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In vitro and *in vivo* Antitrypanosomal Effects of Methanol and Aqueous Extracts of *Picralima nitida*

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Authors' contributions

This work was carried out in collaboration between all authors. Author ACE designed the study, wrote the protocol, performed the statistical analysis and managed the analyses of the study. Authors NGE and CBO did all the laboratory work, managed the literature searches and wrote the first draft of the manuscript. Authors OAO, COU and CUI reviewed the first and final draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

The methanol and aqueous extracts of the leaves, fruits, seeds, stem bark and roots of *Picralima nitida* were studied *in vitro* and *in vivo* for activity against *Trypanosoma brucei brucei* in Swiss albino mice. Phytochemicals studies were also conducted for all the plant extracts. The methanol extracts showed appreciably high *in vitro* and *in vivo* antitrypanosomal activities compared to the aqueous extracts of the plant. The methanol extract of the root exhibited the highest *in vitro* antitrypanosomal activity followed by the methanol extract of seed of *Picralima nitida*. Motility of *Trypanosoma brucei brucei* was stopped by the methanol extract of the root after 10 min, while the methanol extract of the seed of *Picralima nitida* stopped the motility of *Trypanosoma brucei brucei* at 15 min. The methanol extract of the root of *Picralima nitida* showed the highest *in vivo* antitrypanosomal activity at 100 mg/kg body weight. The extract cleared the parasite completely from the *T. brucei brucei* infected Swiss albino mice after day 3 of treatment. There was a statistically significant difference ($p<0.05$) when the level of parasitemia of the animals treated with the methanol extract of the root of *Picralima nitida* were compared with the other treatment groups and the untreated control. The phytochemicals

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detected in these extracts are tannins, flavonoids, alkaloids, steroids, terpenoids, saponins and cyanide glycosides. The *in vitro* and *in vivo* antitrypanosomal activity exhibited by these extracts might be attributed to these phytochemicals.

Keywords: Antitrypanosomal activity; in vitro and in vivo; Picralima nitida; Trypanosoma brucei brucei.

1. INTRODUCTION

The infection of trypanosome which is known as trypanosomiasis is commonly known as sleeping sickness. Transmission of human-infective trypanosomes occurs primarily through insects that feed upon us. The insect vectors however are very specific. The reason is that transmission usually requires the parasite to multiply and undergo specific developmental transitions in the insect. It is transmitted from vector to host. The insect vector for *Trypanosoma brucei* is the tsetse fly [1]. The parasite lives in the mid-gut of the fly (procyclic form), whereupon it migrates to the salivary glands for injection to the mammalian host on biting. The parasite lives within the bloodstream (bloodstream form) where it can re-infect the fly vector after biting [2]. Later, during a *T. brucei* infection, the parasite may migrate to other areas of the host. A *T. brucei* infection may be transferred from human to human via bodily fluid exchange, primarily blood transfer[2].

There are three different sub-species of *T. brucei* which cause different variants of trypanosomiasis. They are as follows: *T. brucei gambiense* which causes slow onset chronic trypanosomiasis in humans. They are most common in Central and Western Africa where humans are thought to be the primary reservoir. *T. brucei rhodesiense* which causes fast onset acute trypanosomiasis in humans. This is most common in Southern and Eastern Africa, where game animals and livestock are thought to be the primary reservoir. In upland Savannas of the East Africa, it is the agent of East African sleeping sickness [3]. Reservoirs for this trypanosome are domestic cattle and wild animals within which the parasites cause severe malnutrition. The trypanomastigote parasites are transmitted through the bite of the fly to humans. The parasite passes through the lymphatic system and enters the blood stream and replicates by binary fission as they pass to other body fluids [3]. *T. brucei brucei* which causes animal African trypanosomiasis along with several other species of trypanosome. *T. brucei brucei* is not human infective due to its susceptibility to lysis by human apolipoprotein L1 [1]. However, as it shares many features of *T. brucei gambiense* ant *T. brucei rhodesiense* (such as antigenic variation), it is used as a model for human infections in laboratory and animal studies.

