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Original article

In vitro-in vivo studies on anti-trypanosomal potentials of *Zapoteca portoricensis*

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Abstract

Objective: Aqueous extracts of *Zapoteca portoricensis* are used traditionally as antidiarrhea agent and in the treatment of diverse gastrointestinal disorders here in Nigeria specifically, the southern part. Similarly, the aqueous extract of the plant is also used traditionally as anticonvulsant, antispasmodic and in the treatment of tonsillitis. Recently too, the anti-inflammatory and antimicrobial activities of the methanol extracts of the root of *Zapoteca portoricensis* was reported. In this research, we are set to investigate the trypanocidal activity of *Zapoteca portoricensis*. **Methods**: The methanol extract of the root of *Zapoteca portoricensis* was investigated for both in vitro and in vivo trypanocidal activity following established models. In summary, phytochemical analysis was carried out on both the crude powdered root and on the methanol extract following standard procedures. The oral acute toxicity test (LD_{50}) of the crude methanol extract was determined according to the method described by Lorke (1983). Albino mice (17g-21g) of either sex were used. The methanol extract was suspended in 3 % v/v tween 85 and administered orally at doses of 10 mg/kg, 100 mg/kg and 1 000 mg/kg to three groups of mice ($n=3$). The animals were observed for 24 hours. Based on the result obtained in this initial test, doses of 4 mg/kg, 6 mg/kg, and 8 mg/kg were administered to three different mice. The LD_{50} was calculated as the geometric mean of the lowest dose killing a mouse and the highest dose showing no death. The *in-vivo/in-vitro* anti-trypanosomal evaluations were carried out in experimental animals and tissue cell culture respectively. **Results**: The result of the in vitro studies shows the inhibitive concentration-50 (IC-50) against Trypanosoma brucei rhodesiense (*T. b. rhodesiense*) to be 0.372 mg/kg, while the control drug melarsoprol was 0.006 mg/kg. On *Trypanosoma brucei brucei* (*T. cruzi*), the IC-50 is 6.42 mg/kg against 0.87 of the reference drug Benznidazole. The cyctotoxicity on L-6 cells exhibited an IC-50 of 0.039 6 mg/kg against the reference drug, podophyllotoxin of 0.01 mg/kg. However, the in vivo study shows that the extract, at the administered doses, could not exhibit appreciable reduction of parasitemia and hence resulted to the death of test animals. **Conclusion**: The present data suggests that *Zapoteca portoricensis* could yield useful leads for the development of potentially potent antitrypanocides.

Keywords: *Zapoteca portoricensis*; *Trypanocidal* effects; *T. b. rhodensiense*; *T. cruzi*; *In vitro/in vivo* model

INTRODUCTION

Plants used in traditional medicine are considered to be potential sources for the development of alternative therapies[1] and offers greater chemical diversity. This is the superiority of nature over other sources of bioactive molecules. As at today, many plant secondary metabolites have been found to be biologically active against numerous diseases afflicting man and animal. While this holds very true for very many disease conditions, scientists have only succeeded in the isolation of few bioactive trypano-

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cidal agents from plants. It is in view of this that intensive search for antitrypanocidal agents become imperative. This is more critical considering the fact that most of the synthetic anti-trypanosomal agents have narrow therapeutic margin of safety and hence precipitates toxicity on administration. *Trypanosoma brucei brucei* (*T. cruzi*) and *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*) are unicellular parasites transmitted by the bite of tsetse fly. It is the causative agent of trypanosomiasis or sleeping sickness in humans and related diseases in animals[2,3]. Trypanosomiasis, over the years, has grown epidemiologically, to become a disease of public health concern. The disease is characterized by fever, which occurs in early stage and lasts about 1 to 3 days and usually, it is simultaneous with peaks of parasitaemia with pronounced hypochromic anemia. Further involvement includes cardiovascular disorders characterized by palpitation, precordal pains with syncope, faint heart sound and low pulse. There is also endocrinological disorders, which are due to abnormalities in mid brain disorder. Furthermore, dermatological effects, psychiatric disorders and chancre are also evidences of established infection.

