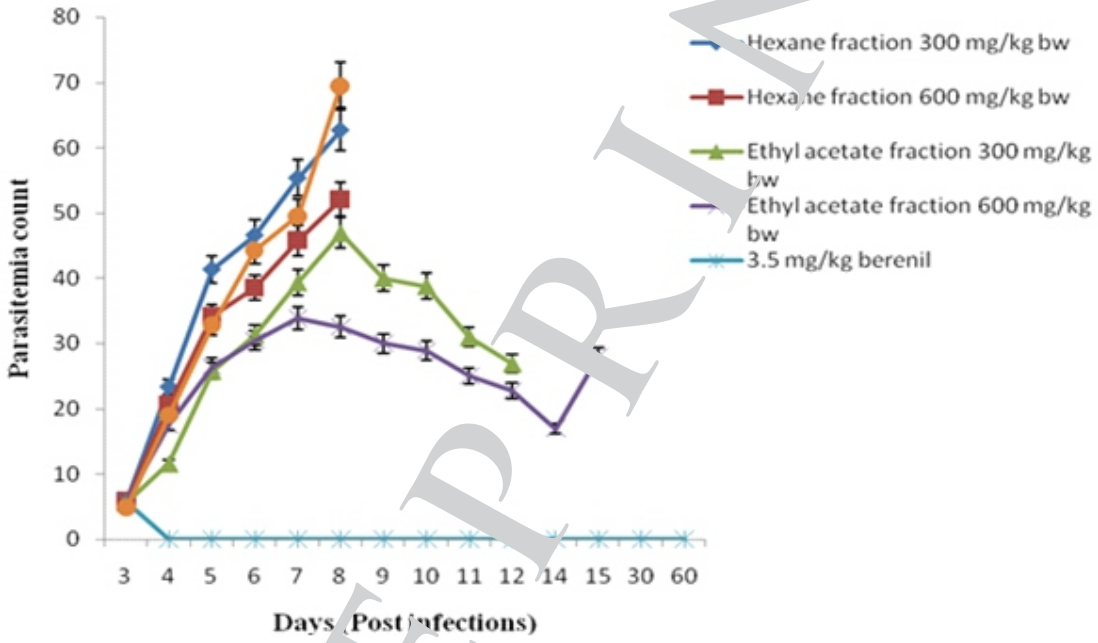

Figure 2: Effect of hexane and ethyl acetate fractions of *Andrographis paniculata* on parasitaemia count in *T. b. brucei*-infected mice

Table 4 Mean survival days and percentage protective effects of crude and solvent fractions of *A. paniculata* leaf

	Average parasitaemia	% P arasite clearance	Mean survival days
Methanol crude 300mg/kg BW	59.08±2.34	15.04	8.05±0.55[a]
Methanol crude 600 mg/kg BW	39.23±4.62	43.58	10.50±0.50[b]
Hexane fraction 300 mg/kg BW	62.66±5.21	9.89	8.94±0.23[ab]
Hexane fraction 600 mg/kg BW	52.05±5.82	25.15	9.53±0.63[b]
Ethyl acetate 300 mg/kg BW	26.89±3.92	61.35	12.50±0.34[c]
Ethyl acetate 600 mg/kg BW	16.94±1.96	75.63	15.76±0.23[d]
Diminazine aceturate	00±0.00	100	60.00±0.00[e]
Untreated	69.54±3.23	-	7.45±0.55[a]

Discussion

The search for active trypanocidal drugs from African natural products, particularly plant extracts, is a concern for many researchers. This study indicated the activity of the crude extract and fractions of *A. paniculata* used, with variation in the trypanocidal effect. The yield obtained from the methanol extract could be an indication of the extraction power of the solvent with respect to the polar components. The high yield from the extracts was found to be active and promising, and further development could add advantages to the commercial production of the plant. The variation in the percentage yields of the hexane and ethyl acetate fractions reflects the presence of different proportions of constituents of different polarity, with mid-polar ethyl acetate-soluble constituents being dominant [17].

According to the results of the phytochemical screening study, various secondary metabolites were identified in the crude methanol extract of *A. paniculata*. The *A. paniculata* extract showed positive results for the presence of alkaloids, flavonoids, saponins, phenols, glycosides, steroids, tannins, and anthraquinones. Except for anthraquinone which was present in our study, the results obtained were in agreement with the findings of [18]. The disparity encountered in this study may be attributed to the age, location, and season of plant collection. The plant used for the current study was collected in Minna, North Central, Nigeria, while that reported in [18] was collected from Southwest, Nigeria. Plants collected under different environmental conditions have different phytochemical profiles [19]. Phenolics and polyphenols have been reported to exhibit anti-trypanosomal potential [20]. Inhibition of trypanosome alternative oxidase (TAO) enzyme is thought to be responsible for the anti-trypanosomal activity of phenolic compounds [21]. Flavonoids and flavonoid-derived natural plant products have long been known to function as free radical scavengers and metal chelators.

