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# *In vitro* and *In vivo* Antitrypanosomal Studies of the Leaf Extract of *Vitexsimplicifolia*

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**ABSTRACT**

*In vivo* and *in vitro* studies were carried out to determine the antitrypanosomal effects of the methanol extract of *Vitexsimplicifolia*using *Trypanosomabruceibrucei* infected mice. The antitrypanosomal effects of the leaf extract was determined using rapid "matching" method. The effect of the extract *in vivo* was determined based on the changes in the level of parasitaemia, packed cell volume (PCV) and weights of the mice, while the *in vitro* assay was determined using the $IC_{50}$ value of 50 µL culture medium of the bloodstream containing $10^4$ of*Trypanosomabruceirhodesiense*. The leaf extract reduced the parasitaemia level, 5 days post infection. The PCV and the weight of the mice decreased upon infection with *Trypanosomabruceibrucei* and improved slightly on commencement of treatment. The acute toxicity of the extract was also determined using standard method. The phytochemical analysis of the leaf extract showed presence of different secondary metabolities.

**Keywords:***In vivo/ in vitro* assay, Trypanocide, *T. bruceibrucei, Trypanosomabruceirhodesiense,Vitexsimplicifolia*, cytotoxicity

## INTRODUCTION

Sleeping sickness threatens millions of people in 36 countries in Sub-Saharan Africa. Many of the affected population live in remote areas with limited access to adequate health services, which hampers the surveillance and therefore the diagnosis and treatment of case [1].

Human African trypanosomiasis (HAT), also known as sleeping sickness is a vector-borne parasitic disease. The parasites concerned are protozoa belonging to the *Trypanosoma genius*. They are transmitted to humans by tsetse fly (Glossina genius) bites, which have acquired their infection from human being or from animals harbouring the human pathogenic parasites.

*Trypanosome brucei gambiense,* found in West and Central Africa accounts for over 95 % of reported cases of sleeping sickness and causes chronic infection. *Trypanosome brucei rhodensiense* is found in Eastern and Southern Africa, accounts for 5 % of the reported cases and causes an acute infection [2].

The two forms of the disease are characterized by an initial haemolymphatic stage, followed by a second central nervous infection when the parasites have crossed the blood-brain barrier.

*Trypanosoma brucei brucei* are unicellular parasites transmitted by the tse-tse fly. They are the causative agent of African Animal Trypanosomiasis (AAT) [3]. The disease results in acute, sub-acute, or chronic disease characterized by intermittent fever, anaemia, occasional diarrhoea, and rapid loss of condition and often terminates in death [4].

Trypanosome infections are known to cause immunosuppression responsible for the host's inability to eliminate the trypanosomes even after administration of trypanocidal drugs [5].

The use of trypanocidal agents in the treatment of sleeping sickness had been limited owing to their

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numerous side effects (tachycardia, dizziness, headache and vomiting) [6].

Studies have also shown that the parasite have developed resistance to convectional antitrypanosomal drugs [7, 8] which led to the search for efficacious alternative chemotherapeutic agents.

Research efforts are therefore currently directed towards alternative means of managing sleeping sickness. This has led to extensive investigation into natural plants for better anti-trypanosomal agents. Studies have revealed that many plants possess potential as sources of novel trypanocidal compounds [9-12].

*Vitex simplicifolia* is a sprawling shrub that can grow as tall as 1.5m in height [13].The leaves are strongly aromatic, intensifying when crushed [14]. The leaves measures 2- 6.5cm in length and 1- 4.5cm in width.

*Vitex simplicifolia* is used traditionally for the treatment of tooth ache, edema, skin troubles and gout (15). The present study aims at evaluating the antitrypanosomal effect of the leaf extract of *Vitex simplicifolia* with a view to authenticating its ethnomedicinal uses.

