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Original Research Article

Phytochemistry and Antitrypanosomal Effects of *Acacia nilotica*, *Tamarindus indica* and *Terminalia avicennioides* Using Drug Incubation Infectivity Test

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ABSTRACT

Trypanosomiasis remains a major constraint to the development of the livestock sector in sub-Saharan Africa with the negative economic impact extending into South America and Asia. The increasing resistance to the available trypanocidal drugs necessitates the need for the discovery of newer and more efficient drug. Therefore, the aim is to screen three important Nigerian ethnomedicinal plants for antitrypanosomal potential. Fifty microliter of 20, 10 and 0.1 µg/µL each of the crude methanol extract of *Acacia nilotica*, *Terminalia avicennioides* and *Tamarindus indica*, and diminazene aceturate was mixed with 50 µL of *Trypanosome congolense*-laden blood (TC-LB) (8.6×10^7 cells per mL of blood) and incubated at 25°C for 5 h. Similarly, wells with 2% tween 80 and TC-LB only served as negative and untreated controls, respectively. The experiment was carried out in triplicate. The contents of each well were inoculated into mice at score 0 and at the end of the experiment for concentrations that did not produce score 0. Phytochemical constituents of each extract were detected by thin-layer chromatography. *T. avicennioides* and *A. nilotica* reached score 0 within 3 and 5 h, respectively, and did not produce infection in the inoculated mice. However, *T. indica* produced significant ($P < 0.05$) reduction in parasite motility at the highest concentration compared to negative control. Alkaloids, phenols, steroids and triterpenes were detected in the three plants. Additionally, *T. avicennioides* also contained anthraquinones. Thus, the plants, particularly *T. avicennioides* and *A. nilotica* offer prospects for the discovery of new antitrypanosomal drugs.

Keywords: Drug discovery, medicinal plant, phytochemical screening, trypanocidal, *Trypanosomal congolense*

Introduction

African Trypanosomiasis is a chronic debilitating disease caused by extracellular flagellated haemoprotozoan parasites called *Trypanosoma species* and it is transmitted primarily by the bite of infected tsetse fly (Diptera: Glossinidae).[1] The disease affects a wide range of mammalian species, including humans. *Trypanosoma vivax* (Dutonella), *T. congolense* (Nanomonas) and *T. brucei* (Trypanozoon) are the three main species of trypanosomes endemic to Africa; with Dutonella and Nanomonas infecting mainly livestock while Trypanozoon affects both humans and animals.[2,3,4] *T. brucei* has three sub-species of which two: *T. b. gambiense*, and *T. b. rhodesiense* infect mainly humans, whereas the third, *T. b. brucei* infects only domestic and wild animals.[5,6] The parasites invade the lymphatic vessels, the blood circulation and eventually the brain of the host, causing a wide range of pathologies; anaemia, weight loss, foetal abortion, cachexia and death if left untreated.[7,8]

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Animal African Trypanosomiasis (AAT) is endemic in sub-Saharan Africa (SSA), affecting an area of approximately 10 million square kilometers of arable land, and reducing the efficiency of productivity of over 150 million cattle and 260 million sheep and goats.[9,10] It renders vast areas of land inhabitable and unsuitable for agriculture. The direct and indirect annual economic losses associated with the AAT alone are estimated at US$ 5 billion.[11] This explains why 21 countries in SSA where trypanosomiasis is endemic are included in the world's 25 poorest countries.[12] Pathogenic trypanosomes of livestock remain an important constraint to the development of the agricultural sector in SSA, and the negative economic impact of the disease is extending into South America and Asia.[13] Although significant progress has been made in the eradication of HAT,[14] AAT remains topmost on the list of infectious diseases that limit the development of the livestock industry in SSA.[15] Control of trypanosomiasis is aimed at the destruction of the arthropod vectors (especially *Glossina species*) and the elimination of the parasite in the infected individuals using chemotherapy.[16] Improvement in the control of trypanosomiasis in Africa will be of immense relief to the endemic areas. The use of trypanocidal drugs for the control of trypanosomiasis and insecticides for vector control would have eradicated the disease or significantly reduced its burden; resistance to these agents reduce their success rate and sustainability.[17] Interestingly, livestock, particularly cattle, remain the most important target for possible eradication of both AAT and zoonotic fatal HAT.[18]

Medicinal plants have been excellent sources of naturally produced compounds which account for a significant proportion of modern therapeutic agents.[19] Natural extracts are complex mixtures rich in bioactive compounds for medicinal uses and their derivatives are

excellent sources of lead structures for synthetic modification and optimisation of bioactivity. It is estimated that 85% of the world population depends directly on plants as medicines.[20] Twenty-five percent of modern medicines are made from plants that were first used traditionally.[21] The main aim has been geared towards alternative drugs to conventional drugs with fewer side effects but greater effectiveness.