Zapoteca portoricensis (family Fabaceae) is widely distributed in West Africa, (Togo; Misahohe) Southern Nigeria (Lagos, Bonny, Aguku, Oban etc) and Ghana (Odumase Aburi). Its distribution is seasonal, with highest distribution occurring mostly between August and October. Interestingly, related species aboriginal to America have been introduced into West Africa. In southern Nigeria, the aqueous and alcohol extracts of *Zapoteca portoricensis* are used traditionally as antidiarrhea agent and in the treatment of diverse gastrointestinal disorders. The aqueous extract of the plant is also used traditionally as anticonvulsant, antispasmodic and in the treatment of tonsillitis. In an effort to validate some of these folkloric uses in traditional medical practice, the anti-inflammatory and antimicrobial activities of the methanol extracts of the root of *Zapoteca portoricensis* have recently been investigated[4]. Plants used in traditional medicine are considered to be potential sources for the development of alternative therapies[1] and offer greater chemical diversity. This is the superiority of nature over other sources of bioactive molecules. In this research, we are set to investigate the trypanocidal activity of Zapoteca portoricensis.

MATERIALS AND METHODS

Plants

The root of the plant (*Zapoteca portoricensis*) was collected by Mr. Ekekwe J. M. C of Departments of botany, Univeristy of Northumara Newcastle (UNN), and authenticated by Mr. Ozoiko Alfred, a taxonomist at Bio-resource Development and Conservation Program(BDCP) Center Nsukka.

Sample preparation and Extraction

The root were cleaned, air-dried under shade and milled. The powdered root was packed in small cloth bags and suspended in methanol for 48 hours. The bag was occasionally agitated. The resulting extract was filtered and evaporated under forced air circulation.

Phytochemical tests

Phytochemical analysis was carried out on both the crude powdered root and on the methanol extract following standard procedures[5,6] as previously report[4].

Acute toxicity tests

The oral acute toxicity test (LD_{50}) of the crude methanol extract was determined according to the method described by Lorke[7]. Albino mice (17-21 g) of either sex were used. The methanol extract was suspended in 3 % v/v tween 85 and administered orally at doses of 10mg/kg, 100mg/kg and 1000 mg/kg to three groups of mice ($n = 3$). The animals were observed for 24 hours. Based on the result obtained in this initial test, doses of 4mg/kg, 6mg/kg, and 8 mg/kg were administered to three different mice. The LD_{50} was calculated as the geometric mean of the lowest dose killing a mouse and the highest dose showing no death.

Determination of *in vitro* antitrypanosomal activity and cytotoxicity

Minimum essential medium (50 μL) supplemented according to Baltz et al.[2] with 2-mercaptoethanol and 15 % heat-inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions were prepared covering a range from 0.123μg/mL to 90 μg/mL. Then 10^4 bloodstream

forms of *Trypanosoma b. rhodesiense* STIB 900 in 50 μL culture medium were added to each well and the plate incubated at 37℃ under a 5 % CO_2 atmosphere for 72 hours. 10 μL of Alamar Blue (12.5 mg resazurin dissolved in 100 mL distilled water) were then added to each well and incubation continued for a further 2-4 hours. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm[8]. Fluorescence development was measured and expressed as percentage of the control. Datas were transferred into the graphic programme Softmax Pro (Molecular Devices) which calculated inhabitive concentration$_{50}$ (IC_{50}) values. Cytotoxicity was assessed using the same assay and rat skeletal myoblasts (L-6 cells).

Determination of *in vivo activity*

Twenty- four albino mice (20-25 g) of both sexes were separated into five groups of four mice each to investigate the effect of the extracts on a *T. b. brucei* infection by the method proposed by Kalu, 1983[9]. The mice were infected by intraperitoneal injection at 10^5 trypanosomes/mouse in normal saline. Three days post-infection (p. i.) the mice were screened individually by tail blood examination for the presence of trypanosomes. The monitoring was done using the rapid matching counting method[10]. Wet blood preparations were covered with a cover slip on a slide and viewed under the microscope (X40) to rate the degree of the infection. The monitoring continued over several days until high level of parasitaemia was established. On day 5 when parasitaemia reached approximately 10^8/mL, treatment with the extract was initiated as follows: Groups I, II and III were treated daily with 4 mg/kg, 6 mg/kg and 8 mg/kg of the extract, respectively, for 4 days intraperitoneally. Group IV which served as the positive control received diminazene aceturate (Berenil, Hoechst AG Frankfurt, Germany) at 7 mg/kg for two days. Group V served as an untreated (negative) control group, and was treated with normal saline. Mice were monitored daily during and after treatments for the level of parasitemia, packed cell volume and weights.