## EXPERIMENTAL

### Plant material

The leaves of *Vitex simplicifolia* were collected between March and April 2010 from Nsukka, Enugu State, Nigeria. The plant material was authenticated by Mr. Alfred Ozioko of the Centre for Biodiversity and Conservation Program (BDCP), Nsukka, Enugu State. The leaves were cleaned, dried under shade and pulverized.

### Laboratory animals

Abino mice (25 – 30 g) of either sex were procured from the Department of Veterinary Parasitology and Enthomology, University of Nigeria, Nsukka. The animals were housed in metallic cages under standard conditions of temperature (28 – 32$^{o}$C), fed with standard palletized feed (Grand Cereals and Oil Mill, Nig Ltd) and allowed access to water *ad libitum.*

The use and care of laboratory animals were conducted in accordance with internationally accepted best practices as contained in the European Community Guidelines (EEC Directive of 1986; 86/609/EEC) and approved by the local Ethics Committee of our institution.

### Extraction

About 500 g of the pulverized dried leaves of *Vitex simplicifolia* were extracted with 2.5 L of methanol at room temperature for 7 days by cold maceration. The methanol extract was filtered with Whatman No1 filter paper and concentrated *in vacuo* using a rotary evaporator.

### Phytochemical tests

The phytochemical analysis of the methanol extracts was carried out using the standard procedure [16].

### Acute toxicity ($LD_{50}$) test

The acute toxicity of the leaf extract was determined according to an earlier described method [17]. Briefly, albino mice (25 – 30 g) of either sex were divided into 3 groups of 3 mice per group. The extract was dispersed in 10 % v/v Tween 80 and administered to the mice at doses of 10, 100 and 1000 mg/kg and the animals monitored for 24 h.

From the result of the first phase, doses of 1000, 1600, 2900 and 5000 mg/kg were administered orally to 4 groups of 1 mouse per group. The $LD_{50}$ was calculated as the geometric mean of the maximum dose that caused 0% death and the minimum dose that caused 100 % death.

### Trypanosome stock

*Trypanosoma brucei brucei* was collected from the Nigerian Institute of Trypanosomes Research (NITR) Vom, Jos Plateau State. The parasites were maintained in the laboratory by continuous passage in mice intraperitoneally.

### Determination of *in vivo* anti-trypanosomal activity

Thirty albino mice (20 – 30 g) of both sexes were divided into 6 groups of 5 mice each to investigate the effects of the extracts on *Trypanosoma brucei brucei* infection using the procedure described by Olunkunle *et al* [18]. The mice were inoculated with 1.0 x $10^6$ trypanosomes in 0.20 ml normal saline except Group VI which served as control (uninfected, untreated). Three days post-inoculation, the mice were screened individually for the presence of *Trypanosoma brucei brucei* infection. The trypanosome count was determined by examination of the wet mount microscopically at x 40 magnification using the "rapid matching" method [19]. This method involves microscopic counting of parasites per field in pure blood or blood appropriately diluted with buffered phosphate saline (PBS, pH 7.2).

With the establishment of parastaemia on the fifth day, animals in Groups (I-III) were respectively treated orally for 8 consecutive days at different dose levels per kg body weight. The dose levels of 50, 100 and 200 mg/kg of the extract were administered to the animals.

Group IV severed as the positive control and received Diminazene aceturate (Veterinary Pharmaceutical products, Holland) at 3.5 mg/kg. Group V (infected) and Group VI (uninfected, untreated) served as untreated control groups and received normal saline.

The *in vivo* activity was determined based on the changes in the levels of parasitaemia, packed cell volume (PCV) and weights of the animals during the experimental period.