The acute oral toxicity of the methanol extract of *A. paniculata* in albino mice up to a dose of 5000 mg/kg body weight (BW) resulted in no mortality. Thus, the median lethal dose (LD_{50}) of the extract was greater than 5000 mg/kg BW. This indicates that the extract is safe for use. Any therapeutic substance with an oral LD_{50} value above 5000 mg/kg BW is regarded as having low toxicity [21]. The results of the current study are in agreement with those in [22]. Parasite motility is a relatively reliable indicator of the viability of most trypanosomes, and a complete elimination or reduction in the motility of trypanosomes when compared to the control could be taken as an index of trypanocidal activity. The results of the *in vivo* efficacy study revealed that the crude methanol extract of *A. paniculata* leaves suppressed the multiplication of parasites in *T. b. brucei*-infected mice compared to the negative control, indicating the presence of anti-trypanosomal constituents in the extract. The ability of the extract to reduce parasite replication and extend the survival period of infected mice was dose-dependent, indicating that the anti-trypanosomal activity of the extract can be improved by using a higher dose, pure fractions, or isolated active compounds in the extract. This finding corroborates a previous study which reported that *A. paniculata* exhibited encouraging *in vivo* trypanocidal activity with a reduction in the level of *T. brucei brucei* parasitaemia in mice [5]. This anti-trypanosomal effect may be attributed to the synergistic effects of the different phytochemical constituents of *A. paniculata*. The methanol crude extract produced 15.04% and 43.58% parasite clearance at 300 mg/kg and

600 mg/kg, respectively, compared to the standard drug that produced 100% cure in mice. The reduced efficacy of the crude extract in clearing trypanosomes from the blood circulation could be due to enzymatic inactivation of active compounds and impaired absorption from the site of administration. In addition, failure to reach target organs with sufficient concentration and duration to effect a cure, as well as the short half-life of the constituents, making them unable to stay long enough to exert a pronounced effect on the parasites, could also be attributed to the failure of the extracts to clear the blood of infected mice from trypanosomes.

In contrast, the ethyl acetate fractions of *A. paniculata* extract produced a significant trypanocidal effect in mice infected with *T. brucei brucei* when compared with the methanol crude extract and n-hexane fraction. The results of this study conform with those of [22] but do not corroborate the findings of [23] and [24], who reported that extracts of plants extracted using different solvents did not produce any significant pharmacological action. The ethyl acetate fraction showed promising trypanocidal activity, although it did not completely eliminate the parasite from the blood but only reduced the level of parasitaemia. Several researchers have made similar observations regarding the reduction in parasitaemia [25]. It was observed that the ethyl-acetate fraction of the plant gave better results (61.33% and 75.33% curative effect) than that of the hexane fraction (9.89% and 25.15 %) at the same dose of either 300 or 600 mg kg-1 BW. The higher concentration of the extracts showed better efficacy than the extract with lower concentration because an increase in concentration results in a supplementary input of different active compounds. It is possible that the low doses used in the present study did not support the attainment and maintenance of adequate plasma concentrations that could produce therapeutic effects.

Conclusion

Phytochemical analyses revealed the presence of alkaloids, flavonoids, saponins, phenols, glycosides, steroids, tannins, and anthraquinones. The acute oral toxicity of the methanol extract of the plant leaves was greater than 5000 mg/kg BW, which is safe for use. Both crude extract and fractions of *A. paniculata* produced significant dose-dependent trypanocidal activity in mice experimentally infected with *T. brucei brucei*. Trypanocidal activity was more pronounced when the ethyl acetate fraction was administered at a higher dose (600 mg kg-1 BW).

Authors' Contributions

IA and OIM conceptualised the study. IA, AKA, OICJ, and ASO designed this study. AM, IA, and OIM participated in the fieldwork and data collection. OICJ, KA, and IA performed data analysis; AKA, AM, and IA interpreted the data. IA and AM prepared the first draft of the manuscript, which was reviewed by OICJ, AKA, and ASO. All the authors contributed to the development of the final manuscript and approved its submission.

Ethical Approval

Ethical approval, with reference number NSVH/2018/VM21/12 was obtained from the Animal Care and Use Committee of the Niger State Ministry of Livestock and Fisheries, Minna.

Conflict of Interest

The authors declare no conflicts of interest.

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