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Evaluation of Anti-trypanosomal Properties of Four Extracts of Leaves, Stem and Root Barks of *Prosopis africana* in Laboratory Animals

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Abstract

Qualitative phytochemical and anti-trypanosomal properties of the petroleum ether, chloroform, methanol and aqueous extracts, obtained by cold extraction from the leaves, stem bark and roots of *Prosopis africana* were evaluated. The methanolic and aqueous extracts of the stem bark and leaves of the plant contained alkaloids, saponins, tannins and flavonoids. Anthraquinone was present in the stem bark methanolic extract and in the methanolic and aqueous extracts of the root as well as the aqueous extract of the leaves. Resins was present in the petroleum ether and chloroform extracts of the stem bark and leaves, while tannins was detected in the methanol and aqueous extracts of the stem bark and leaves of the plant in addition to the petroleum ether and chloroform extracts of the root bark. All the solvent extracts showed strong *in vitro* anti-trypanosomal activity and 2 and 4 mg/ml, but *in vivo* only the methanolic extract of the leaves displayed the most promising anti-trypanosomal effect at 200 mg/kg dose. Hence, *Prosopis africana* extracts possess significant anti-trypanosomal activity to warrant bioassay-guided evaluation and identification of the active principle.

Keywords: Antitrypanosomal effect, Trypanosomiasis, *Prosopis africana*, African mesquite

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INTRODUCTION

Trypanosomiasis is a group of diseases caused by flagellated protozoan, trypanosomes, which belong to the genus *Trypanosoma.* The genus consists of species like *T. brucei, T. evansi, T. equiperdum, T. gambiensi* and *T. vivax* that are transmitted exclusively between the vertebrate hosts by tse tse fly (Glosinna spp). Depending on the trypanosomal parasite involved, Human African Trypanosomiasis (HAT), exist in two potentially fatal clinical forms: a chronic form caused by *Trypanosoma brucei gambiense* affecting countries in west and central Africa, and an acute form, caused by *Trypanosoma brucei rhodesiense in* East and Southern Africa[1-4.]

Similarly, cattle and dogs infected with other species, particularly, *T. vivax, T.congolense* and *Trypanosoma brucei gambiense* are usually chronically ill, demonstrating reduction in milk production, weight gain, and reproductive performance. This causes further reduction in the already limited sources of animal protein in sub-saharan Africa[5]. Because of inadequate chemotherapy and other control measures, the HAT has reached epidemic proportion in countries such as Angola, Southern Sudan, Uganda, and the Democratic Republic of Congo[6-8]. But, in general, the disease is of major health and economic concern in rural sub-saharan Africa[2,5] where it is threatening about 60 million people in over 30 countries (WHO, 1998).

Despite the enormity of the health and economic implication of trypanosomiasis, treatment options are very limited: only two (melarsoprol and efflornithine) out of the four drugs available for treatment of HAT can cross the blood-brain barrier and are useful for the treatment of the late stages. Even for these, relapses and multi-drug resistant population have started to emerge[9-13]. Therefore, the need to source for safer, cheaper and readily available sources of medicaments cannot be over-emphasized.

Efforts in the last two decades[8,14-18] suggest that plants may provide the much needed clue for the emergence of the long awaited new generation of trypanocidal drugs. In line with this trend, we investigated different solvent extracts of different parts of *Prosopis africana* for *in vitro* and *in vivo* trypanocidal activity using *Trypanosoma brucei brucei* as model. Preliminary qualitative phytochemical assessment will also be conducted to provide guide for future detail evaluation of the active ingredient of the plant.

Prosopis africana (Guill & Rich) Taub., also known as African mesquite, *Prosopis oblonga*, Benth and *Prosopis lanceolata*, Benth), belongs to the family, *mimosaceae* (Leguminoceae). In different Nigerian languages it called kiriya (Hausa), kohi (Fulani), sanchi lati (Nupe), kpaye (Tiv), ayan (Yoruba), ubwa (Igbo) and ukpehie (Igala). It is very popular for its seeds, which in fermented form, is used as a food condiment[19]. This is the only Prosopis native to inter-tropical Africa, occurring from Senegal to Ethiopia throughout the Sudanian and Guinean ecozones, reaching the border of the Sahelian ecozones to the north. It is a small to large tree (4-20m), characterized with a deep, fast–growing tap root, probable phreatophyte with very dark and scaly bark which is orange to red brown with white streaks when slashed. The branches and twigs are thornless, leaves alternate with bipinnate leaflets in 9 – 16 pairs, oblong lanceolate (12 – 30 mm) and shortly pubescent.

