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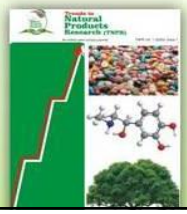

Antitrypanosomal activities of ethanol extract of *Harungana madagascariensis* Lam. ex Poir against *Trypanosoma brucei brucei* - An *in vivo* model

Rolayo Toyin Emmanuel*, Mercy Erutere Okposio, Mohammed Jatto Salawu, Abubakar Ahmad Tijjani, Hauwa Muh'd Sabo

Nigerian Institute for Trypanosomiasis and Onchocerciasis Research, Kaduna, Kaduna State, Nigeria.

Abstract

African trypanosomiasis constitutes public health and veterinary challenges in sub-Saharan Africa. Development of vaccine for its prevention is beset with challenges. Hence, trypanosomiasis control is premised on vector control and treatment of identified cases. Effect of oral administration of ethanol extract of stem bark of *H. madagascariensis* on *Trypanosoma brucei brucei* (Federe strain) was investigated in experimentally-infected rat model. *Harungana madagascariensis* stem bark powder (300 g) was macerated in 1.5 l of 100 % n-hexane for 48 hours. The residue was dried and re-macerated in 1.5 l of 70 % ethanol for 48 hours. The filtrate was dried by evaporation on water bath at 60^{o}C. Phytochemical screening revealed the presence of alkaloids, flavonoids, terpenoids, anthraquinones, saponins, phenols and tannins. Acute toxicity test revealed oral LD_{50} > 5000 mg/kg body weight. Oral administration of the extract did not exert curative effect. Parasitaemia persisted in the extract-treated rats (Group A-1250 mg/kg and Group B-2500 mg/kg body weight) till death. Rats administered 1250 mg/kg body weight outlived all inoculated rats by 2 days despite the significant ($P < 0.05$) rise in parasitaemia. Anaemia was ameliorated in rats taking 1250 mg/kg body weight compared to groups B, C and D that were severely anaemic. Emaciation was ameliorated in both extract-treated groups A and B compared to groups C and D. *Harunga madagascariensis* demonstrated promising prospects as potential source of therapeutic agent for ameliorating the severity of anaemia and African trypanosomiasis.

Keywords
Trypanosomiasis, *Harungana*, procyanidins, ethnomedicine, parasitaemia, anaemia

*Corresponding author:
rolayoemmanuel@gmail.com;+2348093000886
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Introduction

Trypanosomes cause devastating human African trypanosomiasis (sleeping sickness) and animal African trypanosomiasis (nagana), posing danger to human wellbeing and livestock productivity in underserved rural areas. It is transmitted by haematophagous tsetse flies across about 10 million km^2 in thirty-six sub-Sahara African countries (Burri, 2020).

In humans, the disease has distinct haemolymphatic stage where the trypanosomes are confined to the lymph and blood circulatory systems, then advances to cerebrospinal stage, where trypanosomes break the blood-brain barrier to invade the brain and the cerebrospinal fluid. It is characterized by high morbidity, distorted sleep-wake rhythm and fatal if left untreated (Aksoy *et al.*, 2017). Hence, effective control of human African trypanosomiasis (HAT) basically requires early case detection and prompt treatment of confirmed cases.

Fexinidazole is the only approved drug by World Health Organization (WHO) for treating HAT after the prohibition of old toxic drugs which also encounters trypanosome resistance. Fexinidazole is ideal based on its safety and non-invasive route of administration but not readily available in healthcare facilities. Also, its absorption is impaired without food and has not been certified for use in children below 6 years (Steverding, 2010; Lindner *et al.*, 2020). Hence, the search for alternative drugs is imperative.

Medicinal plants are nature's treasury of bio-pharmaceutical resources with plentitude of secondary metabolites which serve as plant defense mechanism and capable of exerting bioactivities *in vivo* and *in vitro*. The use of medicinal plants for disease prevention and cure dates back to prehistoric times. Many botanically-sourced conventional pharmaceutical compounds such as reserpine and vinblastine were derived from *Rauwolfia spp*. and *Catharanthus roseus* respectively, quinine and artemisinin from *Cinchona* and *Artemisia* respectively (Newman *et al.*, 2000; Zhu *et al.*, 2015). *Harungana madagascariensis* Lam. ex Poir is native to Africa and Madagascar, it has historic value in traditional medicine against myriads of illnesses. It is commonly known as dragon blood tree, aliliba rafii (Hausa), asunje (Yoruba) and uturu (Igbo). The plant produces sap with characteristic yellow pigment known as harunganin (Stout *et al.*, 1962). It has been reported to be useful in management of constipation, stomach ache, helminth infestation, cutaneous ailments, ulcer, jaundice, fever and for enhancing lactation (Iwalewa *et al.*, 2008; Afieroho *et al.*, 2019; Happi *et al.*, 2020). It is incorporated into Jubi herbal formula used for curing anaemia (Shorinwa and Monsi, 2019).