Acacia nilotica (L.) Delile, family *Leguminosae*, is a multipurpose tree of this genus found in Africa, Middle East and India.[22] It is rich in polyphenols, especially, condensed tannin and phlobatannin in addition to gallic acid, ellagic acid, catechin, and epigallocatechin-7-gallate.[23] All parts of the plant are used for medicinal purposes in different parts of the world.[24] *Tamarindus indica* L. commonly known as the tamarind tree also belongs to the family *Leguminosae*. It is one of the fruit tree species that is used in traditional medicine.[25] All parts of the plant are used in pharmaceutical, chemical, food and textile industries, and as fodder, timber and fuel.[26] Phenolic compounds like catenin, procyanidin B_2, epicatechin, tartaric acid, mucilage, pectin, arabinose, xylose, galactose, glucose, uronic acid and triterpene have been identified in the plant.[27] *Terminalia avicennioides* Guill. & Perr., family, *Combretaceae*, is one of the most widely used medicinal plants for medicinal purposes worldwide. Ellagic acid, punicalagin, flavogallonic acid and terchebulin are predominant tannin and phenolic phytochemical constituents detected in the plant.[28] The two plants have demonstrated activity against *T. brucei*.[29]

Materials and Methods

Plant materials

The stem-bark of *A. nilotica* and *T. avicennioides* and leaves of *T. indica* were harvested (November, 2018) on the main campus of the Ahmadu Bello University (ABU), Zaria. A sample of each of the plants was taken to the herbarium, Department of Botany, Faculty of Life Science, ABU, Zaria, and the plants were identified by Namadi Sunusi with voucher specimen numbers: 698, 900239 and 602 for *A. nilotica*, *T. avicennioides* and *T. indica*, respectively, deposited at the herbarium. They were dried to constant weight in the laboratory and pulverised with mortar and pestle.

Preparation of plant extracts

Five hundred grams of each pulverized plant was extracted with 2.5 L of absolute methanol using the Soxhlet apparatus. Each distilled extract was decanted through a Whatman filter paper No. 42 into clean appropriately labeled bottles and concentrated to dryness in vacuo using a rotary evaporator. The dried extracts were kept in labelled containers and stored at 4℃ until required.

Trypanosoma congolense

Trypanosoma congolense was obtained from the Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, ABU, Zaria. The parasite was maintained in the laboratory during the experiment through a continuous passage in rat. Each cycle of passage was done when parasitaemia was in the range of 8.4×10^7 parasites per milliliter of blood (64 parasites per field).

Phytochemical analysis

The thin-layer chromatography (TLC) profile of each extract was obtained by spotting each extract on a silica gel 60 TLC_{254} plate and the plate was developed with 6 mL n-butanol + 1 mL acetic acid + 1 mL water (BAW 6:1:1). The plates were sprayed with anisaldehyde/sulphuric acid as detecting reagent followed by heating at 110°C.

Experimental animals

Five pairs of Wistar rats were obtained from the Animal Room of the Department of Veterinary Pharmacology and Toxicology, A.B.U., Zaria for breeding. Outbred rats were used for the study. The rats were fed rat chow and water was provided *ad libitum*. The animals were kept in clean iron cages at room temperature throughout the study and allowed to acclimatise for one week. The bedding was changed twice weekly throughout the duration of the experiment. Ethical approval was obtained from the Ethical Committee on Animal Use and Care, Ahmadu Bello University, Zaria, with reference number: ABUCAUC/2019/005.

Determination of median lethal dose (LD_{50}) of the extracts

The LD_{50} of each extract was determined using the 5,000 mg/kg limit dose described by the OECD guideline 2008 (OECD, 2008).[30] Young adult non-pregnant nulliparous female rats weighing between 150 and 170 g and aged 8-12 weeks were used. The animals were kept in their cages for 5 days to acclimatize before dosing. The rats were taken off feed overnight and their weight was taken. The dose was administered singly through gavage with an intubation cannula. After dosing, feed was withheld for additional 4 h. A total of five rats were dosed for each extract. Briefly, 5,000 mg/kg of each extract was administered to one rat at a time and the rat was observed for a period of 48 h. The survival of the first rat resulted in the sequential dosage of 4 additional rats.