Prosopis africana is one of the many species of *Prosopis*, which have been reported to be of medicinal value. The potential uses of its gum for gels which is used in tablet formulation in pharmaceutical industries have been reported[20-23]. Because of its anti-tyrosine activity, the plant may also be useful in preventing skin whitening or as anti-browning agents[24]. It is listed among the plants used by local farmers in the treatment of trypanosomiasis in northern Nigeria[16]. *In vitro* assessment of the methanolic extract of the stem bark also revealed potent antitrypanosomal activity[17]. Therefore, it was considered necessary to comprehensively evaluate extracts of different parts of the plant for *in vitro* and *in vivo* anti-trypanosomal effect using *T brucei* as a model. *Trypanosoma brucei* was selected for the study because of its role in human animal trypanosomiasis. The qualitative phytochemical components of the extracts obtained from the Prosopis variety

studied from this particular environment were also determined.

MATERIALS AND METHODS

Plants, Test Parasite and Standard Drug

Prosopis africana was collected from Samaru-Zaria in Kaduna State of Northern Nigerian in March 2004. The identity of the plant was confirmed at The Crop Residue Program of the National Animal Production Research Institute (NAPRI), Ahmadu Bello University, Zaria, Nigeria where a specimen was deposited with voucher Number 1712. *Trypanosoma brucei brucei* used for the study was obtained from stabilates maintained at the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Plateau State, Nigeria. *Diminal*^{R} *(445mg diminazene diaceurate*+ 555mg phenazone /g, Eagle Chemical Company LTD, Ikeja, Nigeria) was used as the standard trypanocidal drug.

Sample Preparation and Extraction

The leaf, root and stem barks of *Prosopis africana* plant were collected and dried under the shade. Dried materials were grinded in laboratory mortar and pestle into small particles. The collection of the different parts of the plant was to establish whether the part used in folkoric medicine is indeed more active than other parts of the plant. Fifty grams (50g) of the resulting powder was weighed and extracted three times with150ml methanol each on Wrist Action Shaker by shaking for 3 hours during each extraction. Prior to this, samples were first extracted in similar manner with petroleum ether followed by chloroform. These solvents were selected because they extract different classes of phytochemicals, and hence rendered extract less heterogeneous (relatively purer). The combined extracts were dried under electric fan, and traces of the solvents removed by heating on water bath at 50 ^{o}C for about an hour before storage in a refrigerator at 4^{o}C until required.

Furthermore, the residue obtained after methanol extraction was shaken for 2 hr with water, filtered through glass wool and drying in water bath maintained at 50 ^{o}C to obtain the aqueous extract. In all cases, first extraction with each solvent was conducted following soaking in the appropriate solvent and storing in the refrigerator for 18 – 24 hours.

Animal infection with Trypanosoma brucei brucei

Trypanosoma brucei brucei was maintained in the laboratory by continuous passage in rats aged 60 – 90 days until required. Passage was considered necessary when parasitaemia was in the range of 16 – 32 parasites per field (usually 3 - 5 days post infection in rats and 8 – 12 days in mice; Mice was mainly used to maintain the parasite stock and in vivo studies, while rats was mainly used for the *in vitro* assay). In passaging, 1 x 10^4 parasites were introduced intramuscularly into rats in 0.1 - 0.2ml blood / PBS solution. For several passages, approximately 90% blood solution (v/v) was obtained by cardiac puncture into 1ml syringe containing 0.1ml EDTA (1%w/v). About 0.1 - 0.2ml of the blood collected as described above or blood (diluted with PBS to contain approximately 1 x 10^4 Parasite / ml) was injected into uninfected animals acclimatized under laboratory condition for at least two weeks. For mice used in the *in vivo* experiment, about 0.05 ml of the inoculants was used to avoid sudden upsurge in parasitaemia.

Determination of Parasitaemia

Parasitaemia was monitored in the blood obtained from the tail of rats or mice, pre-sterilized with methylated spirit. The number of parasites was determined microscopically at X 400 magnification using the "Rapid Matching" method described by Herbert and Lumsden[25]. Briefly, the method involved microscopic counting of parasites per field in pure blood or blood appropriately diluted with buffered phosphate saline (PBS, pH 7.2). Logarithm values of these counts obtained was matched with the table of Herbert and Lumsden[25] and converted to antilog to provide absolute number of trypanosomes per ml of blood.

