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In vitro antitrypanosomal activity of ethnopharmacologically selected Beninese plants

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Abstract

The in vitro antitrypanosomal activity of methylene chloride, methanol and aqueous extracts of the leaves and twigs of five plant species traditionally used in Benin for the treatment of sleeping sickness were evaluated on *Trypanosoma brucei brucei* and their selectivity was analysed on *Leishmania mexicana mexicana* and J774 macrophage-like murine cells. The results showed that the four most active extracts had MIC values ≤19 μg/ml (*Hymenocardia acida* twig and leaf, *Strychnos spinosa* leaf, *Trichilia emetica* leaf methylene chloride extracts). All these extracts had a lower activity on *L. m. mexicana* and J774 cells. Determination of the IC_{50} values of the methylene chloride leaf extracts on two strains of trypanosomes (*T. b. brucei* and *T. b. rhodesiense*) and two mammalian cell lines (L6 and J774 cells) showed that all extracts possessed some antitrypanosomal activity with IC_{50}'s ranging from 1.5 to 39 μg/ml. All were also toxic to the mammalian cells, but usually with higher IC_{50}'s. The only exception was the *S. spinosa* methylene chloride leaf extract which had no toxicity on J774 cells. Although tannins have been identified in most of the species studied, they could not be detected in the most active extracts, just as alkaloids. The presence of flavonoids and quinones may at least in part explain the observed activities of some of the active extracts.

Keywords: Antitrypanosomal activity; *Cassia sieberiana*; *Hymenocardia acida*; *Pericopsis laxiflora*; *Strychnos spinosa*; *Trichilia emetica*

1. Introduction

African trypanosomes are protozoan parasites responsible for human African trypanosomiasis and nagana in cattle and are transmitted by the bite of an infected tsetse fly. *Trypanosoma brucei brucei*, the causative agent of nagana, is closely related to *Trypanosoma brucei rhodesiense* (East to South Africa) and *Trypanosoma brucei gambiense* (West and Central Africa) which cause human African trypanosomiasis, or sleeping sickness. Sleeping sickness currently affects about half a million people in sub-Saharan Africa and an estimated 60 million people are at risk of contracting this disease, which is fatal if untreated (WHO, 1998; Barrett, 1999). However, the currently available treatments are far from being ideal. The few registered trypanocides are frequently toxic, require lengthy parenteral administration, lack efficacy and are unaffordable for most of the patients (Keiser et al., 2001; Legros et al., 2002). Therefore, there is an urgent need for new, safe, effective and cheap molecules and for new leads with new mechanisms of action.

In countries where sleeping sickness occurs, plants have traditionally been used for centuries and are still widely used to treat this illness and other parasitic diseases. It is estimated that two-third of the world population rely on traditional medical remedies due to the limited availability and affordability of pharmaceutical products (Tagboto and Townson, 2001). Furthermore, several well-known drugs, such as quinine and artemisinin used as antiprotozoal agents, have their origins in nature (Kirby, 1996; Camacho et al., 2000a; Tagboto and Townson, 2001). The present study investigates the in vitro antitrypanosomal activity of crude extracts from five plants which are traditionally used in Benin to fight sleeping sickness and for which this traditional use has been described in the literature (Neuwinger, 2000). Those five plants are commonly used in African traditional medicine for a wide spectrum of other medical indications

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including schistosomiasis and protozoan diseases such as malaria (Neuwinger, 2000).

2. Materials and methods

2.1. Plant material

Candidate plants (Table 1) were selected on an ethnopharmacological basis. They were collected in December 1999 in the savannas of the Zou province in Benin and identified by one of us (V.A.). Voucher specimens were deposited at the Herbarium of the Belgian National Botanical Garden, at Meise (see Table 1 for voucher specimen numbers).

2.2. Preparation of crude plant extracts

Plant extracts were prepared by percolating 20–50 g of dried and powdered plant material (leaves or twigs) sequentially with methylene chloride, methanol and Milli-Q water. Each extraction was performed for about 24 h at room temperature. The quantity of solvent used for each extraction was at least 10 times the quantity of plant material used. Thus, three extracts of increasing polarity were obtained for each plant part. After filtration, methylene chloride and methanol filtrates were evaporated to dryness at 30 °C under reduced pressure while the water filtrates were freeze-dried to powder. The solvent-free extracts were stored frozen. Yields for each percolation are indicated in Table 2.