Sleeping sickness itself is identified as a neurological disorder proceeded by an acute lymphatic infection. The disease garners its name from the effect it has in creating severe fatigue in a patient, followed by bouts of mania and eventually causing coma and death. However, it is not readily transmittable between mammalian hosts and facilitates the use of an insect vector: the flies of the genus Glossina, otherwise known as the tsetse flies[1]. These flies are endemic to 36 Sub-Saharan countries and are found, unsurprisingly within the range of trypanosomiasis infections.

The severity of the infection vary depending on which sub-species of *T. brucei* are causing the infection [4]. More than 60 million people living in 36 Sub-Saharan African countries are at risk of contracting sleeping sickness caused by *Trypanosoma brucei gambiense* and *T.*

*brucei rhodesiense*5. It is estimated that between 300,000 and 500,000 people were infected in 2001, with 50,000 deaths annually [5,6].

Current chemotherapeutic options in the treatment of trypanosomiasis are very limited and far from ideal. Some of the trypanocidal drugs in the market are very expensive and most trypanosomes have developed resistance to some of the trypanocidal drugs. This has led the diversification of trypanosomiasis research into medicinal plants [7] and thus this study. Therefore, this study is aimed at assessing the *in vitro* and *in vivo* antitrypanosomal effects of methanol and aqueous extracts of *Picralima nitida* plant.

Picralima nitida staphf is a member of Apocynaceae family. The plant is popularly used in West Africa as an antipyretic and ant malarial [8,9].

2. MATERIALS AND METHODS

2.1 Plant

The plant *Picralima nitida* was identified by Mr. Ibendukwe of the Department of Forestry and Wild Life Technology (FMT), Michael Okpara University of Agriculture, Umudike, Nigeria. The plant voucher number is (FHI) 29271.

2.2 Plant Extract Preparation

The leaves, stem bark, fruits, roots and seeds of the plant *Picralima nitida* were harvested from Umudara-Nwaneri Umuezeala, Awo-Omamma in ORU East loca government area of Imo State, Nigeria.

All the plant parts were dried in the laboratory at room temperature for about seven weeks. The dried plant parts were ground into powdered form using a mortar and pestle. Thirty gram (30g) of the ground plant samples were weighed separately into Soxhlet extractor and extracted respectively by reflux with 250ml of methanol and 250ml of deionized water. The extracts were dried by heating in a water bath at 45ºC for about 24 h. The extracts were then stored in the refrigerator at -4ºC until required.

2.3 Animals and Animal Husbandary

Forty (40) Swiss albino mice which were used for this study were purchased from the University of Nigeria, Nsukka. They were transported to the Department of Biochemistry, Federal University of Technology, Owerri, Nigeria where the research was carried out between August and November, 2012. The animals weighed between 20 and 30g. The animals were fed adlibitum with vital feed starter (feed stock) and water. The protocol for the use of animals was approved by the ethics committee of the Federal University of Technology, Owerri, Nigeria.

2.4 Test Organism

Trypanosoma brucei brucei was obtained from the Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The parasite was maintained in the laboratory by continuous passage in mice and rats until required. Passage was considered necessary when parasitemia was in the range of 16-32 parasites

per field. About 0.1 to 0.2ml of the parasitized blood diluted with phosphate buffered saline (PBS) to contain approximately 1 x10^3 parasites/ml was injected into clean mice or rats which were acclimatized under laboratory conditions for about two weeks [7].

2.5 Determination of Parasitemia

Parasitemia was monitored in the blood obtained from the tail vein of the infected mice. A drop of blood was placed on a glass slide and covered with a cover slip. This was then viewed under the microscope at x400 magnification using the "Rapid Matching" method of Herbert and Lumsden [8]. This entails microscopic counting of parasites per field in pure blood or blood approximately diluted with phosphate buffered saline (PBS, pH 7.2). Logarithm values of these counts obtained by matching the table of Herbert and Lumsden[8] was converted to antilog to provide absolute number of trypanosomes per ml of blood.

