Tropical Medicine and International Health

VOLUME 1 NO. 6 PP 765–771 DECEMBER 1996

In vitro antitrypanosomal activity of African plants used in traditional medicine in Uganda to treat sleeping sickness

Franziska Freiburghaus[1], Elizabeth N. Ogwal[2], Mayunga H. H. Nkunya[3], Ronald Kaminsky[1] and Reto Brun[1]

[1] *Swiss Tropical Institute, Basel, Switzerland*
[2] *Department of Botany, Makerere University, Kampala, Uganda*
[3] *Department of Chemistry, University of Dar Es Salaam, Tanzania*

Summary In Uganda, as in many other African countries, herbal treatment of various diseases is still common. In the present study, 9 plant species collected from Tanzania and Uganda and used by traditional healers in southern-eastern Uganda for the treatment of human African trypanosomiasis (sleeping sickness) were extracted and screened for their *in vitro* activity against *Trypanosoma brucei rhodesiense*, one of the two causative agents of sleeping sickness. Eight lipophilic extracts of 5 plants revealed very promising antitrypanosomal activity with IC_{50} values below 1 μg/ml; among them were extracts prepared from *Albizia gummifera* (2), *Ehretia amoena* (1), *Entada abyssinica* (2), *Securinega virosa* (1) and *Vernonia subuligera* (2). Activity with IC_{50} values between 1 and 10 μg/ml was determined for 15 further extracts. Cytotoxicity of active extracts, tested on a human fibroblast cell line (WI-38), was found to be high, and therefore selectivity indices resulted in less favourable ranges than those for the few commercially available drugs. Nevertheless, the results confirm the potential of ethnobotanically selected plants as remedies against sleeping sickness and call for phytochemical studies.

keywords sleeping sickness, *Trypanosoma b. rhodesiense*, traditional medicine, *in vitro* activity, medicinal plants

correspondence Dr R. Brun, Swiss Tropical Institute, PO Box, CH-4002 Basel, Switzerland

Introduction

Sleeping sickness caused by the flagellated parasites *Trypanosoma brucei rhodesiense* and *T. b. gambiense* occurs in 36 African countries. Over 50 million people are at risk of acquiring the infection which occurs focally (Kuzoe 1993). The estimated prevalence in 1993 was 250 000 to 300 000 (WHO 1994) with a high mortality because the disease is fatal if untreated. Treatment remains extremely problematic since the number of available drugs is limited, the duration of treatment is long and side-effects are usually severe (Kuzoe 1993; Doua & Yapo 1993).

Plants used in indigenous medicine are considered to be potential sources for the development of alternative therapeutics (Cox & Balick 1994). Since herbal treatment for various diseases in Africa is still widespread (Anokbonggo 1992), an ethnobotanical approach in collaboration with traditional healers may prove to be a rich source of drug discovery. On the other hand, herbal remedies are the only source for day-to-day healthcare for a large number of the world's population in rural areas (Farnsworth *et al.* 1985), so it is a vital task to identify those plants suitable for use, focusing on efficacy and toxicity.

Various regions of Uganda are affected by tsetse flies and in many of those areas herbal treatment of sleeping sickness is common. However, so far no studies for the identification of plants used for the treatment of sleeping sickness have been carried out. The 9 plants investigated in the present study were selected on the basis of information from traditional healers on their curative effect in the treatment of sleeping sickness. For performing the *in vitro* assays, aqueous extracts were prepared according to the methods used by traditional healers. In addition, consecutive extraction using organic solvents was performed with the aim of a polarity-based pre-separation of compounds present in the plants. All extracts produced were tested for their activity against *T. b. rhodesiense in vitro* and active extracts ($IC_{50} \leq 10$ µg/ml) for their cytotoxicity to a human fibroblast cell line (WI-38).

Materials and methods

Plant materials

Information on plants used for the treatment of sleeping sickness, the preparation of extracts and the route of administration was obtained by one of the authors (E. N. Ogwal) from traditional healers in south-eastern Uganda. Small amounts (10–50 g) of plants were collected in Uganda together with traditional healers (identified by Mrs E. N. Ogwal) and large amounts (kg) in Tanzania (identified by Mr L. B. Mwasumbi). Voucher specimens are preserved at the Herbaria of Makerere University, Kampala (Uganda), University of Dar Es Salaam (Tanzania) and University of Basel (Switzerland).

Preparation of crude plant extracts

Crude plant extracts were prepared for primary *in vitro* screening by extracting 20–50 g of dried and powdered plant material from stem, root, etc. sequentially with petroleum ether, dichloromethane, methanol and distilled water by percolation. A ten-fold quantity of solvent in relation to plant material was used for the extraction. For each solvent extraction was performed 3 times at room temperature, each time for 8 hours. Thus, 4 extracts of increasing polarity were obtained from each plant part. In addition, extracts according to the traditional method of preparing herbal remedies were produced from some plants. For that purpose, dried plant materials were pulverized and extracted for 30 minutes either with boiling (*Garcinia huillensis*, *Entada abyssinica*) or cold (*Capparis elaeagnoides*, *Vernonia auriculifera*, *V. subuligera*) tap water. All extracts were filtered through a filter paper (Schleicher and Schuell, Germany); the filtrates were then concentrated on a rotary evaporator (Büchi, Switzerland) at 50°C under reduced pressure and then freeze-dried. The solvent-free extracts were stored at 4°C until use.