It is noteworthy that African trypanosomiasis is a disease ravaging remote neglected rural communities with poor healthcare facility and healthcare personnel coverage. It is important that drug discovery projects target drug candidate that requires minimal professional supervision, non-parenteral administration and treatment protocol with shortest hospitalization period. This study evaluated the anti-trypanosomal activity of orally-administered ethanol extract of *Harungana madagascariensis* against *Trypanosoma brucei brucei* in Wistar rat model.

Materials and methods

Preparation of ethanol extract

The stem bark of *Harungana madagascariensis* was collected from Ogbomosho, Oyo State, Nigeria. The identity of the plant was authenticated at the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. The stem bark was rinsed under running tap to remove dust, then dried under shade at room temperature to a constant weight and milled into fine powder. About 300 g of powder was soaked in 1.5 l of 100% n-hexane for 24 hours. The supernatant was decanted and the residue air-dried at room temperature. Dried residue was re-macerated in 1.5 l of 70% ethanol for 48 hours. The filtrate was concentrated on water bath at 60ºC. The yielded extract was stored in airtight glass vial in refrigerator at 4ºC

Qualitative phytochemical screening

Phytochemical analyses were in accordance with Harborne's methods (Harborne, 1998).

Animals

Thirty-six adult albino rats were purchased from the laboratory animal colony of Nigerian Institute for Trypanosomiasis and Onchocerciasis Research, Kaduna, Nigeria. The animals were maintained on standard grower's pellets (Pfizer Plc., Lagos, Nigeria) and water *ad libitum*. Animal handling was in adherence to Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animal in Research (Committee on Animal Research and Ethics, 2012).

Trypanosomes

Trypanosoma brucei brucei (Federe strain) was obtained from Vector and Parasitological Research Department, Nigerian Institute for Trypanosomiasis (and Onchocerciasis) Research, Kaduna, Nigeria. The parasite was maintained in Wistar rats.

Acute toxicity study

Lethal dose (LD_{50}) was determined using Lorke's method (Lorke, 1983). The animals were observed over a period of 7 days for signs of toxicity such as salivating, dilated pupil, sneezing, protruded tongue, dyspnea, lethargy, convulsion, foaming, hyperactivity and death.

Experimental design for in vivo antitrypanosomal study

Twenty rats were randomly divided into five groups A, B, C, D and E of 4 rats each. Groups A, B, C and D were inoculated via intra-peritoneal route with 1 x 10^3 *Trypanosoma brucei brucei* (Federe strain). Treatment with extract commenced on day 3 post-inoculation for 3 consecutive days as stated below:
Group A: Oral administration of 1250 mg/kg b.w. of extract.
Group B: Oral administration of 2500 mg/kg b.w. of extract.
Group C (positive control): Intra-muscular administration of single dose of 3.5 mg/kg b.w. of Diminazene aceturate (Berenil, Merck & co., USA).
Group D (negative control group): Infected, given oral placebo of distilled water only.
Group E: Not infected, given oral placebo of distilled water only.

Determination of parasitaemia

Parasitaemia was monitored at 72 hours' interval from day 3 post-inoculation (dpi). A drop of blood from rat tail was placed on a microscopy glass slide, then covered with a microscopy cover slip and examined under the light microscope at x400 magnification. The number of trypanosomes per microscopy field was recorded and estimated as number of trypanosomes/ml using the rapid 'matching' method (Herbert and Lumsden, 1976).

Determination of packed cell volume

The packed cell volume (PCV) was determined using the microhaematocrit technique (Mondal and Lotfollahzadeh,2024).Heparinized microhematocrit capillary tubes was filled to 75% capacity with rat tail blood. One end of the tube was sealed with clay sealant and centrifuged at 12000 rpm for 5 min in a microhaematocrit centrifuge. The packed cell volume (PCV) for each sample was determined with the aid of a microhaematocrit reader, and the values expressed as percentages.