In Vitro Test for Anti-trypanosomal Activity

Exactly 10mg of the different plant extracts were weighed into Eppendorf tubes and first dissolved in 0.1ml of 10% dimethylsulfoxide (DMSO) in PBS, before further dilution with PBS to produce extract solutions of 20.0mg/ml (stock). Extract concentration of 10.0 mg/ml was prepared from the 'stock' extract solution by appropriate dilution with PBS. Extract solutions were freshly prepared before use.

Assessment of *in vitro* anti-trypanosomal activity was performed in triplicates in 96 well micro titer plates (Flow laboratories Inc., Mclean, Virginia 22101, USA). In wells of micro titer plates, 20 µl of blood containing about 20-25 parasites per field obtained as described under "determination of parasitaemia" was mixed with 5 µl of extract solution of 20.0mg/ml and 10.0mg/ml to produce the two effective test concentrations of 4mg/ml and 2mg/ml respectively, so that result can be compared with existing reports[17]. To ensure that the effect monitored was that of the extract alone, a set of control was included which contained the parasite suspended in 2% DMSO only. For reference, tests were also performed with the same concentrations of *Diminal*^{R} - a standard commercial trypanocidal drug. All analyses were performed in triplicates.

After 5 minutes incubation in closed Eppendorf tubes maintained at 37 ^{o}C, about 2µl of test mixtures were placed on separate microscope slides and covered with cover slips and the parasites observed every 5 minutes for a total duration of sixty minutes. Cessation or reduction in motility of the parasites in extract-treated blood compared to that of parasite-loaded control blood without extract was taken as a measure of anti-trypanosomal activity[17]. This is a relatively simple, cheap but reliable first step in the screening of natural products for anti-trypanosomal activity before the more expensive *in vivo* animal evaluation is adopted as done in this study.

Animal Grouping and In Vivo Assay

Following *in vitro* studies, the chloroform extract of the leaves and the stem bark, and petroleum ether, methanol and aqueous extracts of the roots which displayed anti-trypanosomal activity *in vitro* were selected for *in vivo* evaluation. Mice inoculated with *Trypanosoma brucei brucei* were intramuscularly treated with 100, 200 or 300 mg/kg of the extracts when average parasitaemia was approximately one parasite per field. The treatment continued daily with continuous monitoring of parasitaemia for 10 days. After withdrawal of treatment, parasitaemia was also monitored daily for the next four days (14th day), and thereafter monitoring was reduced to thrice a week, for surviving animals. Six animals were used per treatment group. An infected but untreated group was included as a negative control, while another control group in which the animals were treated with a standard drug (*Diminal*^{R}) was also included

Phytochemical Evaluation

Basically, the methods outlined by Farnsworth and coworkers[26,] as well as Trease and Evans[27] were utilized. Alkaloids were tested with Dragendorff's Mayers and Wagner's reagents, while carbohydrate and steroidal rings / triterpenes were evaluated with Molisch test and Salkowski/ Lieberman Burchard's tests respectively. Antraquinones and saponin derivatives were confirmed by Borntranger's and Frothing tests, respectively. The presence of flavonoids were established by a battery of tests, including , sodium hydroxide test, Ferric chloride test, lead acetate test and Shinod's test, while tannins were determined by a combination of gelatinous salt block test on 5% infusion, ferric ammonium citrate test, lead sub-acetate test and bromine water test. Presence of resins was evaluated with acetic anhydride-sulphuric acid reaction. All analyses were performed in duplicates.

RESULTS

The methanolic extracts of the leaf, stem bark and root bark of *Prosopis africana* all contained alkaloids, tannins and flavonoids, while those of the stem bark and leaf also contained saponins. Anthraquinone was detected in the methanol and aqueous extracts of the root, stem bark and leaf, respectively. Resin was detected in the petroleum ether and chloroform extracts of the stem bark and leaf (Table 1).

All extracts of different parts of the plant showed significant *in vitro* anti-trypanosomal activity, except the lower dose (2mg/kg) of methanol and petroleum ether extracts of the leaf and stem bark, respectively. The aqueous and chloroformic extracts of all parts of *P. africana* greatly reduced parasitamia under *in vitro* condition (Table 2).

