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Original Research Article

Evaluation of *In vivo* and *In vitro* Screening of the Antitrypanosomal Properties of Methanol Leaf Extract and Fractions of *Trichilia heudelotii* Oliv. (Meliaceae)

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ABSTRACT

Trypanosomiasis is a serious parasitic disease with high morbidity and mortality particularly in the tropics. *Trichilia heudelotii* Oliv. (Meliaceae) is an important medicinal plant used widely in ethno medicine in treatment of wound sores, gastro-intestinal complaints, dizziness and parasitic diseases. This study evaluated the antitrypanosomal potentials of methanol extract of *T. heudelotii* using Swiss mice. The antitrypanosomal activity of the methanol extract of *T. heudelotii* leaf (METH) was investigated using rapid matching method in mice. Cytotoxicity and *in vitro* antitrypanosomal test were also done using the extract and fractions. Phytochemical screening was done using standard procedures. The extract (100, 200 and 400 mg/kg), diminazene aceturate as positive control (3.5 mg/kg) were administered in the mice. The extract showed dose-dependent inhibition on percentage parasitemia. There was significant ($P < 0.05$) reduction in the parasitemia at all administered doses. METH exhibited significant ($P < 0.05$) increase in packed cell volume (PCV) at 400 mg/kg similar to diminazene aceturate. There was also a significant ($P < 0.05$) increase in body weight at (100, 200 and 400 mg/kg) when compared to the control. In the *in vitro* study the *n*-hexane fraction revealed the highest IC_{50} (2.88 µg/mL) while dichloromethane fraction has the highest selectivity (13.84). Phytoconstituents present include alkaloids, glycosides, saponins, tannins, flavonoids and carbohydrates. The results revealed that the extract possess antitrypanosomal activity in Swiss mice and this justifies the folkloric use of *T. heudelotti* leaves in the treatment of African trypanosomiasis.

Keywords: *Trichilia heudelotti*, Antitrypanosomal activity, Diminazene aceturate, Cytotoxicity, African trypanosomiasis.

Introduction

Trypanosomes are microscopic unicellular protozoa that are parasites of insects, birds, amphibians and mammals and transmitted mainly by insect *Glossina morsitans* (tse tse fly).[1,2] *Trypanosome brucei gambiense and Trypanosome brucei rhodesiense* infect humans while *T. congolense* infects animals. About ninety eight percent (98 %) of the clinical symptoms is caused by *Trypanosoma brucei gambiense.* [3-4] Trypanosomiasis manifests in clinical stages known as the haemolytic and neurological phase.[5] Generally, the symptoms range from headache, joint pains, fever, itchiness, reduced coordination, insomnia, fatigue, confusion and death. Medication used in treatment of trypanosomiasis are eflornithine, pentamidine, suramin, and melarsoprol.[6] Trypanocides are faced by numerous challenges which range from drug toxicity, drug resistance by the parasite. These factors emphasize the need for researchers to seek out for more comprehensive, formidable and cheaper sources of trypanocides. [7]

Trichilia heudelotii an ethno-medicinal plant, and its bark is commonly used in traditional medicine. It is commonly called Ako (Yoruba) and Otanduru (Ghana).[8] It is widely used in ethnomedicine in the treatment of wounds, heart complaints, dysentery, skin infections, sooth cough and parasite infections.[9-10] It has been reported to show significant analgesic property,[10] moderate antimalarial properties,[9] antimicrobial, antipyretic.[11] The study aimed at the determination of the phytoconstituents and antitrypanosomal activity of the extract and fractions of the leaves of *Trichilia heudelotii*.

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Materials and Methods

Plant materials

The leaves of the plant were collected from Orba, Nsukka in April, 2016 and Mr. A.O, Ozioko a taxonomist with the Bioresources Development and Conservation Program (BDCP) Centre, Nsukka identified and authenticated the plant. The voucher number PCG/UNN/0346 was deposited in the herbarium for further reference. The plant material was dried under the shade and milled with a clean milling machine and the powdered plant material was used for extraction using standard analytical grade methanol.

Experimental animals

Inbred Swiss mice, (28.0 - 36.0 g) were kept in steel cages housed in the Department of Anatomy animal house, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The mice were acclimatized for fourteen (14) days (two weeks), water and feed were provided for them before the experiment was started under standard conditions (temperature: 25-34 ºC, humidity: 30-70 %, adequate light and ventilation). Animal handling was in compliance with the approved protocols (FVUNN.IACUC.2019.0816) of the University of Nigeria Ethical Committee on the Care and Use of Laboratory Animals and International guidelines and regulations on the use of animals for experimentation.

