

Anti-Trypanosomal Activity of Aqueous Leaf Extract of *Annona senegalensis* against *Trypanosoma congolense*

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Abstract

This study investigated *in vitro* and *in vivo* anti-trypanosomal activity of cold (25°C) and hot (80°C) aqueous extracts of *Annona senegalensis* leaf against *Trypanosoma congolense*. The phytochemical screening showed presence of flavonoids, phlebotannins, resins, and saponins. Hot aqueous extract revealed presence of tannins and glycosides which were absent in cold aqueous extract. But alkaloids were absent in the two extracts. The median lethal doses (LD₅₀) for both cold and hot aqueous leaf extracts were estimated at dose level of > 16,095 mg/kg. The *in vitro* study revealed inhibition of parasites' motility at 100 mg/ml concentration of hot aqueous extract 30 minutes post application of the extract while 200 mg/ml concentration of the same extract inhibited parasites' motility at 50 minutes post application of the extract. Cold aqueous extract inhibited parasites' motility 50 and 20 minutes post application of 100 mg/ml and 200 mg/ml concentration respectively. *In vivo* screening of anti-trypanosomal activity showed that both cold and hot aqueous extracts were not effective at the dose level of 161 mg/kg but showed anti-trypanosomal activity at the dose levels of 322 mg/kg and 483 mg/kg body weight respectively. Hence *Annona senegalensis* may serve as a resource plant for discovery of drug(s) that can be used for the treatment of trypanosomosis caused by *Trypanosoma congolense*.

Keywords: Anti-trypanosomal Activity; *Annona senegalensis*; Cold Extract; Hot Extract; Trypanosomosis; *Trypanosoma congolense*

Introduction

Trypanosomosis is a potentially fatal disease of humans and domestic animals in tropical Africa and South America [1]. The disease is transmitted by tse-tse flies and covers approximately ten million square kilometres in thirty-eight African countries [2]. It is probably the only disease which has profoundly affected the settlement and economic development of a major part of the African continent [3]. Unfortunately, the existing drugs for trypanosomosis are either toxic and/or expensive [4]. Therefore, the need to search for cheaper, more effective, easily available and less toxic trypanocidal drugs cannot be over-emphasized.

In the recent past, the possibility of sourcing for new generation of trypanocidal agents has received adequate attention [5]. Freiburghaus, *et al.* [6] evaluated several medicinal plants of Tanzania and Uganda origin for their *in vitro* trypanocidal activity. The results revealed that plants could indeed be a good source of trypanocidal drugs. *Annona senegalensis* is used as anthelmintic [7] antidiarrhoeic, anticonvulsant, antibacterial, antifungal, antitussive, anti-inflammatory, anti-pyretic, anticancer including leukemia [8], pediculosis, pasteurellosis, cough and headache. It contains anonaine, mucilage, anorcine [9], α -phellandrene, limonene, α -pinene, Z-sabinol and P-cymene, elemol, β and γ -eudesmols, bornyl acetate, 1,8-cineole, terpinen-4-ol, lauric acid, hexadecanoic acid, myristic acid and oleic acid [10]. Ogbadoy, *et al.* [11] investigated *in vivo* trypanocidal activity of aqueous leaf extract of some plants including *Annona senegalensis*

against *Trypanosoma brucei brucei* in an attempt to validate the folkloric claim made by traditional medicine practitioners on the use of the plants for the treatment of sleeping sickness. In this report, anti-trypanosomal activity of aqueous leaf extracts of *Annona senegalensis* against *Trypanosoma congolense* is presented.

Materials and Methods

Collection and extraction of *Annona senegalensis* leaves

The leaves of *Annona senegalensis* were collected in Makurdi, the head quarter of Makurdi Local Government Area of Benue State, Nigeria in the month of January 2010 and identified by a botanist in the Forestry Department, University of Agriculture, Makurdi, Nigeria. The plant has been given voucher No. 2010001. The leaves were air dried at room temperature to constant weight and ground into powder using pestle and mortar. Hundred (100) grammes each of *Annona senegalensis* leaf powder was placed in two separate containers, each containing 3 litres of hot (80°C) and cold (25°C) water solvent respectively. The two mixtures were thoroughly agitated intermittently throughout the period of extraction using glass rod stirrer, and then allowed to stand overnight. Thereafter the mixtures were filtered with Whatman filter paper No 1 into measuring cylinder and concentrated at 45°C in an incubator and stored in a refrigerator at 4°C until required.

Phytochemical analysis

The phytochemical analyses of the cold and hot aqueous extracts were carried out as described by Odebiyi and Sofowora [12] to test for presence of tannins, resins, glycosides, flavonoids, saponins, alkaloids, and phlebotannins.