RESULTS

The LD_{50} of the methanolic extract of *Zapoteca potoricensis* was calculated to be 8.9 mg/kg body weight intraperitoneally and in mice. The results of the different evaluations on the methanolic extract are as shown in tables 1 to 4.

Table 1 *In vitro* screening of the methanolic extract of *Zapoteca portoricensis*.

Trypanosome	Strain	Stage	Reference Drug	IC-50 (mg/mL)	
				Extract	Reference drug
T. b. rhodensiense	STIB 900	Trypomastigotes	Melarsopol	0.37	0.01
T. cruzi	Tulahem	Amastigotes	Benznidazole	6.42	0.87
L. donaxen	Mitom-ET-67/L82	Amastigotes	Miltefosine	5.49	0.08
P. falc. KI	KI	IEF	Chloroquine	2.37	0.40
Cytotoxic L6	L6	L6	podophyllotoxin	3.01	0.01

Table 2 Mean level of parasitaemia after treatment.

Drug	Dose (mg/kg)	No. of animal	Mean level of parasitaemia (mean ± S. E. M)				
			5 th day	8 th day	11 th day	15 th day	20 th day
Zapoteca portoricensis	4	4	7.6 ± 0.30	8.1 ± 0.00	8.0 ± 0.06	7.9 ± 0.13	8.1 ± 0.50
root extract	6	4	7.6 ± 0.08	8.1 ± 0.06	7.6 ± 0.12	8.1 ± 0.08	8.2 ± 0.10
	8	4	7.9 ± 0.23	8.0 ± 0.12	7.86 ± 0.10	8.0 ± 0.12	8.2 ± 0.10
Berenil®	7 *	4	6.8 ± 0.71	7.9 ± 0.30	6.4 ± 0.06 *	5.4 ± 0.00 *	5.4 ± 0.00 *
Negative Control	Distilled water	4	7.8 ± 0.22	8.2 ± 0.08	8.3 ± 0.06	8.3 ± 0.06	8.6 ± 0.00

* $P < 0.05$

Table 3 Mean packed cell volume.

Drug	Dose (mg/kg)	No. of animal	Mean packed cell volume (mean ± S. E. M)				
			5th day	8th day	13th day	17th day	20th day
Zapoteca portoricensis root extract	4	4	49.5 ± 0.75	46.2 ± 1.50	36.0 ± 1.90	32.5 ± 0.50	37.0 ± 1.90
	6	4	47.2 ± 2.30	43.2 ± 2.40	41.5 ± 2.40	35.5 ± 1.40	80.0 ± 3.50
	8	4	54.0 ± 1.40	47.2 ± 1.60	43.2 ± 1.02	36.7 ± 1.70	33.5 ± 0.53
Berenil®	3	4	49.5 ± 1.10	47.0 ± 1.84	38.5 ± 2.72	37.5 ± 2.35 *	37.0 ± 2.20 *
Negative Control	Distilled water	4	48.7 ± 2.95	44.2 ± 2.80	40.5 ± 2.10	34.5 ± 1.40	25.0 ± 0.00

* $P < 0.05$

Table 4 Mean weight (g) after treatment.

Drug	Dose (mg/kg)	No. of animal	Mean weight (g) (mean ± S. E. M)			
			5 th day	8 th day	17 th day	20 th day
Zapoteca portoricensis root extract	4	4	30.5 ± 2.60	33.3 ± 1.12	33.9 ± 2.40	31.6 ± 3.80
	6	4	24.6 ± 0.80	27.3 ± 1.13	27.3 ± 1.05	26.2 ± 0.22
	8	4	28.2 0.10	28.0 ± 0.77	28.1 ± 0.97	25.8 ± 0.41
Berenil®	3	4	36.6 ± 2.13	36.4 ± 2.20	35.7 ± 0.20 *	35.0 ± 2.30 *
Negative Control	Distilled water	4	32.17 ± 0.72	32.0 ± 0.53	33.0 ± 0.69	32.0 ± 0.00