Table 1: Phytochemical constituents of *Prosopis africana*

Compounds	Stembark				Root				Leaf			
	Petroluem Spirit Extract	Chloroform Extract	Methanol Extract	Aqueous Extract	Petroluem Spirit Extract	Chloroform Extract	Methanol Extract	Aqueous Extract	Petroluem Spirit Extract	Chloroform Extract	Methanol Extract	Aqueous Extract
Alkaloids	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve
Saponins	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve
Flavonoids	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve
Anthraquinones	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve
Resins	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve
Tannins	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve

Table 2: *In vitro* antitrypanosomal effect of *Prosopis africana* extracts

Plant Part	Time (minutes) after which motility ceased with different concentrations of extract							
	Petroluem spirit		Chloroform		Methanol		Aqueous	
	(4mg/ml)	*(2mg/ml)*	*(4mg/ml)*	*(2mg/ml)*	*(4mg/ml)*	*(2mg/ml)*	*(4mg/ml)*	*(2mg/ml)*
Leaf	45	30**	25	35	30**	NA	30	35
Stem bark	35	NA	25	30	30	45	35	40
Root	30	45	50	20**	30	50*	30	40

*N.B: * = very slightly reduced motility, ** = slightly reduced motility, NA = no noticeable effect on motility after 60minutes*

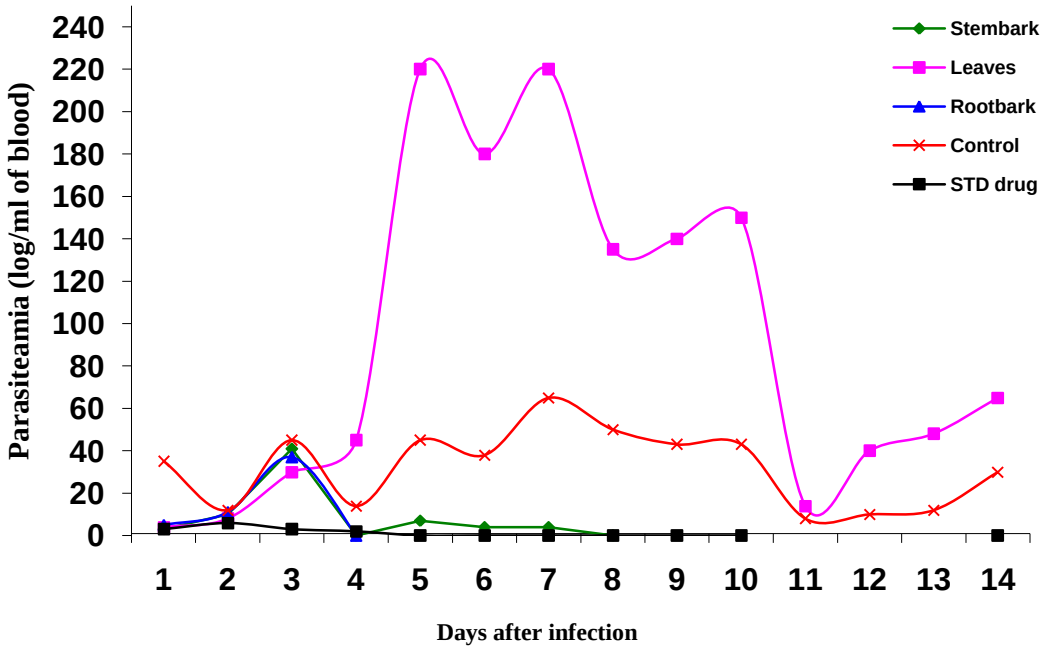

Fig. 1: Level of parasitamia in *T. brucei* – infected mice administered aqeous extract of different parts of *Propis africana* mice at 200mg/kg