2.3. Parasites, cell lines and media

Trypanosoma brucei brucei (strain 427) bloodstream forms were cultivated in vitro in a modified Iscove's medium containing 10% heat-inactivated fetal calf serum (HI-FCS) and bloodstream form supporting factors: 0.05 mM bathocuproine sulfonate, 1.5 mM L-cysteine, 1 mM hypoxanthine, 0.2 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.16 mM thymidine known as HMI 9 (Hirumi and Hirumi, 1994).

Trypanosoma brucei rhodesiense bloodstream forms (STIB 900) were grown in vitro in Minimum Essential Medium supplemented according to Baltz et al. (1985) with 2-mercaptoethanol (0.2 mM) and 15% heat-inactivated horse serum.

Leishmania mexicana mexicana promastigotes (MHOM/BZ/84/BEL46) were grown in vitro in a semi-defined medium (SDM-79) (Brun and Schönenberger, 1979) supplemented with 10% HI-FCS.

The macrophage-like cell line, J774G8, derived from a BALB/c murine reticulum cell sarcoma, was cultivated in vitro in RPMI 1640 with L-glutamine supplemented with 10% HI-FCS prior to use. L6 cells, rat skeletal muscle myoblasts, were cultivated in the same medium.

All cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C except the *Leishmania* promastigotes which were incubated at 28 °C.

Table 1
Plants assayed for their antitrypanosomal activity

Botanical name	Family	Voucher specimen number
Cassia sieberiana DC	Caesalpiniaceae	BR S.P. 848100
Hymenocardia acida Tul.	Hymenocardiaceae	BR S.P. 848107
Pericopsis laxiflora (Bentham ex Baker) van Meeuwen	Papilionaceae	BR S.P. 848102
Trichilia emetica Vahl subsp. *suberosa* J.J.F.E. DeWilde	Meliaceae	BR S.P. 848104
Strychnos spinosa Lam.	Loganiaceae	BR S.P. 848106

2.4. In vitro test for antitrypanosomal and antileishmanial activity and cytotoxicity

The assay performed, long incubation low inoculation test (LILIT), was previously described by Brun and Lun (1994) with minor modifications by Räz et al. (1997). To evaluate the selectivity of the in vitro antitrypanosomal activity, in vitro cytotoxicity tests were also performed as well as evaluation of the effect on *Leishmania* promastigotes, a closely related parasite. The same general procedure was used for the different biological assays to facilitate comparison of the results.

The plant extracts were initially dissolved in DMSO and/or water and diluted with the medium after a sterilizing filtration for the aqueous solutions. The highest concentration of DMSO after serial dilution with complete culture medium was 0.3%.

Each extract was tested in 96-well microtiter plates in three-fold serial dilutions; each concentration was tested in duplicate. For the preliminary screening, each extract was tested once. For the more detailed study of the leaf methylene chloride extracts, each extract was tested at least twice.

Fifty microlitre of a cell suspension (100 μl for L6 cells) were added to each well at a cell density adapted so that after 72 h of incubation, in the control wells, the cells in suspension were at the end of the logarithmic growth phase and the adherent cells formed a confluent monolayer.

Control wells without plant extracts were included as well as control wells with DMSO. Background fluorescence of the drug-containing medium was determined for each drug concentration.

After 68 h of incubation, each plate was examined with an inverted microscope to determine the Minimum Inhibitory Concentration (MIC) which is the concentration at which no cell with a normal morphology and/or motility was found in comparison to the control cultures. Afterwards, the fluorochrome Alamar Blue™ was added into each well and the fluorescence was quantified after a total incubation time of 72 h with a fluorescence plate reader (Millipore Cytofluor 2300 for *T. b. brucei* and J774 or SpectraMax Gemini XS

Table 2
Antitrypanosomal, antileishmanial and cytotoxic activity of plant extracts