* $P < 0.05$

DISCUSSIONS

In the present work, we have applied the ethnopharmacological approach to the screening of the crude methanol extract of the root of *Zapoteca portoricensis* for both *in vitro* and *in vivo* trypanocidal activity. Farnsworth *et al*[11] showed that out of 119 important plant derived drugs used in one or more countries, 88 (77%) were discovered by employing the ethnopharmacological approach. This approach mainly involves two separate disciplines: ethnobotany and pharmacognosy. Anti-parasitic drug discovery based on an ethnobotanical approach is most promising and recently resulted in the successful identification of artemisinin for the treatment of malaria[12]. There is increasing search for drugs from natural sources and the results so far achieved are commendable[12]. Trypanosomiasis or sleeping sickness is a very serious medical problem of great public health concern affecting both human and animals. Synthetic drugs have been the mainstay of therapy with its attendant side effects[12, 13]. Currently, four drugs are available for the treatment of trypanomiasis[14]. It is therefore, worthwhile to seek for bioactive molecules against trypanosomes from plants and other natural sources. This is the current trend in antitrypanosomidal research[15,16]. The observed *in-vitro* trypanocidal effect (Table 1) is in agreement with findings of previous workers on plants of similar class which were found to exhibit such actions[17-21]. The methanolic extract, which supposedly contains both polar and non-polar constituents showed activity *in vitro* but not in vivo (Table 2). This is expected as previous reports have shown that natural agents active against trypanosomiasis which are alkaloids, flavanoids, phenolics and/or terpenes could be degraded *in-vivo* to inactive metabolites[16]. Preliminary phytochemical analysis had already been carried out on this plant root, and it was shown the root extract contained alkaloid, flavonoids, saponins, reducing sugars, steroidal aglycones and proteins[4]. As presently convincing as it appears, the absence of *in vivo* activity cannot be completely explained by the present data. There is still need to establish the exact reason why the extract could not exhibit *in vivo* trypanocidal activity. On the day-three post infection, the presence of the typanosomes was detected in the blood of few mice but they appeared in all mice on day-four post infection. The level of parasitemia was high enough on day five post infection for commencement of treatment in all the groups. The presence of trypanosomes was detected in the blood of all the groups of the experimental animals, day-four post infection. There was no presence of *T. brucei* in group VI as they were not infected. The level of parasitemia persistently increased in methanolic extract treated group (I, II, III) and negative control group (V) till death started occurring from day-17 post infection. The level of parasitemia in-group IV started to decrease after treatment until day-nine post infection when the whole mice became parasitized as shown by the microscope. The standard drug

used, Berenil® at a dose of 3mg/kg cleared the trypanosome completely. In group V the parasite was still present because they were infected and untreated. This resulted to the death of some mice; day-20-post infection while in the group VI, there was no trace of any parasite in their blood, as they were not infected. The result of parasitaemia determination in the five groups of mice is recorded in Table 2. The base 10-logarithm value represents the concentration of organisms per milliliter of blood[10]. There was no decrease in PCV in the methanolic extract treated group (I, II, III) and negative control group (V) respectively within the period of post infection. In the positive control Group (IV) PCV initially decreased but after 3 days of post treatment the PCV gradually increased but the level differed due to the difference in their recovering. While in group V their PCV continued to decrease, as there was no treatment till death started occurring to them. In group VI the PCV remained constant throughout the course of the practical. The result of the PCV was shown in table III. Generally, PCV and body weight of animals are useful indices in the monitoring of infection progression and overall treatment response. The present *in-vivo* data is an indication that the extract lacked activity since an increment in PCV for an infected animal indicates regression of infection and convalescence.

In conclusion, *Zapoteca portoricensis* has been shown to possess appreciable *in-vitro* anti trypanosomal activity when compared to standard drugs. There was no meaningful or significant *in-vivo* activity. Further work is needed to isolate and characterize the main active *in-vitro* trypanocidal principles from the plant root. This future work should involve the elucidation of possible mechanism of action of the crude methanol extract. Moreover, the exact reason for the absence of *in-vivo* anti trypanosomal activity in *Zapoteca portoricensis* is a future worthwhile research.

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