**Determination of *in vitro* antitrypanosomal activity and cytotoxicity ($IC_{50}$)**

The *in vitro* antitrypanosomal activity of the extract was determined by a known method [20], modified by a recent contribution by Nwodo *et al* [10]. Briefly, minimum essential medium (50 µL) supplemented with 2-mercaptoethanol and 15 % heat-inactive horse serum was added to each well of a 96-well micro liter plate. Serial drug dilutions were prepared covering a range from 90 to 0.123 g/mL. Then $10^4$ bloodstream forms of *Trypanosomabrucei rhodesiense* (Stage:trypamastigotes) STIB 90 in 50µL culture medium was added to each well and the plate incubated at 37°C under a 5 % $CO_2$ atmosphere for 72 h. 10 µL of Alamar Blue (12.5 mg resazurin dissolved in 100 ml distilled water) was then added to each well and incubation continued for a further 2-4 h. The plate was then read in a Spectramax Geminin XS microplate fluorometer (Molecular Devices Cooperation,Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm [21]. Fluorescence development was measured and expressed as percentage of the control. Data were transferred into the graphic programme Softmax Pro (Molecular Devices) which calculated $IC_{50}$ values. Cytotoxicity was determined using the same assay and rat skeletal myoblast (L-6 cells). Melarsoprol was used as standard drug for the antitrypanosomal assay and podophyllotoxin as standard for cytotoxicity determination.

**Determination of packed cell volume (PCV)**

The packed cell volume was determined using the microhaematocrit method [22]. Blood samples were collected before and after treatment from the tail end of each mouse and mixed with heparin in tubes. The mixed blood was allowed to fill the capillary tube and the unfilled end of the tube sealed and centrifuged at 10,000 rpm for 5 mins, for determination of the PCV.

**Determination of body weight**

The body weight of the mice was determined before the animals were infected and during treatment using Haus top-loading balance.

**Statistical analysis**

The results were expressed as means ± SEM using student t-test. Differences between means were considered significant at $P < 0.05$ using one way ANOVA [23].

## RESULTS

**Table 1: Results of the *in vitro* and cytotoxicity assay of the leaf extract**

| *Sample* | *T. b.br* $IC_{50}$ | *Cytotoxicity L-6* $IC_{50}$ |
|---|---|---|
| Extract | 14.2 µg/ml | 13.6 µg/ml |
| Melarsoprol | 0.001µg/ml | 0.005 µg/ml |