2.6 *In vitro* Test of Extracts for Trypanocidal Activity

Assessment of methanol and aqueous extracts of *Picralima nitida* for *in vitro* trypanocidal activity was performed in triplicates in 96 well micro titre plates as described by Ene et al. [7] The extracts doses of 20.0 10.0 and 2.0 mg/ml were prepared and neutralized by dissolving 20.0, 10.0 and 2.0 mg of the extracts respectively in 1 ml of PBS. Blood (20µl) of *T. brucei brucei* infected blood of mice was mixed with 5µl of extract solution of 20.0, 10.0 and 2.0mg/ml to produce effective test concentrations of 4, 2 and 0.4 mg/ml respectively. To ensure that the effect monitored was that of the extract alone, a set of control was included which contained the parasites suspended in normal saline which was used to reconstitute the extracts. For reference, tests were also performed with the same concentrations of Diaminal (445mg diminazine diaceturate + 555mg phenazone/g, Eagle Chemical Company LTD, Ikeja, Lagos, Nigeria. This diaminal is a commercial trypanocidal drug.

The test mixtures were incubated for 5min in the covered micro titre at 37ºC. Then, 2µl of the incubated test mixtures were placed on separate microscope slides and covered with cover slips and the parasites observed every 5min for a total duration of 60min [7]. Cessation or drop 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 trypanocidal activity.

2.7 *In vivo* Test of Extracts for Antitrypanosomal Activity

Swiss albino mice were used for this study. The mice were divided into groups according to the number of extracts and the control groups. Three mice were used for each of the test/treatment groups, diaminal standard and untreated negative control groups. All the mice were infected with *T. brucei brucei* as earlier described.

Parasitemia was confirmed in the mice after 48h of infecting them with *T.brucei brucei*. The various plant extracts of methanol and aqueous base were then administered to the experimental group animals (groups 1-10) at a dose level of 100 mg/kg body weight daily for 7 days [7] (i.e. treatment commenced after 48 h of infection). This is because at this time, the parasites were not numerous and can still be counted. Diaminal standard drug was also administered to the standard control group at a dose of 3.5 mg/kg for one week. The negative control mice were not treated. The administration of the extracts and the standard drug was done through the intraperitoneal route. Parasitemia was monitored in all the treatment and control groups as earlier described.

2.8 Phytochemical Analysis of the Various Plant Extracts

The phytochemical screening of the various plant extracts of *Picralima nitida* was carried out to determine the secondary metabolites present in them using standard procedures [9,10,11].

2.9 Statistical Analysis

All the data generated in this study were analyzed using Analysis of Variance (ANOVA) at 5% level of significance.

3. RESULTS

The yield of extracts showed that for methanol extracts, the same quantity of extracts were obtained from fruit and stem bark followed by root, seed and leaves respectively Table 1. For aqueous extract of different parts of *Picralima nitida*, more extracts were obtained from stem bark followed by fruit and seed (the same yield), then leaves and root respectively Table 2. The aqueous solvent yielded more extracts than the methanol.

Table 1. Percentage yield of crude extracts from different parts of *Picralima nitida* using methanol

Samples	Methanol (ml)	Sample weight (g)	Weight of extract (g)	Percentage yield of extract (%)
Leave	600	100	10	15.15
Fruit	600	100	16	24.24
Seed	600	100	11	16.67
Stem bark	600	100	16	24.24
Root	600	100	13	19.70

Table 2. Percentage yield of crude extracts from different parts of *Picralima nitida* using aqueous solvent

Samples	Deionized H_2O (ml)	Sample weight (g)	Weight of Extract (g)	Percentage yield of extract (%)
Leave	800	100	21	17.36
Fruit	800	100	33	27.27
Seed	800	100	23	19.01
Stem bark	800	100	25	20.66
Root	800	100	19	15.70

Methanol and aqueous extracts of *Picralima nitida* showed appreciable antitrypanosomal activity *in vitro*. The highest *in vitro* antitrypanosomal activity was exhibited by the methanol extract of the root of *Picralima nitida* followed by the methanol extract of the seeds of *P. nitida* Table 3. The methanol extract of the root at 4 mg/ml caused complete cessation of *T. brucei brucei* motility in 10 min, while the methanol extract of the seeds caused cessation of motility of the parasite in 15min at 4 mg/ml, as compared to the complete cessation of motility of the parasite by the other extracts at the same drug concentration Table 3. The standard trypanocidal drug, diaminal eliminated motility of the parasite within 25min even at the lowest concentration tested Table 3.