Plant	Part[a]	Solvent[b]	Yield (%)	Activity[c] on		
				Tbb	Lmm	J774
Cassia sieberiana	LF	CH_2Cl_2	5.8	+	–	+/–
		CH_3OH	20.3	+/–	–	+/–
		H_2O	5.5	+	+/–	+
	TW	CH_2Cl_2	1.1	+	+/–	+/–
		CH_3OH	11.4	+/–	–	+/–
		H_2O	2.3	+	–	+
Hymenocardia acida	LF	CH_2Cl_2	5.0	++	+	+
		CH_3OH	13.7	–	–	–
		H_2O	7.1	+/–	–	+
	TW	CH_2Cl_2	0.5	++	+	+
		CH_3OH	8.7	+/–	–	+/–
		H_2O	3.3	+	+	+
Pericopsis laxiflora	LF	CH_2Cl_2	1.7	+	+/–	+/–
		CH_3OH	11.3	+/–	–	–
		H_2O	6.4	+/–	–	+/–
	TW	CH_2Cl_2	1.1	+	+/–	+/–
		CH_3OH	14.5	+/–	–	–
		H_2O	4.3	+/–	–	+/–
Strychnos spinosa	LF	CH_2Cl_2	3.3	++	+/–	–
		CH_3OH	8.3	–	–	–
		H_2O	14.8	+/–	–	–
	TW	CH_2Cl_2	0.8	+	+/–	+/–
		CH_3OH	6.5	–	–	–
		H_2O	4.5	–	–	–
Trichilia emetica	LF	CH_2Cl_2	6.4	++	+/–	+
		CH_3OH	12.8	–	–	–
		H_2O	5.2	+	–	+/–
	TW	CH_2Cl_2	0.6	+	+/–	+/–
		CH_3OH	5.5	–	–	–
		H_2O	3.7	+/–	–	+/–
Suramin (μg/ml)				0.1		>1
Diminazene aceturate (μg/ml)				0.1	0.9	>10
Colchicine (μg/ml)						0.2

[a] Plant part used: LF = leaves, TW = twigs.
[b] Extraction solvent.
[c] Activity = MIC (minimum inhibitory concentration) = concentration at which no cell with a normal morphology and/or motility is found in comparison to the control (++) MIC ≤ 19 μg/ml; (+) MIC: 19–56 μg/ml; (+/–) MIC: 56–167 μg/ml; (–) MIC ≥ 167 μg/ml. Tbb = *Trypanosoma brucei brucei* bloodstream forms, Lmm = *Leishmania mexicana mexicana* promastigotes, J774 = macrophage-like murine cells.

for *T. b. rhodesiense* and L6) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Fluorescence development was expressed as percentage of the control, considered as 100%, and IC_{50} values (concentration of extract that reduced fluorescence intensity by 50%) were calculated by linear interpolation according to Hills et al. (1986). Assays with commercial drugs were also performed to have reference values (melarsoprol (Aventis), diminazene aceturate (Hoechst AG), suramin (Sigma), colchicine (Sigma)).

3. Results

Table 2 presents the MIC of all 30 extracts on *T. b. brucei* bloodstream forms, *L. m. mexicana* promastigotes and J774 cells. Concerning the antitrypanosomal activity, we observed that four extracts could be considered as active with MIC values ≤19 μg/ml, 10 were moderately active with MIC values between 19 and 56 μg/ml, 10 had MIC values between 56 and 167 μg/ml and 6 were inactive with MIC values ≥167 μg/ml. We also observed that the MIC values were overall higher for *Leishmania* promastigotes and for mammalian cells. Table 3 shows the IC_{50}'s obtained with the leaf methylene chloride extracts of the five candidate plants when tested on two strains of trypanosomes and on two mammalian cell lines. All five leaf methylene chloride extracts showed some activity on the two African trypanosomes with IC_{50}'s ranging from 1.5 to 39 μg/ml. All extracts also showed activity against both tested mammalian cell lines, with higher IC_{50}'s as compared to the trypanosomes (Table 4). The only excep-

Table 3
In vitro antitrypanosomal activity and cytotoxicity of five leaf methylene chloride extracts