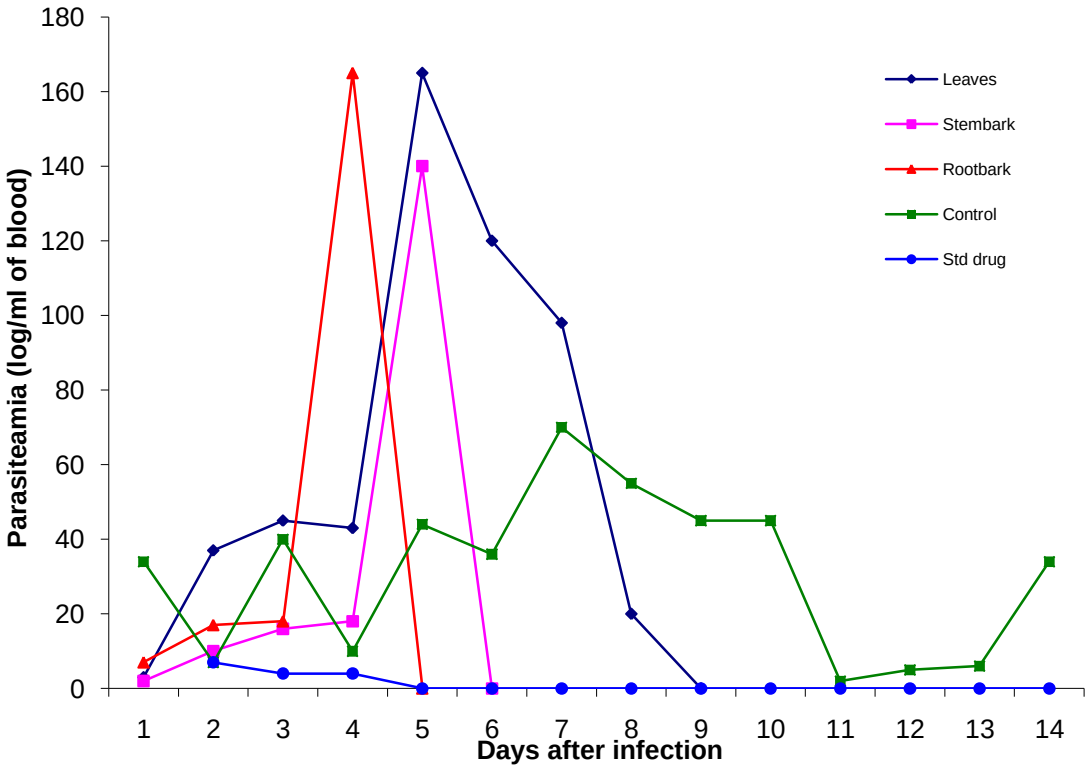

Fig. 2: Effects of methanolic extracts of different parts of *Propis africana* on parasiteamia in mice infected with *T. brucei* following daily intramuscular dose of 200mg/kg

For dose-dependent *in vivo* experiments, it was observed that at the preliminary stages those animals that received dose of 300mg/kg of all extracts died in less than one week, while little or no anti-trypanosomal effect was observed when dose of 100mg/kg was administered for all extracts. Therefore, attention was focused on the 200mg/kg dose for the aqueous and methanol extracts of the leaves, stem bark and root bark.

Figure 1 compares the anti-trypanosomal effect of aqueous extracts of different parts of the plant at 200mg/kg. The aqueous extracts of the stem bark suppressed parasitaemia much more than the aqueous extracts obtained from the leaves, but the root aqueous extract killed all the animals within four days of administration. In the case of methanol extracts, no noticeable effect of stem and root bark on parasitaemia was observed when all the animals in the groups died within 3 – 5 days of administration (Figure 2). However for the group on methanol extract of the leaf, parasitaemia was eliminated within 9 days of extract administration, and remained so for another 2 days when all the animals died. In general, methanol extract of the leaves was more effective than the aqueous extract, while the toxicity of the stem and root bark extracts were comparable (Figs. 1&2).

DISCUSSION

The reported *in vitro* trypanocidal activity of the stem bark methanol extract of the *P. africana*[17] is confirmed by this study. However, the death of animals administered 300mg/kg dose during the preliminary experiment suggest that the high *in vitro* anti-trypanosomal activity observed (Table 2), might be due to toxic agents that are not specifically selective for the parasites, a factor that is of paramount consideration in drug development.

As expected, the results of phytochemical screening (Table 1) did not give any clear picture of the exact bioactive component responsible for the observed anti-trypanosomal activity. However, the fact that the extracts that displayed some activity contain alkaloids, saponins, flavonoids, anthraquinones and tannins, suggest that these bioactive

compounds may be acting individually or synergistically to bring about the observed trypano-suppressive activity.

Information on the general phytochemical constituents of *Prosopis africana* in the internationally accessible literature are rare, but scattered information on the presence of the two alkaloids, prosopine and prosopinine [28,29] and tannins which constitutes about 18% of the stem bark extract Earlier workers have demonstrated that, alkaloids, tannins and anthraquinones, which were also detected in most of the extracts (Table1) may possess antitrypanosomal and antitryprotozoal effects[4,30,31].

The results of this investigation suggest that extracts of different parts of *Prosopis africana* possess enough anti-trypanosomal activity to warrant further pharmacological, toxicological and therapeutic studies utilizing bioassay-guided techniques and spectroscopic methods to evaluate if the active ingredient can provide chemical lead for development of a new generation of anti-trypanosomal drugs.

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