In vitro antitrypanosomal study

Fifty microliters of 20, 10 and 0.1 µg/µL of each extract and diminazene aceturate were pipetted into separate wells of a 96-well microtitre plate and 50 µL of trypanosome-laden blood from donor rat sacrificed at greater than 72 parasites per field (8.6×10^7 per mill of blood) was added; giving final concentrations of 10, 5 and 0.05 µg/µL, respectively. The mixture was rocked gently and incubated at 25°C for 5 h. Similarly, 2% tween 80 was put in separate wells and served as negative control; while *T congolense*-laden blood in another well served as untreated control. The experiment was carried out in triplicate. The antitrypanosomal effect of the treatment was assessed at 1 h intervals by taking a drop of the mixture from each well onto a microscope slide, covered with a cover slip and observed with a light microscope at ×400 magnification. The differences in the rate of immobilisation (cessation) or reduction of parasite motility compared to control groups were scored (Table 1) and used as a measure of efficacy.[31,32]

Drug incubation infectivity test

A drug incubation infectivity test (DIIT) was carried out as described by Kaminsky et al.[33] Any concentration from the *in vitro* antitrypanosomal study that produced cessation of parasite motility (score 0) was immediately aspirated and inoculated into a mouse. Similarly, all the concentrations with scores 1 to 6 at the end of 5-h incubation period were equally inoculated into mice. The mice were observed daily for possible development of parasitaemia by nipping the tail and collecting a drop of blood onto a microscope slide, covered with a cover slip and observed with a light microscope at ×400 magnification.

Data analysis

Values were expressed as mean ± standard error of the mean and the means were compared with one way analysis of variance using GraphPad Prism version 5. Values of $P < 0.05$ were considered significant.

Table 1: Score chart for *in vitro* antitrypanosomal study (Tauheed et al., 2020; 2021)

Trypanosomes per field	Score	Remark
0	0	No motile (active) parasite in ≥ 20 fields
1 - 2	1	Moribund parasites in ≥ 20 fields
3 - 5	2	Motile parasites in ≥ 3 fields
6 - 10	3	Motile parasites in ≥ 3 fields
11 - 20	4	Motile parasites in ≥ 3 fields
21 - 40	5	Motile parasites in ≥ 3 fields
> 40	6	Motile parasites in ≥ 3 fields

Results and Discussion

Thin-layered chromatographic screening of the crude methanol extracts of the three plants showed that all the plants contained alkaloids, phenols, steroids and triterpenes. In addition, *T. avicennioides* also contained anthraquinones (Table 2). Secondary metabolites are organic compounds produced by plants and animals but are not directly required for their growth, development or reproduction.[34] These metabolites are often required by the plant to ward off potential attacks from animals and the environment; and they are responsible for the pigmentation, fragrance and toxicity of some of these plants.[35] Plant secondary metabolites are important sources of lead compounds for drug discovery. It has been reported that antitrypanosomal effect of alkaloids is due to the inhibition of protein synthesis and intercalation of deoxyribonucleic acid.[36,37] Jatrorrhizine, palmatine, columbamine and βsitosterol alkaloids isolated from the stem-bark of *Enantia chlorantha* demonstrated significant trypanocidal effect *T. brucei* in addition to antiplamodial and antileishmanial effects.[38] Furthermore, Rozenkranz and Wink[39] postulated that the trypanocidal effect of alkaloids could be due to their ability to inhibit mitochondrial microtubule formation, disturbance of membrane fluidity, intercalation of DNA and inhibition of protein synthesis with resultant induction of programmed cell death (apoptosis). Natural products rich in phenolic compounds have been reported to possess potent antitrypanosomal properties.[40,41] Anthraquinone, aloin, and its derivative demonstrated dose-dependent *in vitro* and *in vivo* antitrypanosomal effects against *T. congolense* field isolate.[42] The antitrypanosomal mechanism of action of anthraquinones was reported to be due to their ability to suppress the expression of paraflagellum rod protein subunit 2 (PFR-2) with resultant cell cycle alteration and induction of apoptosis in the bloodstream form of trypanosomes.[43]