Parasites

The parasites used for the study was the flagellate protozoa *Trypanosoma brucei.* This was obtained from an infected mouse obtained from the Parasitology Department of the Faculty of Veterinary Medicine University of Nigeria, Nsukka. The rat was bled from the tip of its tail and its blood collected with a heparinized capillary tube. The blood was diluted with normal saline and kept to be used in infecting the mice for the study.

Solvents, chemicals and reagents

The solvents, chemicals and reagents were of analytical grades. Methanol (JHD Chemical Reagent Co., Guangzhou China), *n*-hexane (JHD Chemical Reagent Co., Guangzhou China), dichloromethane (JHD Chemical Reagent Co., Guangzhou China), ethylacetate (JHD Chemical Reagent Co., Guangzhou China), diminazine aceturate (Techno Pharmachem, India), distilled water, normal saline 0.5 litre (Juhel Pharmaceutical, Nigeria), Water for injection (Juhel Pharmaceutical, Nigeria).

Extraction

Methanol extract

The powdered leaf of *Trichilia heudelotti* (700 g) was extracted with 2500 mL of analytical grade methanol by cold maceration. The mixture was shaken intermittently for forty eight (48) hours. The extract was filtered and was evaporated *in vacuo* using rotary evaporator at 40ºC. The semi-solid extract was weighed and stored in a refrigerator prior to experimentation.

Successive fractionation

n-hexane, dichloromethane and ethyl acetate were used for successive fractionation. 20 g of the methanol extract was dispersed in 90% methanol and successively partitioned with *n*-hexane (900 mL), dichloromethane (800 mL), ethyl acetate (900 mL) to obtain the different fractions.

Acute toxicity test

The acute toxicity evaluation of the methanol extract of *Trichilia heudelotii* (METH) was according to the method of Lorke.[12] The index of the test was the LD_{50} (Lethal median Dose). 13 mice were divided into two stages. In stage one, nine mice were used, they were grouped into three groups of three (3) mice and were administered (10, 100, and 1000 mg/kg) orally respectively. The crude extract was dissolved in a solution of 1% v/v Tween 80 in water. They were watched for 24 hours for behavioural changes and death. The remaining 4 animals were given 1000, 1600, 2700 and 5000 mg/kg METH orally and also observed for 24 hours for behavioural changes and death.

Phytochemical screening of the extracts

Phytochemical screening was done using standard procedure as described by Harbourne.[13] The methanol extract of *Trichilia heudelotti* was screened for alkaloids, glycosides, saponin, tannins, terpenoids, steroids, flavonoids and carbohydrates.[14]

Anti trypanosomal screening

The antitrypanosomal activity of METH was evaluated using 30 mice. They were grouped into six groups of 5 mice each. The infected mice were administered orally 1% v/v Tween 80 in water (negative control), extract (100, 200, and 400 mg/kg) and diminazene aceturate 3.5 mg/kg respectively. The mice were infected with 0.2 ml of the infected blood in saline intraperitoneally. The animals were well cared for by constant provision of food and water. The infection period lasted for five days before initiation of treatment while daily treatment lasted for 12 days.

Study parameters

The degree of infection was assessed using three parameters: level of parasitemia, body weight and packed cell volume (PCV).

Weighing of animals

The mice were weighed before passaging the parasite into the mice. The weights of the animals were taken on days 0, 5, 8, 11, 14 and 17.

Packed cell volume (PCV) determination

The packed cell volume (PCV) value was determined using the micro hematocrit method, with the aid of heparinized micropipette, blood was withdrawn from the tip of tail of the mice into the micropipette and the ends were carefully sealed with plasticine. The micropipette was spun in the hematocrit centrifuge, at 1000 rpm for 5 minutes. The packed cell volumes of the mice were checked on the days 0, 5, 9, 13, and 17. PCV values were taken before passaging the parasite into the mice. They were infected with the parasite obtained from the infected rat, watched and screened for the presence of parasite in their blood and the screening continued until the level of parasitemia became high but not too high to kill the animals. Treatment commenced after the establishment of experimental parasitemia.