The *in vivo* studies showed that the methanol extracts of the root exhibited the highest antitrypanosomal effect at 100 mg/kg body weight of the test animals compared to the other extracts treatment groups and the untreated control group Table 4. In the methanol root extract treated group, the parasitemia was drastically reduced in day 3 of treatment and completely eliminated in day 4 of treatment. Parasitemia was reduced from 8.67±0.53 in day 0 to 0.26±0.83 in day 3 and completely cleared in day 4. A statistically significant difference ($p<0.05$) was observed between the level of parasitemia of *T. brucei brucei* infected mice treated with the root methanol extract of *Picralima nitida* and the other treatment groups/untreated control Table 4. The aqueous extracts of the roots and seeds and the

Table 3. *In vitro* effect of methanol and aqueous extracts of *Picralima nitida* plant parts on motility of *Trypanosoma brucei brucei*

S/N	Treatment	Plant part	Extract concentration/*Time (min)		
			4 mg/ml	2 mg/ml	0.4 mg/ml
1	Methanol	Leaves	25 (CT)	35 (CT)	55 (CT)
2	Methanol	Fruits	45 (CT)	50 (CT)	SRM
3	Methanol	Seeds	15 (CT)	25 (CT)	45 (CT)
4	Methanol	Stem bark	35 (CT)	45 (CT)	SRM
5	Methanol	Root	10 (CT)	20 (CT)	40 (CT)
6	Aqueous	Leaves	50 (CT)	55 (CT)	SRM
7	Aqueous	Fruits	45 (CT)	55 (CT)	SRM
8	Aqueous	Seeds	25 (CT)	30 (CT)	50 (CT)
9	Aqueous	Stem bark	35 (CT)	45 (CT)	SRM
10	Aqueous	Root	25 (CT)	30 (CT)	50 (CT)
11	Diaminal standard		05 (CT)	10 (CT)	25 (CT)
12	Untreated control		>60 (VM)	>60 (VM)	>60 (VM)

*Data shows *time (min) after which motility ceased or reduced slightly with different effective concentrations of extracts. Key: CT = Complete reduction in motility, SRM = Slightly reduced motility, VM = Very motile*

methanol extract of the fruit of *P. nitida* also showed appreciable *in vivo* antitrypanosomal activity Table 4. The methanol extract of the fruit reduced the parasitemia from 6.80±0.77 in day 0 to 0.66±0.12 in day 3 and later cleared the parasite in day 5. The aqueous extract of the root reduced the parasitemia from 7.00+1.00 in day 0 to 1.66±0.23 in day 3 and cleared it on day 6. The aqueous extract of the seed on the other hand reduced the parasitemia from 8.93±0.70 in day 0 to 2.06±0.90 in day 3 0f treatment Table 4. There was no recrudescence of parasitemia at days 7 and 14 of the infected animals treated with the methanol extracts of the roots and fruits. At days 7 and 14 also, there was no recrudescence of parasitemia of the infected animals treated with the aqueous extract of the roots of *Picralima nitida* and those treated with diamial standard trypanocidal drug. After recrudescence, the animals may die due to the high level of parasitemia. However, no significant differences were observed between all the groups on day 0 of treatment. These comparisons were made at days 0, 3, 7 and 14 of treatment Table 4. Diaminal at the standard dose of 3.5 mg/kg body weight cleared the parasitemia in the *T. brucei brucei* infected mice after two (2) days of drug administration Table 4. The *T. brucei brucei* infected mice that were not treated died after seven days of infection. The results of the phytochemical screening of the methanol and aqueous extracts of *Picralima nitida* plant parts show that all the extracts contain steroids and terpenoids Table 5. There is absence of saponins in the methanol extracts of the roots, leaves, fruits and stem bark, but it is present in the other extracts. Tannins were absent in the methanol extracts of the roots, fruits and stem bark, but present in the others. Flavonoids

were absent in the methanol extracts of the fruits and seeds, but present in others. Alkaloids were present in all the extracts except the aqueous extracts of the leaves and fruits Table 5.