Plant	Antitrypanosomal activity (IC_{50}, μg/ml) average ± standard deviation		Cytotoxicity (IC_{50}, μg/ml) average ± standard deviation	
	Tbr	Tbb	L6	J774
Cassia sieberiana	25.8 ± 4.5	20.0 ± 11.3	nd	44.5 ± 0.7
Hymenocardia acida	9.1 ± 4.9	5.0 ± 1.4	12.2 ± 7.4	28.0 ± 1.3
Pericopsis laxiflora	11.8 ± 0.8	39.2 ± 1.6	32.2 ± 13.5	83.5 ± 10.6
Strychnos spinosa	16.4 ± 8.8	1.5 ± 0.9	25.9 ± 22.4	>100
Trichilia emetica	14.9 ± 10.7	8.6 ± 5.9	8.9 ± 5.9	36.5 ± 2.1
Melarsoprol	0.002 ± 0.001	nd	nd	nd
Suramin	0.06 ± 0.007	0.049 ± 0.025	nd	nd
Diminazene aceturate	0.002 ± 0.0005	0.011 ± 0.006	nd	nd
Colchicine	nd	nd	0.006 ± 0.002	0.067 ± 0.028

Tbb = *Trypanosoma brucei brucei* bloodstream forms. Tbr = *Trypanosoma brucei rhodesiense* bloodstream forms. J774 = macrophage-like murine cells. L6 = rat skeletal muscle myoblasts. nd = not determined.

Table 4
Selectivity indices of five leaf methylene chloride extracts

Plant	Selectivity index[a]			
	L6/Tbr	L6/Tbb	J774/Tbr	J774/Tbb
Cassia sieberiana	nd	nd	1.7	2.2
Hymenocardia acida	1.3	2.4	3.1	5.6
Pericopsis laxiflora	2.7	0.8	7.1	2.1
Strychnos spinosa	1.6	17.3	>6.1	>66.7
Trichilia emetica	0.6	1.0	2.4	4.2

nd = not determined.
[a] Selectivity index = $IC_{50\,(L6\text{ or }J774)}/IC_{50\,(Tbb\text{ or }Tbr)}$.

tion was *Strychnos spinosa* which showed no toxicity on J774 cells.

4. Discussion and conclusions

In our preliminary screening, three plants out of five had at least one extract, often the leaf methylene chloride extract, with MIC values ≤19 μg/ml for trypanosomes: *Trichilia emetica*, *Strychnos spinosa*, *Hymenocardia acida*. In general, the extracts were more active on trypanosomes than on *Leishmania* promastigotes and the mammalian cell line, J774. The methylene chloride extracts were equally or more active on trypanosomes than the water extracts which were often more active than the methanol extracts. Based on those results and the fact that the leaves are the plant part traditionally used in the treatment of sleeping sickness, we analysed in greater detail the antitrypanosomal activity and cytotoxicity of the leaf methylene chloride extracts of the five plants.

All five extracts showed some activity for *T. b. brucei* and *T. b. rhodesiense* and no major differences in the sensitivities of the two strains were observed except for *S. spinosa* and *P. laxiflora*. Nevertheless, the IC_{50} values for the active extracts were high compared to the values obtained for commonly used trypanocidal drugs such as suramin and diminazene aceturate. However, since the crude extracts have a very complex composition, purification might lead to pure compounds with highly increased activity.

All extracts also showed activity on both mammalian cell lines, usually with higher IC_{50}'s as compared to the ones for trypanosomes but we did not observe a high selectivity. Nevertheless, as crude extracts contain many different compounds, a higher selectivity can be expected for isolated molecules. The most interesting result represents the *S. spinosa* extract which showed no cytotoxicity for J774 cells and a high activity on *T. b. brucei* resulting in a selectivity index >65.

Some of the candidate plants have already been investigated for their antitrypanosomal activity in other studies. Our results support those of Freiburghaus et al. (1996) who showed that leaf extracts of *S. spinosa* and *H. acida* were active in vitro on *T. b. rhodesiense*. The activity of *S. spinosa* leaf extracts were close to those observed in our study, while the range of MIC values were quite different for *H. acida* leaf extracts. Such differences between our results and those of other authors may be due to the known variation in the chemical composition of plants according to the geographical area and the time or season of collection. We also noted variations in the antitrypanosomal activity of the candidate plants depending on the time and place of collection (data not shown).