$$\text{PCV (\%)} = \frac{Packed\ RBC\ column\ height}{Total\ blood\ volume\ height}$$

Determination of parasitemia

The estimation of the level of parasitemia was done using rapid matching method to match the parasite seen on the field of the microscope under view to that on the matching paper. The level of parasitemia was checked using rapid matching method on the days 5, 7, 8, 11, 14, 17 after infection. The percentage parasitemia was determined using the formula:

$$\text{Percentage Parasitemia (\%)} = \frac{\text{Number of parasitized RBC}}{Total\ number\ of\ RBC} \times 100$$

Determination of in vitro antitrypanosomal activity and cytotoxicity.

Determination of *in vitro* antitrypanosomal activity and cytotoxicity was done using standard procedure as described by Boris *et al.*[15] The methanol crude extract and fractions of *Trichilia heudelotti* were screened for cytotoxicity and inhibitory concentration.[16] The selectivity index was calculated by dividing the mean cytotoxic values with the mean effective concentrations.

Statistical analysis

Data were analyzed using SPSS version 20, One-way ANOVA followed by Dunnett's multiple comparisons post-hoc test. Results are presented as mean ± standard error of mean (SEM) and "n" represents the number of animals per group. Statistically significant were considered at $P < 0.05$.

Results and Discussion

Phytochemical studies of *Trichilia heudelotii* leaves showed the presence of tannins, flavonoids, alkaloids, saponins, glycosides and carbohydrate (Table 1).

Antitrypanosomal properties of medicinal plants have been found to be due to alkaloids, tannins, flavonoids.[17-18] The plant owes its medicinal properties to these chemical constituents.[19,20] The antitrypanosomal properties may be due to the presence of flavonoids as many other plants with trypanocidal activities normally contain flavonoids.[21]

The toxicity test (LD_{50}) showed that the leaves of the plant is relatively safe at a dose of 5000 mg/kg without any death or severe signs and symptoms of toxicity in the mice. METH is considered safe as postulated by Lorke.[12]

Diminazene aceturate shows no steady effect on the weight of the animals when compared to the extracts, the changes in body weight of the animals when compared with the mice treated with different doses of the extract and those treated with diminazene aceturate showed no significant difference (Figure 1).

It was observed that PCV, treated with the extract (100, 200, 400 mg/kg) and diminazene aceturate (3.5 mg/kg) dropped significantly

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Table 1: Qualitative phytochemical screening of methanol extract of *T. heudelotii*

Phytoconstituents	Relative presence
Alkaloids	+
Glycosides	+
Saponins	+
Tannins	+
Flavonoids	+
Resins	-
Proteins	-
Steroid	-
Terpenoids	-
Carbohydrates	+

- = Absent, + = Present

after the seventh day post infection. The haemolytic destruction of the red blood cells of the mice by the invading parasites may be responsible for it. During treatment, it was observed that the PCV of group treated with 100, 200 and 400 mg/kg of the extract and untreated mice respectively continued to decrease progressively within the period (Figure 2). The extract failed to return the PCV values of the mice to normal value. This may be attributed to the fact that the dose of the extract used may be sub-optimal and may need to be increased. However, diminazene aceturate (3.5 mg/kg) was not able to prevent PCV reduction until the fifth day, suggesting its failure to clear the parasite. Similar observations have been reported in previous studies.[22-24] The PCV values of mice treated with diminazene aceturate increased on the fifth day into the treatment and returning to its normal values just before the end of treatment (Figure 2). Pyrexia in trypanosomiasis is caused by trypanolytic crisis which enhances red blood cell damage and destruction leading to anemia.[25] For the parasitaemia level, there is significant decrease ($p < 0.05$) between the parasitaemia level of mice treated with diminazene aceturate and other infected groups on day 17 (Figure 3). Also it was observed that reduction in parasitemia was dose dependent.