In vitro antitrypanosomal study

The *in vitro* antitrypanosomal result of the three plants is shown in Figure 1. *Terminalia avicennioide*s caused complete cessation of the parasite motility (score 0) within 3 h at the highest concentration of 10 µg/µL; while *A. nilotica* exhibited moderate *in vitro* antitrypanosomal activity and completely immobilised the parasite motility (score 0) within 5 h at the same concentration. However, *T. indica* showed the least inhibitory activity as it only caused a significant ($P < 0.05$) reduction in the number of motile parasites (score 1) at the highest concentration compared to the negative control. Paradoxically, diminazene aceturate (positive control) did not cause a complete cessation of parasite motility even at the highest concentration. Nonetheless, it demonstrated some levels of effect by significantly reducing the number of motile parasites at the highest concentrations when compared to the negative control. There has been a growing call in the scientific community to domesticate the principle of 3 Rs (replacement; refinement and reduction) in research. The replacement relies on the use of *in vitro* techniques that are cheap, sensitive and reproducible. According to Hartung and Daston,[44] *in vitro* techniques require small set-ups with few test substances and, therefore, have the advantages of low cost, use of high number of replicates, miniaturisation, automation and fewer ethical requirements.

Drug incubation infectivity test

The highest concentration of 10 µg/µL of *T. avicennioides* and *A. nilotica* that was inoculated into the mice following the attainment of score 0 did not produce infection in the mice and the mice survived the 4-week experimental period. Similarly, mice inoculated with 5 µg/µL of *T. avicennioides* at the end of 5-h incubation period and at score 1 did not produce infection and yielded a 100% survival rate (Table 3). However, 5 µg/µL of *A. nilotic*a inoculated into mice at the end of 5-h incubation period produced infection in one mouse and the mouse succumbed to the infection, giving a 66.7% survival rate. All other treatment groups and concentrations of *T. avicennioides* and *A. nilotica* developed infection with 100% mortality (zero survival rates).

DIIT is an extension of *in vitro* antitrypanosomal study used to confirm the efficacy of *in vitro* enumeration of cultured trypanosomes.[32] It does not require trypanosomes pre-adapted to an expensive culture environment.[33] The 100% survival rates recorded for the groups and concentrations that completely immobilized the parasites showed that the parasites were killed. Furthermore, it could also mean that the enumeration of trypanosomes under a microscope that produced score 0 (no motile parasites in at least 20 fields) could be relied upon for rapid, inexpensive *in vitro* antitrypanosomal study. The DIIT results showed that the course of infection of mice inoculated at score 3 with the highest concentration of *A. nilotica* which exhibited significant *in vitro* antitrypanosomal effect (enumeration of trypanosomes) did not differ from other concentrations that did not demonstrate *in vitro* antitrypanosoal effect. From the foregoing, it follows that a screening test that achieves a score of 1 (Table 1) does not require mouse inoculation. This will be of particular advantage for studies involving trypanosomes that do not grow in rodents. It will also help reduce the number of mice to be inoculated for DIIT since this may only be considered for the concentrations that attain score 2 and thus help achieve the aim of "reduction" (one of the 3 Rs in research).

Intermediate (24 to 48 h) and long (greater than 48 h) incubations are required for diamidines to exert their full *in vitro* chemotherapeutic efficacy.[45] Therefore, the poor efficacy of diminazene aceturate reported in the present study compared to the plant extracts may be due to the ultra-short duration of incubation (5 hours) used. Several authors have reported greater *in vitro* activity of medicinal plants than diminazene.[31,46,47] However, Tauheed et al reported greater *in vivo* efficacy of diminazene than plant extract despite the latter greater *in vitro* effect and they postulated that plant extract may exert a cytotoxic effect on trypanosome during *in vitro* assay.[31]

Table 2: Phytochemical constituents of the crude methanol extracts of the three plants

Plants	Dragendoff (Alkaloids)	Bontragers (Anthraquinones)	Ferric chloride (Phenolic compounds)	Liebermann-Burchard (Steroids and triterpenes)
Acacia nilotica	+	-	+	+
Terminalia avicennioides	+	+	+	+
Tamarindus indica	+	-	+	+

+ means present; - means absent

Table 3: Survival rate of mice under drug incubation infectivity test

Concentration (µg/µL)	TI	TA	AN	DA	PBS	T-LB	T80
	Survival rate (%)						
0.05	0	0	0	0	0	0	0
5	0	100*	66.7*	0	0	0	0
10	0	100	100	0	0	0	0