Acute toxicity study

Male rats of about ten weeks' old that weighed 135 ± 2.5 g were obtained from the Experimental Unit of National Veterinary Research Institute, Vom, Plateau State, Nigeria. The animals kept in plastic cages were fed Commercial feed (Vital feed[®]) and clean water was provided ad libitum. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in CIOMS [13] guidelines. The "Up and Down" method of median lethal dose estimation was adopted. The initial dose of 5,000 mg/kg body mass of *Annona senegalensis* extracts were administered orally to 5 mice each for cold and hot aqueous extracts. The mice were observed for signs of toxicity which include behavioural changes, increased respiratory rates and death within 48 hours. The main test was carried out dosing 1 animal at 48 hours interval. The initial dose of 5,000 mg/kg was administered and dose progression or regression using Logarithm of 3.2 was used.

In vitro anti-trypanosomal screening

Trypanosoma congolense was obtained from stabulates maintained at University of Nigeria, Nsukka, Nigeria. The parasite was maintained in our laboratory by serial passaging of mice. *In vitro* anti-trypanosomal activity of aqueous extract of *A. senegalensis* was carried out as follow. Prepared respectively, were 100 mg/ml and 200 mg/ml concentrations of cold and hot aqueous extracts of *Annona senegalensis* leaf powder, in buffered phosphate saline, and 10 µL of each of the two extracts was mixed with 60 µL of infected blood and the mixtures were incubated at 37°C for 10 minutes in Wells of Microtitre plates using water bath. Phosphate buffered saline was used as the control. After incubation, the parasites were observed under x40 objective lens for cessation of motility at 0, 10, 20, 30, 40, 50 and 60 minutes intervals.

In vivo anti-trypanosomal screening

In vivo screening for anti-trypanosomal activity of both hot and cold aqueous extracts of *A. senegalensis* was carried out thus. A total of 24 mice were used for this experiment. Mice were randomly divided into eight groups of three mice each. Method of Yamba, *et al.* [14] was adopted for therapeutic dose selections. The administered parasite inoculums were determined according to the body weight of each

mouse. Every 1g body weight of mouse was intraperitoneally inoculated 0.01 ml of inoculum containing 10¹ *Trypanosoma congolense*. The method of Herbert and Lumsden [15] was used to monitor daily level of parasitaemia using blood collected from the tail vein. Groups I, II and III were treated with cold aqueous extract of *A. senegalensis* at varying doses of 161, 322 and 483 mg/kg body weight respectively. Whereas, hot aqueous extract was administered to mice in groups IV, V and VI at varying doses of 161, 322 and 483 mg/kg respectively. The extracts were administered intramuscularly while group VII served as the positive control treated with diminazene aceturate at 3.5 mg/kg body weight intramuscularly. Group VIII served as the negative control. The level of parasitaemia was checked at intervals of 0 hour, 12th hour, 1st, 2nd, 3rd, 4th, 5th, 6th and 7th day of experimentation by obtaining blood from the tail vein of each mouse and at least 30 fields were observed under x40 objective lens of light microscope to determine the presence of *Trypanosoma congolense* in blood film.

Statistical analysis

Willcoxon’s matched-pairs signal-rank test was used to analyse the results of *in vitro* and *in vivo* anti-trypanosomal assay [16].

Results

Extraction yield

Cold aqueous extraction yielded 33.5g extract of *A. senegalensis* leaf after oven dried at 45°C.

$$\begin{aligned} \text{Percentage yielded extract} &= \frac{\text{weight of extract after drying}}{\text{weight of leaf powder}} \times 100\% \\ &= 33.5\text{g}/150\text{g} \times 100\% \\ &= 22.3\% \text{ w/w} \end{aligned}$$

Hot aqueous extraction yielded 28g extract of *A. senegalensis* after oven dried at 45°C.

$$\begin{aligned} \text{Percentage yielded extract} &= \frac{\text{weight of extract after drying}}{\text{weight of leaf powder}} \times 100\% \\ &= 28\text{g}/100\text{g} \times 100\% \\ &= 28\% \text{ w/w} \end{aligned}$$

Phytochemical components of *Annona senegalensis*

Phytochemical analysis of the crude aqueous extracts revealed the presence of flavonoids, phlebotannins, resins, and saponins. Alkaloids were absent in all the extracts. But hot aqueous extract revealed presence of glycosides and tannins in addition. Unextracted leaf powder revealed presence of flavonoids and saponins but other test phytochemicals were absent (Table 1).

Phytochemicals	Inferences		
	Cold Aqueous	Hot Aqueous	Unextracted Leaves
Alkaloids	-	-	-
Flavonoids	+	++	++
Glycosides	-	++	-
Phlebotannins	++	+++	Not tested
Resins	++	+	-
Saponins	+	++	+
Tannins	-	+	-

Table 1: Phytochemical components of *Annona senegalensis* leaf extract.