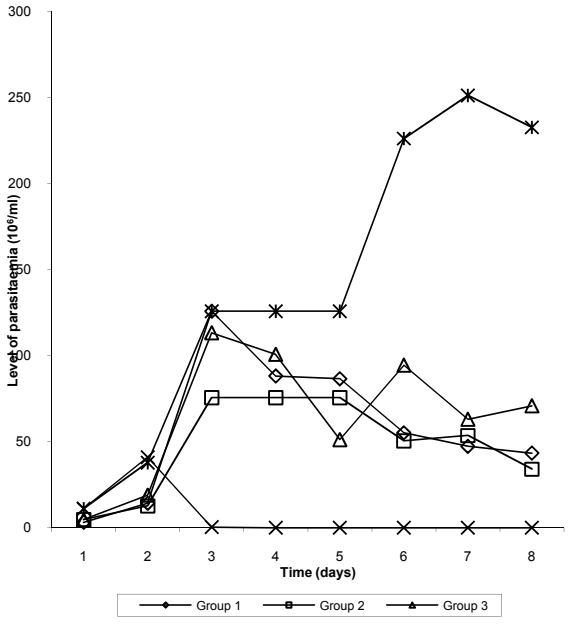


**Fig. 1 Effects of Extract on the parasitaemia on mice infected with T. brucei brucei (P<0.05)**

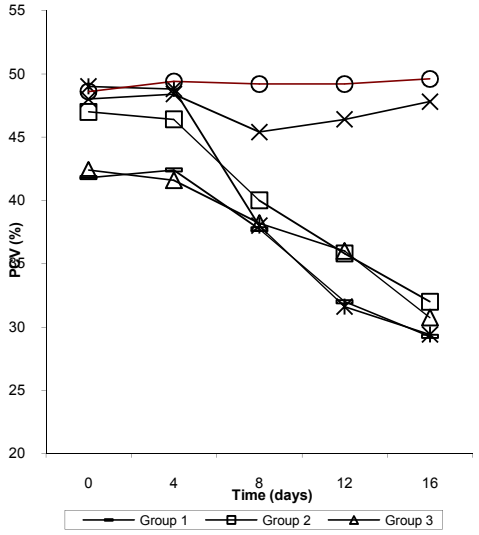


**Fig. 2: Effect of the Extract on the Packed Cell Volume of the Mice (P<0.05)**

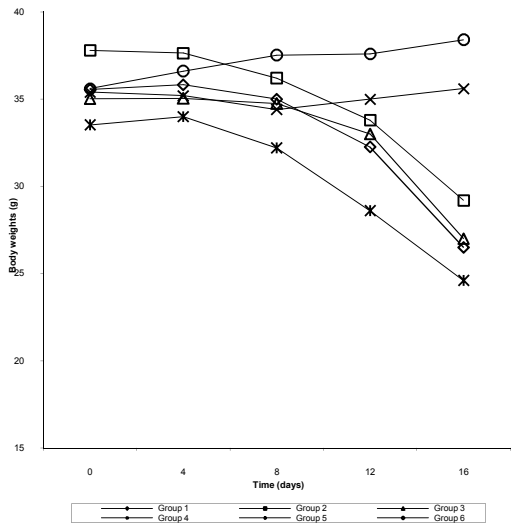


**Fig. 3: The Effect of the Extract on the Body Weight of the Mice (P<0.05)**

## DISCUSSION

The result of the present study revealed that *Vitex simplicifolia* leaf extract exhibited mild antitrypanosomal activity *in vivo* and moderate activity *in vitro.*

The extract at the dose levels administered reduced the parasitaemia level from the 4th post commencement of treatment (Fig.1). The reduction in the level of parasitaemia relapsed on the 6th day of treatment for mice treated with 50 mg/kg. The extract at the dose levels administered failed to clear the parasites in the blood of the mice.

The mild in *vivo* activity of plant extracts have been reported [24], and may be attributed to the metabolism of the active principle through various metabolic processes in the animal. The high level of parasitaemia in the infected mice could also be a factor. Also the moderate *in vitro* activity (Table 1) of the extract may results from the animal's metabolic processes to activate extract with little or no activity *in vitro* [25].

Many natural products exhibit 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 [26]. This is because natural products possess structures capable of generating radicals that may cause peroxidative damage to enzymes that are very sensitive to alterations in redox balance [27].

Diminazene aceturate had total clearance of parasitaemia from the 3rd post treatment without relapse of infection throughout the treatment duration, while there was about 35 % reduction of the parasiteamia between day 5-7 treatment periods. The statistical analysis showed that difference between the treated group and the control group is P<0.05 in all the cases.

The changes in the PCV levels for the various treatment groups are presented in (Fig. 2). There was a gradual fall in the mean PCV levels starting from the 4th day post treatment in all infected mice. However, the reduction continued throughout the treatment period in the mice treated with the extract. The PCV of the mice treated with Diminazene aceturate increased steadily from the 8th day of treatment.

The inability of the extract to lower the parasitaemia level could account for the reduction in the PCV levels of the mice treated with the extract and this explains the anaemic status of the mice throughout the experimental period, since the degree of parasitaemia has been linearly linked to the severity of anaemia [28].

All trypanosome-infected mice except those treated with diminazene aceturate showed a decline in body weight throughout the experimental period (Fig. 3). The reduction in body weight was drastic in the groups treated with the extract. However, the mice treated with the standard drug showed a gradual increase in body weight, from the 8th day post treatment.

## CONCLUSION

The effect of the leaf extract of *Vitex simplicifolia* was investigated in this present study. The results

showed that the extract exhibited moderate activity against *Trypanosomabrucei rhodesiensein vitro* and mild effects on the level *Trypanosomabrucei brucei in vivo.* Work on the effects of the solvent fractions of the plant is ongoing in order to determine the active principle responsible for the *in vitro* effect. To the best of our knowledge no work has been reported on this plant to this effect.

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