4. DISCUSSION

The variation in the percentage yield of the extracts of methanol and aqueous solvents might be attributed to the volumes of solvent used for the two extractions. However, the yield was low when compared with the amount of pulverized plant part used for extraction. The aqueous extracts have higher yield than the methanol extracts, suggesting a high proportion of water-soluble components in the plant parts of *Picralima nitida*.

The *in vitro* antitrypanosomal activity exhibited by the crude extracts of *Picralima nitida* plant parts is in agreement with the previous findings of other researchers who established that the extracts of different plants may exhibit *in vitro* antitrypanosomal activity [7,12,13]. The antitrypanosomal effect exhibited by these extracts may be attributed to the presence in them of some secondary metabolites like flavonoids, tannins, alkaloids, saponins, steroids, cyanide glycosides and terpenoids [7].

The high *in vivo* antitrypanosomal effect of the methanol extract of the root and fruits, and the aqueous extracts of the roots and seeds of *Picralima nitida* can be attributed to the presence of the above secondary metabolites in them. In a similar study, Shaba et al. [14] Screened the leaves and fruits of *Brassica oleracea* (cabbage) for *in vitro* and *in vivo* antitrypanosomal effects. They stated that the methanol extracts of the leaves and fruits of *Brassica oleracea* exhibited *in vitro* and *in vivo* trypanocidal activity which ranged from immobilization, reduction and to the killing of trypanosomes. They equally stated that as the concentration of the extract increases, the trypanosomes counts and mobility decreased in concentration and time dependent manner. This is in agreement with the result of the *in vitro* evaluation of the methanol and aqueous extracts of *Picralima nitida* in this study.

In another study, Ene et al. [7] evaluated the petroleum ether, chloroform and methanol extracts of the whole plant of *Artemisia maciverae* for *in vivo* antitrypanosomal effects at 100 mg/kg body weight. They stated that the chloroform extract of this plant showed the highest *in vivo* antitrypanosomal activity at 100 mg/kg body weight. This is in agreement with the result of the *in vivo* evaluation of the methanol and aqueous extracts of *Picralima nitida* in this study where 100 mg/kg body weight of the extracts were used.

Investigation of antitrypanosomal activity of medicinal plants have been a major area of contemporary research focus. Natural products such as alkaloids, terpenes, quinines and polyphenols found in these plants extracts have been shown to be potent growth inhibitor of *T. cruzi* [15]. Triterpenoids and sterols from the plants are reported to possess antitrypanosomal activity [16]. The antitrypanosomal activities of alkaloids like actinodopamine, dicentine and cassythine isolated from *Cassytha filiformis* [17] are found in *B.buonopozense* and several other alkaloids [18] displayed significant *in vitro* antitrypanosomal activity. The DNA intercalation in combination with partial biosynthesis inhibition is reported to be the mechanism of action responsible for the *in vitro* antitrypanosomal effect of the active alkaloids [18]. The trypanocidal activity of several flavonoids such as quercetagetin [19], hispidulin and Santin [20] have been reported. The active components in the present study might be any of these secondary metabolites and their mechanisms of action similar.