-: Absent; +: Low, ++: Moderate; +++: High.

Median lethal dose of *Annona senegalensis*

Median lethal dose estimation adopting the “Up and Down” method with initial dose of 5,000 mg/kg produced no observable clinical signs after the expiration of 48 hours. Also, there were no observable clinical signs from dose progression using Logarithm of 3.2 until a dose level of 16,095 mg/kg was reached. The median lethal dose (LD₅₀) was therefore estimated at dose level of > 16,095 mg/kg for both hot and aqueous extracts.

***In vitro* anti-trypanosomal findings**

The *in vitro* study revealed inhibition of parasites’ motility at 100 mg/mL concentration of hot aqueous extract 30 minutes post application of the extract while 200 mg/mL concentration of the same extract inhibited parasites’ motility at 50 minutes post application of the extract. Cold aqueous extract inhibited parasites motility 50 and 20 minutes post application of 100 mg/mL and 200 mg/mL concentration respectively (Table 2).

Time	Extracts				PBS
	Hot Aqueous		Cold Aqueous		
	10%	20%	10%	20%	
0 minute	+++	+++	+++	+++	+++
10 th minute	+	++	++	++	+++
20 th minute	+	++	++	X	+++
30 th minute	X	+	+	X	+++
40 th minute	X	+	+	X	+++
50 th minute	X	X	X	X	+++
60 th minute	X	X	X	X	+++

Table 2: *In vitro* anti-trypanosomal activity of *Annona senegalensis* leaf extracts.

+: Highly Sluggish; ++: Moderately Sluggish; +++: Active; X: No Motility; PBS: Phosphate Buffered Saline.

***In vivo* anti-trypanosomal findings**

The result of *in vivo* screening for anti-trypanosomal activity showed that both cold and hot aqueous extracts were not effective at the dose level of 161 mg/kg. However, at the dose levels of 322 and 483 mg/kg body weight, anti-trypanosomal activity was observed for both extracts. The results further showed that, there was resurgence on the 6th and 7th days post treatment with hot aqueous extract at dose levels of 322 and 483 mg/kg respectively (Table 3).

Treatment Regimens	Groups	Level of Parasitaemia in relation to time of examination									
		0 hour	12 th hour	1 st day	2 nd day	3 rd day	4 th day	5 th day	6 th day	7 th day	
Cold Aqueous Extract	1	a	10/10	10/10	10/10	10/10	10/10	X	X	X	X
		b	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
		c	10/10	10/10	10/10	10/10	10/10	10/10	X	X	X
	2	a	10/10	10/10	10/10	10/10	10/10	10/10	10/10	X	X
		b	10/10	10/10	10/10	10/10	10/10	10/10	0/10	0/10	0/10
		c	10/10	10/10	10/10	10/10	10/10	10/10	10/10	X	X
	3	a	10/10	10/10	10/10	10/10	X	X	X	X	X
		b	10/10	10/10	X	X	X	X	X	X	X
		c	10/10	10/10	10/10	3/10	0/10	0/10	0/10	0/10	0/10

Hot Aqueous Extract	4	a	10/10	10/10	10/10	X	X	X	X	X	X	
		b	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	
		c	10/10	10/10	10/10	10/10	10/10	X	X	1	X	X
	5	a	10/10	10/10	10/10	10/10	X	X	X	X	X	X
		b	10/10	10/10	10/10	10/10	10/10	0/10	0/10	0/10	0/10	2/10
		c	10/10	10/10	10/10	10/10	X	X	X	X	X	X
	6	a	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
		b	10/10	10/10	10/10	10/10	10/10	10/10	X	X	X	X
		c	2/10	2/10	2/10	2/10	2/10	2/10	0/10	0/10	0/10	0/10
Diminazene aceturate	7	a	10/10	2/10	1/10	1/10	0/10	0/10	0/10	0/10	0/10	
		b	10/10	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	
		c	10/10	2/10	1/10	0/10	0/10	0/10	0/10	0/10	0/10	
Normal Saline	8	a	10/10	X	X	X	X	X	X	X	X	
		b	10/10	X	X	X	X	X	X	X	X	
		c	10/10	X	X	X	X	X	X	X	X	

Table 3: *In vivo* anti-trypanosomal activity of *Annona senegalensis* leaf extracts.

a: 1st Animal; b: 2nd Animal; c: 3rd Animal; X: Animal Died.