Statistical Analyses

One-way analysis of variance (ANOVA) and Newmann Keul's post hoc test were used to compare difference between data using Statistical Package for Social Sciences version 25 software. Results were expressed as mean±standard error of the mean (mean±SEM). Differences in means were considered statistically significant at $P \leq 0.05$.

Results

Qualitative phytochemical screening revealed the presence of alkaloids, flavonoids, anthraquinones, saponins, tannins, phenols, glycosides and terpenoids (Table 1). There were no mortality or observable physical signs of toxicity among animals administered with 1250 mg/kg bw, 2500 mg/kg bw and 5000 mg/kg bw of the extract. The PCV of the group A rats decreased from 45.35 ± 1.7% to 45.50 ± 8.4% on day 3 post-inoculation ($P > 0.05$), decreasing further to 28.50 ± 8.4% by day 6 post-inoculation ($p < 0.05$). Group B rats have slight decreases in PCV from 45.75 ± 2.2% to 43.50 ± 2.3% ($P > 0.05$) which progressed to severe anaemia by day 6 post-inoculation. There was a significant drop in PCV of rats in group C and group D from 44.25 ± 1.5% and 40.25 ± 1.9% pre-inoculation to 32.50 ± 6.4% and 33.51 ± 1.5% on day 3 post-inoculation ($P < 0.05$) respectively. Rats in group C were severely anaemic

Table 1: Qualitative analyses of phytochemical constituents of *Harungana madagascariensis* stem bark ethanol extract

Phytochemical	Test reagent/Method	Analyses outcome
Alkaloids	Dragendroff's test	+
	Mayer's test	+
Flavonoids	Hydrochloric acid	+
Anthraquinones	Benzene/Ammonium solution	+
Saponin	Frothing test	+
	Fehling's test	+
Tannins	Ferric chloride solution	+
Terpenoids	Salkowski's test	+
Phenols	Folin-Ciocalteu reagent	+
Glycosides	Libermann's test	+

Keys: + = Present - = Absent anaemic while those in group D were all dead by day 6 post-inoculation

All inoculated groups presented parasitaemia on day 3 post-inoculation (Table 3). Group A showed increase in parasitaemia from 43.33 ± 3.3 × 10^3 trypanosomes/ml on day 3 post-inoculation to 48.52 ± 0.2 × 10^3 trypanosomes/ml) on day 6 post-inoculation. However, rats in groups A and B were relatively active and not emaciated compared to rats in groups C and D despite the increase in parasitaemia observed among group A. Furthermore, parasitaemia persisted until all the inoculated rats died. Group A outlived groups B, C and D by 2 days despite the rise in parasitaemia and severe anaemia.

Table 2: Effects of administration of *Harungana madagascariensis* stem bark ethanol extract on packed cell volume

Group/Treatment	Packed Cell Volume (%)		
	Day 0	Day 3	Day 6
A (1250 mg/kg bw extract)	45.35 ± 1.7	45.50 ± 8.4	28.50 ± 8.4*
B (2500 mg/kg bw extract)	45.75 ± 2.2	43.50 ± 2.3	+++
C (3.5mg/kg bw Berenil)	44.25 ± 1.5	32.50 ± 9.4*	+++
D (Distilled water)	40.25 ± 1.9	33.51 ± 1.5*	All dead
E (Uninfected/untreated)	45.33 ± 1.6	43.67 ± 1.1	43.67 ± 1.7

Note: Results are expressed as Mean ± SEM (n=4). * $P < 0.05$. +++ means extremely anaemic.

Table 3: Effects of administration of *H. madagascariensis* stem bark ethanol extract on parasitaemia

Groups/Treatment	**Parasitaemia (×10^3 trypanosomes/ml)**		
	Day 0	**Day 3**	**Day 6**
A (1250 mg/kg bwt extract)	0	43.33 ± 3.3[a]	48.52 ± 0.2[a]
B (2500 mg/kg bwt extract)	0	31.67 ± 1.7[a]	++++
C (3.5mg/kg bw Berenil)	0	31.33 ± 3.2[a]	+++
D (Distilled water)	0	41.00 ± 0.6[a]	All dead
E (Uninfected/untreated)	0	0	0

Results are expressed as Mean ± SEM (n=4). * ($P<0.05$). +++ means extremely anaemic.