Freiburghaus et al. (1996) tested rootbark extracts of *S. spinosa* as well as root extracts of *H. acida* which were also active in vitro and analysed the selectivity of these root extracts on human fibroblasts. In our studies we focused on the leaves which are the plant part used in traditional medicine and we examined the effects of these leaf extracts on another protozoan parasite (*L. m. mexicana*) and on mammalian cells. Freiburghaus et al. (1996) also investigated the activity of leaf extracts of *T. roka*, which is now named *T. emetica* subsp. *emetica* (White, 1986), which were inactive. In our work, we showed that the *T. emetica* leaf extracts (methylene chloride and water) possess interesting activities. The plant we collected in Benin is *T. emetica* subsp. *suberosa*, another subspecies. These results support

the taxonomic differentiation of these two subspecies, showing different biological properties probably linked to different chemical composition. This is further supported by the results obtained on *Plasmodium falciparum* where *T. roka* (=*T. emetica* subsp. *emetica*) had no activity while *T. emetica* (=*T. emetica* subsp. *suberosa*) was active (El Tahir et al., 1999; Traore-Keita et al., 2000; Prozesky et al., 2001). Our studies analysed for the first time the in vitro activity of *P. laxiflora* and *C. sieberiana* on trypanosomes, leishmania and mammalian cells. In an in vivo study with mice infected with *T. b. gambiense*, root water decoction and maceration of *C. sieberiana* were found to be inactive (Youan et al., 1997), while we found an activity in vitro for the leaf extract, which according to Neuwinger (2000) is used in traditional medicine.

It also has to be mentioned that the plants we tested have already been investigated for other antiparasitic activities. Methanolic and methylene chloride extracts of the leaves of *H. acida* were active on *P. falciparum* (Vonthron-Senecheau et al., 2003). Stem bark decoction of the same plant species has shown in vitro effect against *Entamoeba histolytica*, as well as rootbark decoction but with a lower activity (Tona et al., 1998). An ethanol bark extract of *S. spinosa* was inactive on *P. falciparum* (Frederich et al., 1999). *T. emetica* (undefined subspecies) was also studied for its in vitro antihelminthic activity: it was very active on schistosomes and also active on cestodes (Sparg et al., 2000; Mølgaard et al., 2001).

To obtain information on the type of compounds which could be responsible for the antitrypanosomal activities, we reviewed the literature but only little is known about the leaf composition of the selected species.

Tannins have been identified in the leaves of all species except *S. spinosa* (Watt and Breyer-Brandwijk, 1962; Persinos and Quimby, 1967; Duquenois and Anton, 1968; Kerharo and Adam, 1974). However, our phytochemical screening did not allow us to identify them in our methylene chloride extracts, so the observed activity must be due to other classes of compounds. One candidate could be flavonoids which were identified previously in *C. sieberiana*, *P. laxiflora* and *S. spinosa* (Duquenois and Anton, 1968; Kerharo and Adam, 1974; Sultana and Ilyas, 1987). A literature survey indicated that several flavonoids have antitrypanosomal activity, some showing an interesting selectivity such as quercetagetin (IC_{50} = 800 nM, selectivity index = 571) (Räz, 1998; Camacho et al., 2000b; Tarus et al., 2002). *C. sieberiana* was also shown to contain anthracenic derivatives (Duquenois and Anton, 1968) which could also explain a part of the observed activity as a recent study has shown that azaanthraquinone has interesting effects on *Trypanosoma congolense* (Nok, 2002). Very low concentrations of alkaloids have been reported in the leaves of *P. laxiflora* and *S. spinosa* (Bisset and Phillipson, 1971; Kerharo and Adam, 1974; Oguakwa et al., 1980; Ohiri et al., 1983). As a recent paper analysed the antitrypanosomal activity of alkaloids from different types, some of which being active, we tried to detect alkaloids using thin-layer chromatography but failed to demonstrate their presence in these two crude methylene chloride leaf extracts (Merschjohann et al., 2001). Nevertheless, due to the complex composition of our extracts, it is not possible at this stage to identify the compounds which may be responsible for the observed activities. This should be done by bio-guided fractionation and isolation and characterisation of pure compounds.

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