Table 4. *In vivo* effect of methanol and aqueous extracts of *Picralima nitida* plant parts on *Trypanosoma brucei brucei*

Treatment	Plant part/ Drug dose	Level of parasitemia (Days)			
		D0	D3	D7	D14
Methanol	Root (100 mg/kg)	8.67±0.53[a]	0.26±0.15[b]	0.00±0.00[b]	0.00±0.00[b]
Methanol	Fruit (100 mg/kg)	6.80±0.77[a]	0.66±0.12[b]	0.00±0.00[b]	0.00±0.00[b]
Methanol	Stem bark (100 mg/kg)	7.00±1.00[a]	14.25±1.20[e]	20.00±0.00[ef]	20.00±0.00[ef]
Methanol	Leaves (100 mg/kg)	7.66±0.42[a]	9.20±0.52[e]	7.33±0.96[e]	10.25±1.00[e]
Methanol	Seeds (100 mg/kg)	6.33± 0.50[a]	13.40±0.71[e]	20.00±0.10[ef]	20.00±0.10[ef]
Aqueous	Root (100 mg/kg)	7.00±1.00[a]	1.66±0.23[b]	0.00±0.00[b]	0.00±0.00[b]
Aqueous	Fruit (100 mg/kg)	6.33± 0.57[a]	9.85±0.45[e]	17.15±0.30[ef]	20.00±0.00[ef]
Aqueous	Stem bark (100 mg/kg)	6.40±0.25[a]	8.06±0.34[e]	6.73±0.48[e]	15.88±0.17[ef]
Aqueous	Leaves (100 mg/kg)	8.66±0.70[a]	11.86±0.58[e]	20.00±0.10[ef]	20.00±0.10[ef]
Aqueous	Seeds (100 mg/kg)	8.93±0.70[a]	2.06±0.90[b]	0.13±0.10[b]	0.13±0.10[b]
Diaminal std control	3.5m g/kg	9.06±1.00[a]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]
Untreated control		8.80±0.80[a]	20.00±0.10[ef]	28.00±0.25[ef]	-

- *The parasitemia were compared with each other at days 0, 3, 7 and 14*
- *Values with different superscripts vertically differ statistically*
- *Values are mean±SE from 3 animals in each group*
- *indicates death of animals after seven days*

Table 5. Phytochemical screening of aqueous and methanol extracts of different parts of *Picralima nitida*

Test	Specific test	Result/Inference									
		Aq. leave	Aq.Root	Aq. Stem bark	Aq. Seed	Aq. Fruit	Met. Leave	Met. Root	Met. Stem bark	Met. Seed	Met. fruit
Tannins	Ferric chloride test	+	+	+	+	+	+	-	-	+	-
Saponins	Frothing test	+	+	+	+	+	-	-	-	+	-
Flavonoid	Lead chloride test or sodium hydroxide test	+	+	+	+	-	+	+	+	-	-
HCN		-	-	+	+	+	+	+	+	+	+
Terpenoids	Salkowskill test	+	+	+	+	+	+	+	+	+	+
Steroids		+	+	+	+	+	+	+	+	+	+
Alkaloids	Wagner's test Mayer's test Dragendroff's test	+	+	+	+	-	+	+	+	+	+

Where Aq represents aqueous, met represents methanol, HCN represents Hydrogen cyanide, + represents present, - represents not present

The results of the present study confirmed that medicinal plants and natural products derived from them are potential sources of new drugs for the treatment of important tropical diseases caused by trypanosomes. The high antitrypanosomal activity values obtained from some of the extracts of *Picralima nitida* render them candidates for the isolation of antitrypanosomal compounds which could be developed into new lead structures for drug development. Therefore, the extracts of *Picralima nitida* which showed positive antitrypanosomal activity are currently being fractionated and characterized using chromatographic and spectroscopic techniques to isolate the pure active components responsible for the antitrypanosomal activity. This study is in conformity with the existing literature judging from the pattern of the *in vitro* and *in vivo* antitrypanosomal activity of the *Picralima nitida* plant extracts.

5. CONCLUSION

The *in vitro* and *in vivo* evaluation of the methanol and aqueous extracts of *Picralima nitida* against *Trypanosoma brucei brucei* in Swiss albino mice showed that the methanol extracts exhibited appreciably high *in vitro* and *in vivo* antitrypanosomal activities compared to the aqueous extracts of the plant. It is therefore concluded that the methanol extracts of the various parts of this plant possess better antitrypanosomal properties.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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