*Score 1 at the point of inoculation into the mice

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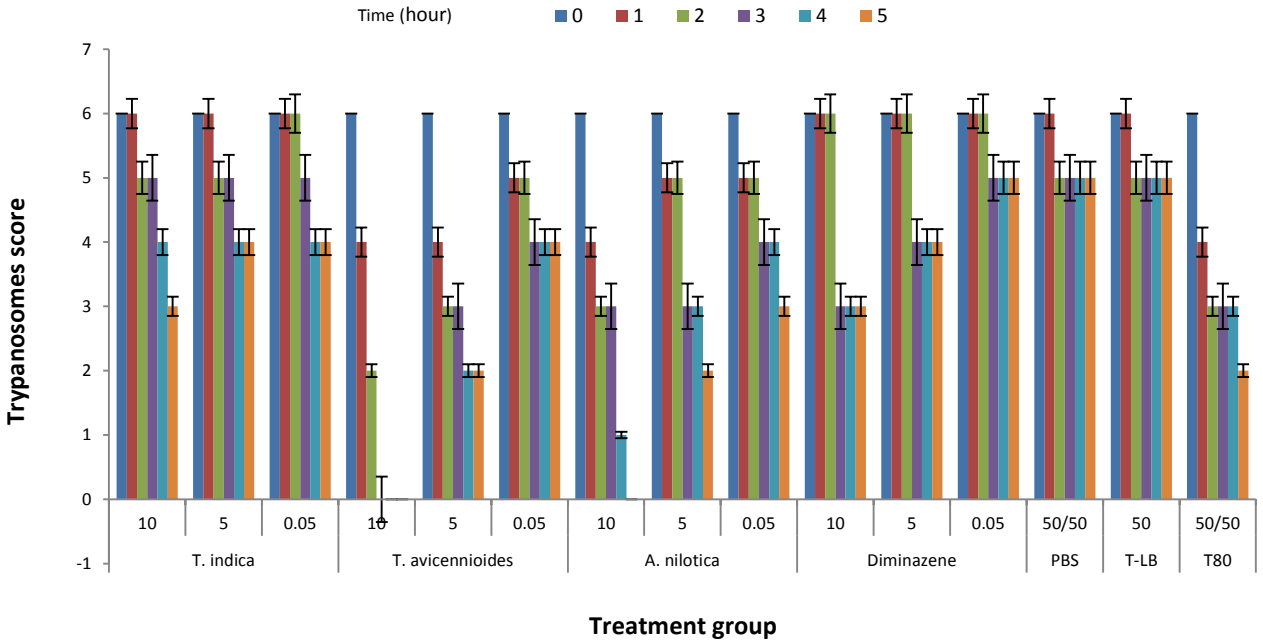

Figure 1: *In vitro* antitrypanosomal studies of the crude methanol extracts of the three plants

The greater antitrypanosomal activity observed in the group treated with the extract of *T. avicennioides* compared to the group treated with the extract of *A. nilotica* could be due to the synergistic effects of alkaloids and anthraquinones. Only the extract of *T. avicennioides* was positive for the presence of anthraquinones while alkaloids are found in the two plants. From the foregoing discussion, the mechanism of action of antitrypanosomal effect of alkaloids and anthraquinones is mediated through the intercalation of DNA and inhibition of protein synthesis[36] and the presence of these phytoconstituents in the extract of *T. avicennioides* may have resulted in synergistic antitrypanosomal effect. Combination of alkaloids affecting different molecular targets (ranging from intercalation of DNA, induction of apoptosis, inhibition of protein biosynthesis and inhibition of microtubule assemblage or combination of any of these targets) of the trypanosomes with digitonin, that affects the integrity of cell membrane resulted in synergistic (enhanced) antitrypanosomal effect.[36] Comparing antittrypanosomal effects of *T. avicennioides* and *T. indica*, the promising antitrypanosomal effects observed in the *T. avicennioides*-treated group may be due to the presence of antraquinones in the extract of *T. avicennioides* which was not detected in the extract of *T. indica*.

Conclusion

Although the three plants demonstrated antitrypanosomal effect, *Terminalia avicennioides* and *Acacia nilotica* exhibited the greatest trypanocidal activity and offer prospect for the discovery of potential lead compound(s) against *T. congolense*. The greatest antitrypanosomal effect observed in the group treated with the extract of *T. avicennioides* could be explained by the presence of anthraquinones in the extract.

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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