Table 2: *In vitro* screening of extract and fractions of *Trichilia heudelotii* leaf

Drugs	IC_{50} (µg/mL) 1	IC_{50} (µg/mL) 2	IC_{50} (µg/mL) Mean	CC_{50} (µg/mL) 1	CC_{50} (µg/mL) 2	CC_{50} (µg/mL) Mean	SI (IC_{50}/CC_{50})
Melarsoprol	0.005	0.006	0.006				
$METH_1$	38.80	14.70	26.75	>100	59.9	59.9	2.24
TSH_2	3.88	1.88	2.88	22.8	18.8	20.8	7.22
$TSDC_3$	12.60	4.84	8.72	56.8	47.8	52.3	13.84
TSE_4	4.64	2.92	3.78	>100	75.5	75.5	8.66
Podophyllotoxin				0.006	0.004	0.005	

$METH_1$ = Methanol extract, TSH_2 = n-hexane fraction, $TSDC_3$ = Dichloromethane fraction, TSE_4 = Ethylacetate fraction, SI = Selectivity Index, CC = Cytotoxicity, IC = Inhibitory Concentration (µg/mL)

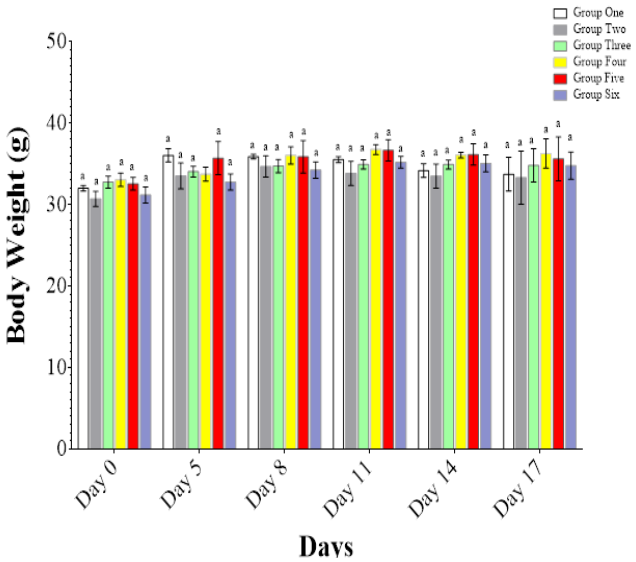

Figure 1: shows the body weight of mice against days of treatment.
Data are expressed as mean ± SEM; n = 5; $p < 0.05$ relative to negative control (group V). a-d indicate significant difference $p < 0.05$. Group one: treated with 100 mg/kg of crude extract per body weight, Group two: treated with 200 mg/kg of crude extract per body weight, Group three: treated with 400 mg/kg of crude extract per body weight, Group four: treated with 3.5 mg/kg of diminazene aceturate per body weight, Group five: negative control (administer orally 1% v/v Tween 80 in water), Group six: not infected and not treated

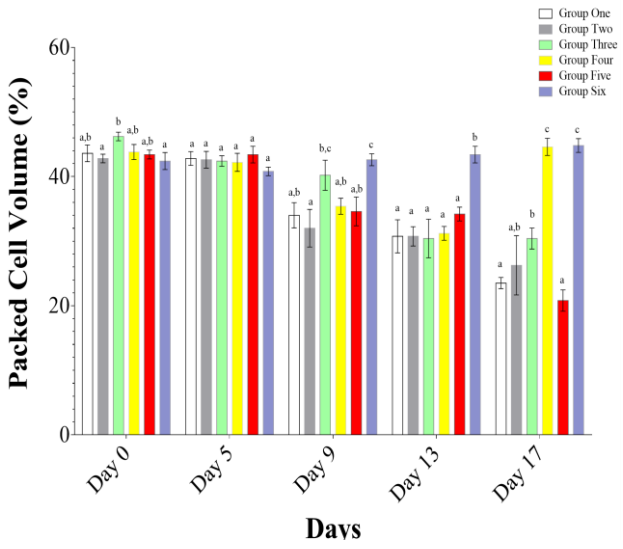

Figure 2: shows the Packed Cell Volume (PCV) against days of treatment.
Data are expressed as mean ± SEM; n = 5; $p < 0.05$ relative to negative control (group V). a-d indicate significant difference $p < 0.05$. Group one: treated with 100 mg/kg of crude extract per body weight, Group two: treated with 200 mg/kg of crude extract per body weight, Group three: treated with 400 mg/kg of crude extract per body weight, Group four: treated with 3.5 mg/kg of diminazene aceturate per body weight, Group five: negative control (administered orally 1% v/v Tween 80 in water).