Discussions and conclusion

Medicinal potential of a plant is determined by its phytochemical constituents. The therapeutic properties of *H. madagascariensis* meriting its listing among ethnomedicinal remedies can be attributed to proven bioactivities of its phyto-constituents. The presence of alkaloids, flavonoids, terpenoids, anthraquinones, saponins, phenols and tannins corroborate previous researches (Eze and Airouyuwa, 2014; Ngbolua, 2020). Plant phyto-constituents exert adverse effects when consumed in certain doses or via specific route of administration. Hence, the relative safety of oral administration of *H. madagascariensis* extract to experimental rats with LD_{50} >5000 mg/kg bw agrees with the findings of Etame *et al.* (2017) which reported LD_{50} >5000 mg/kg bw for methanol extract, and Biapa *et al.* (2013) who reported LD_{50} of 6250 mg/kg bw.

Decrease in PCV among infected groups indicates progress of trypanosomiasis. Anaemia is a cardinal indicator of African trypanosomiasis attributable to deleterious effects of biological substances produced by proliferating trypanosomes and infection-induced free radicals on sialic acid component of the erythrocytes glycocalyx, iron sequestration, platelet aggregation, distortion of erythropoiesis, haemodilution, alterations of redox balance of the red blood cell membrane and eventually erythrolysis inferred by decrease in PCV (Igbokwe and Anosa, 1989; Boada-Sucre *et al.*, 2016). Dose-dependent decrease in PCV among extract-treated groups especially group B presenting severe anaemia may be attributed to higher concentration of phytochemicals such as saponin in larger dose of extract administered to group B which is capable of exerting pronounced effect on the red blood cells but unobvious at lower doses containing lesser concentration of saponin. Several studies demonstrated the deleterious effects of saponin on red blood cells leading to haemolysis and consequently lowered packed cell volume (Podolak *et al.*, 2010; Savage, 2016). Similarly, tannins and saponins are incriminated in essential nutrients sequestration (South and Miller, 1998; Savage, 2016), despite tannins' antioxidant potency, it chelates with nutritional minerals such as dietary iron in intestinal lumen impairing its bioavailability for erythropoiesis (Karamać, 2009; Hurrell and Egli, 2010; Jaramillo *et al.*, 2015). Shortage in bioavailability of iron could have been exacerbated by underlying trypanosomal infection.

Conversely, the extract ameliorated the severity of anaemia among group A and emaciation among groups A and B when compared to groups C and D. This validates the claim that extracts of *H. madagascariensis* stem bark possess anti-anaemic and red blood cells protective properties (Iwalewa *et al.*, 2009; Nku-Ekpang *et al.*, 2018). Ameliorative effect observed in severity of infection and anaemia in groups A and B may also be owing to the antioxidant potency of *H. Madagascariensis* (Iwalewa *et al.*, 2008; Antia *et al.*, 2015; Koné *et al.*, 2023) conferred by its richness in procyanidins, capable of scavenging trypanosomiasis-induced free radicals (Llorent-Martinez *et al.*, 2020).

The extract did not exert curative effect on infected rats similar to the findings of Adelodun *et al.* (2013). However, it exhibited trypano-suppressive effect similar to *in vivo* malaria-suppressive activity reported by Iwalewa *et al.* (2008). This may be due to the extreme virulence of the trypanosome strain (Federe strain) used for the study (Ihedioha *et al.*, 2010). Furthermore, the bioactivity of medicinal plant extracts depends on the concentration of the phytoconstituents available in a biological system in stable active form. Bioavailability and bioactivity may therefore be impacted by phytoconstituent

metabolism in the liver, binding to plasma proteins, and excretion (Ghuman *et al.*, 2005; Teng and Chen, 2019; Hu *et al.*, 2023). Hence, the absence or insufficient concentration of anti-trypanosomal phyto-constituents administered doses is a plausible explanation for lack of curative effect (Calonico and De La Rosa-Millan, 2023; Hu *et al.*, 2023).
Therefore, detailed qualitative and quantitative bioassay piloted study of various bioactive constituents of *H. madagascariensis* is required to further explore its anti-trypanosomal potential. Also, alternative route of administration may be considered in subsequent studies.

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Conflict of Interest

Authors declare that there is no conflict of interest.

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