Discussion

The phytochemicals present in the aqueous leaf extracts of *Annona senegalensis* as revealed by our study are flavonoids, phlebotannins, resins and saponins in addition to tannins and glycosides identified in hot aqueous extract. Our findings are in agreement with the report of Celestine [10] indicating that *Annona senegalensis* contained a wide range of phytochemicals including essential oils which contain sesquiterpenes (elenol, β and γ eudesmols), diterpenes and monoterpenes (α -pinene, β -phellandrene, terpinen-4-ol), aliphatic acid (lauric acid, hexadecanoic acid, myristic acid and oleic acid) [10].

The median lethal dose (LD₅₀) for both cold and hot aqueous leaf extracts of *Annona senegalensis* estimated at dose level of > 16,095 mg/kg body weight in mice shows that *Annona senegalensis* is relatively harmless or practically safe. Clarke and Clarke [17] were of the opinion that any substance whose LD₅₀ in mice is above 15,000 mg/kg is relatively harmless or practically safe. The wider use of the plant to treat a myriad of diseases may be attributable to safeness of the plant.

The *in vitro* anti-trypanosomal study of the extracts of this plant reveals that the motility of *Trypanosoma congolense* parasites was effectively inhibited. Hot aqueous extract of 100 mg/mL concentration inhibited the motility of the parasites 30 minutes post application of the extract as opposed to the inhibitory effect of 200 mg/mL concentration 50 minutes post application of the extract. For cold aqueous extract, 100 mg/mL concentration caused inhibitory effect 50 minutes post application of the extract while 200 mg/mL concentration inhibited parasites' motility 20 minutes post application. Trypanosomes that were not treated with the extracts (control) were actively motile throughout the period of the experiment. The result of this *in vitro* study is in concordance with the report of Wosu and Ibe [18] indicating that plant extracts could be used to treat trypanosomiasis in animals. Freiburghaus, *et al.* [19] had earlier reported that African medicinal plants have potential *in vitro* trypanocidal activity against different species of trypanosomes including *Trypanosoma congolense*. Sawadago, *et al.* [20] has earlier reported *in vitro* anti-trypanosomal activity of the methanolic extracts of *Lantana ukambensis*,

Xeoderris sthulmanii, *Pavinaria curatelifolia*, *Ozoma insignis* and *Ficus platyphylla* between 1.5 and 25. µg/ml. Other plants that exhibited *in vitro* anti-trypanosomal activity are *Cedrela odorata*, *Aristolochia pilosa* [21], *Cassia sieberiana*, *Hymenacardia acta*, *Pericopsis laxiflora*, *Strychnos spinosa* and *Trichilia emetica* at concentration ranging from 1.5 to 39 µg/ml [22].

In vivo screening of anti-trypanosomal activity of the cold and hot aqueous extracts of *A. senegalensis* at dose levels of 322 mg/kg and 483 mg/kg respectively agrees with the report of Igweh and Onabanjo [23] indicating that *A. senegalensis* has anti-trypanosomal activity but on *Trypanosoma brucei brucei*. Freiburghaus, *et al.* [6] reported that flavanol could be responsible for anti-trypanosomal activity of *Annona senegalensis* at 200 mg/kg body weight which perhaps completely cleared experimental *Trypanosoma brucei brucei* from blood without producing another infection of trypanosome within 60 days when blood and cerebrospinal fluid of treated mice were sub-inoculated into mice. This finding is in contrast with our findings that showed resurgence of the infection on the 6th and 7th day after having treated the infected mice with 322 and 483 mg/kg of *A. senegalensis* leaf extract. Both *in vitro* and *in vivo* anti-trypanosomal activity of both hot and cold aqueous extracts of *Annona senegalensis* agree with the report of Saganuwan [20] indicating that traditional people use the plant to treat trypanosomiasis in animals, the active principles of the plants are steroids, lignans, polyketides, glycoalkaloids and flavonoids [24]. Medicinal plants have trypanolytic activity against the epimastigote form and trypanocidal activity against the blood stream and metacyclic trypomastigote form of *Trypanosoma cruzi* in culture medium prepared in micromolar concentration [25]. Other anti-trypanosomal phytochemicals are bromopyrrole alkaloids, loganide B and dibromopalaupamine, terpenoids, furanoterpenes and sesquiterpene lactones [26,27].

Conclusion

The *in vitro* and *in vivo* anti-trypanosomal activities of aqueous leaf extracts of *A. senegalensis* justify the folkloric use of the plant for the treatment of sleeping sickness in animals. The findings suggest that there is potential discovery of novel anti-trypanosomal agent(s) from *Annona senegalensis*. The plant is safe having LD₅₀ of > 16,095 mg/kg. The phytochemical principles which include flavonoids, glycosides, phlebotannins, tannins, saponins and resins might be responsible for the anti-trypanosomal activity of the aqueous extracts of *Annona senegalensis* against *Trypanosoma congolense*.

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