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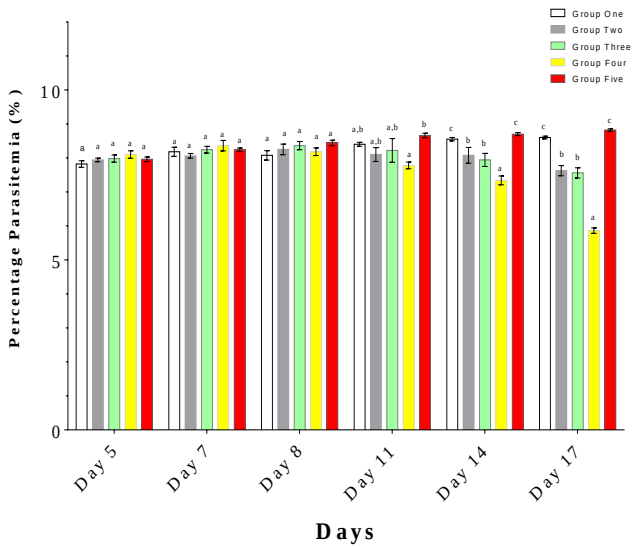

Figure 3: shows the percentage parasitemia (%) against days of treatment.
Data are expressed as mean ± SEM; n = 5; $p < 0.05$ relative to negative control (group V). a-d indicate significant difference $p < 0.05$. Group one: treated with 100 mg/kg of crude extract per body weight, Group two: treated with 200 mg/kg of crude extract per body weight, Group three: treated with 400 mg/kg of crude extract per body weight, Group four: treated with 3.5 mg/kg of diminazene aceturate per body weight, Group five: negative control (administer orally 1% v/v Tween 80 in water).Group six: not infected and not treated.

Reduction in the parasites level of mice treated with diminazene aceturate 3.5 mg/kg was significantly different ($p < 0.05$) compared to doses of 400 and 200 mg/kg. Reduction in parasitaemia obtained in 400 mg/kg was comparable to that obtained in 200 mg/kg. Highest reduction in parasite level was observed in (diminazene aceturate 3.5 mg/kg) compared to other groups. Diminazene aceturate acts by selectively blocking the replication of kinetoplast DNA but the primary target is not known.[1,26] Therefore, the reduction in parasitemia levels between the five infected groups can be summarized as follows starting from the dose that showed highest reduction to the dose that showed the lowest reduction in parasite levels after treatment. 3.5 mg/kg of diminazene aceturate > 400 mg/kg of METH, >200 mg/kg of METH > 100 mg/kg of METH. The extract showed potentials as an anti trypanosomal agent and may produce effect similar to that of diminazene aceturate at increased dose. It was also observed that diminazene aceturate did not give complete eradication of the parasites on the last day of treatment as against that which is seen in the literature, this may be due to drug resistance.[27-30]
The *in vitro* screening of METH and three fractions for antitrypanosomal and cytotoxicity showed that the leaf extract had moderate activity when compared with melasoprol. The Greater activity was seen with the activity order was *n*-hexane fraction with IC_{50} average of 2.88 µg/ml followed by dichloromethane fraction with IC_{50} average of 3.78 µg/ml and then ethyl acetate fraction with IC_{50} of 8.72 µg/mL while the crude extract showed the least trypanocidal activity (26.75 µg/mL). From the cytotoxicity assay, the crude extract and ethyl acetate fractions produced the highest mean cytotoxic values of 59.9 and 75.5 respectively while dichloromethane fraction gave 52.3, *n*- hexane fraction gave the least mean cytotoxic values of 20.8 (Table 2). The crude extract had the least selective index (2.24) while the dichloromethane fractions gave the highest selective index (13.84), though *n*-hexane and ethylacetate fractions gave values of 7.22 and 8.66 respectively (Table 2). The crude extract with the least selectivity index is expected to have the least trypanocidal activity and has the potential of harming the host cells easily. The dichloromethane fractions with the highest selectivity index showed the best activity compared to other fractions. From previous study carried out by Badisa *et al*., [31] the higher the selectivity index value of dichloromethane fraction (13.84), the more selective the parasite is to the fraction.

Conclusion

The *in vivo* and *in vitro* methanol extract and fractions of *Trichilia heudelotii* (METH) possesses moderate antitrypanosomal activity. There was reduced parasite burden, relative increase in PCV and reduced body weight. The selectivity index showed that the dichloromethane fraction has the highest selectivity while the crude extract showed the least selectivity.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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