Trypanocidal drugs

The following commercial drugs for the treatment of sleeping sickness were tested: pentamidine isethionate [bis(*p*-amidinophenoxy)-1,5-pentane; Pentacarinat] from Rhône-Poulenc Rorer (Dagenham, UK) and suramin [*m*-aminobenzoyl-*m*-amino-*p*-methylbenzylnaphthylamino-l trisulphonate-sodium-4,6,8; Germanin] from Bayer (Leverkusen, Germany). Stock solutions were freshly prepared in sterile distilled water and diluted in complete culture medium.

Trypanosome stock

Trypanosoma brucei rhodesiense STIB 900 (Swiss Tropical Institute Basel) was isolated in 1982 from a male patient in Ifakara, Tanzania. After several passages in Swiss ICR mice and generation of clones, one clone was finally adapted to axenic culture conditions. Stabilates were prepared containing 10% (v/v) glycerol and stored in liquid N_2. After initiation of a culture with a stabilate the trypanosome population was kept for a maximum of 4 months in culture.

Culture medium

The culture medium for the axenic cultivation of the bloodstream forms consisted of minimum essential medium with Earle's salts (MEM; Gibco-BRL No. 072-1100, powder) supplemented with 1 g/l additional glucose, 25 mM HEPES (Calbiochem), 2.2 g/l $NaHCO_3$, and 10 ml/l MEM non-essential

amino acids (× 100). This stock medium was further supplemented with 10% (v/v) heat-inactivated horse serum, 0.2 mM 2-mercaptoethanol, 2 mM sodium pyruvate and 0.1 mM hypoxanthine (Baltz *et al.* 1985).

Determination of *in vitro* activity of crude plant extracts

The activity of plant extracts was assessed after a 66-hour exposure time using two different evaluation procedures. First, the MIC (minimum inhibitory concentration) was determined microscopically with an inverted microscope (× 200) according to Brun and Lun (1994). The MIC was defined as the lowest concentration of crude plant extract in which no trypanosomes with normal morphology or motility could be found. Secondly, the fluorochrome BCECF-AM was used to measure viability of the exposed trypanosomes. The non-fluorescent dye BCECF-AM is taken up by viable cells and cleaved by unspecific esterases into a fluorescent product. Activity of the extracts was expressed as IC_{50}, according to Obexer *et al.* (1995).

Before performing an assay, all solutions of crude plant extracts were freshly prepared from the freeze-dried material. The lipophilic extracts were dissolved in 10% DMSO, the methanolic extracts were dissolved in 10% methanol (96%) and the water extracts in sterile distilled water. All water extracts were sterilized by filtration (0.2 μm). After serial dilution with complete culture medium for use, the highest concentration of DMSO or methanol did not exceed 0.8%. Each extract was tested 2 or 3 times in duplicate in 96-well microtitre plates (Costar, USA) in threefold serial dilutions ranging from 500 to 0.07 μg/ml crude plant extract. Trypanosomes were inoculated at a density of 2×10^2 per well in a final volume of 100 μl. Control wells without plant extract and wells with solvent were included. After incubation at 37°C for 66 hours in a humidified incubator containing 5% CO_2, the MIC and MTC (maximum tolerated concentration) were determined. For all extracts which showed MIC values <56 μg/ml, 100 μl of a solution containing 4 μM BCECF-AM (2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein-pentaacetoxy-methylester) (Calbiochem, Switzerland) was added to each well. After incubation of another 45 minutes, fluorescence units were read by a fluorescence plate reader (Cytofluor 2300, Millipore Corp. Bedford, USA) at 485 nm excitation and 530 nm emission wavelength. The IC_{50} (concentration of plant extract that reduced fluorescence intensity by 50%) was calculated according to Hills *et al.* (1986). For reference, tests with commercial drugs (pentamidine, suramin) were performed.

Cytotoxicity assay

WI-38 (human fibroblastoid) cells were seeded in 96-well microtitre plates at a density of 2.2×10^5 cells/ml in 50 μl MEM per well supplemented with 10% heat-inactivated FBS (fetal bovine serum). A threefold serial dilution ranging from 500 to 0.07 μg/ml of crude extract in 50 μl test medium was added. Plates with a final volume of 100 μl/well were incubated as described for testing of antitrypanosomal activity. After 66 hours, the MTC (maximum tolerated concentration) was determined microscopically. The MTC was defined as the highest concentration of crude plant extract which did not affect growth of WI-38 cells. Selectivity indices (SI) were then calculated by dividing MTC for WI-38 cells by MIC for *T. b. rhodesiense* (Kaminsky *et al.* 1996).

Results

The botanical names of the plants used by traditional healers and investigated in our study are listed in Table 1. Information on traditional methods for preparing extracts of some of these plants was obtained from healers. The common way of administering the extracts is by the oral route, except for *Lantana camara* extracts which are applied topically (Table 1).

Antitrypanosomal activities of plant extracts are presented in Table 2. Out of a total of 72 extracts tested, 23 from 8 plants (32%) were active with MIC values $\leqslant$ 19 μg/ml, 18 (25%) showed moderate activity with MIC values around 56 μg/ml and 31 (43%) showed no activity with MIC values $\geqslant$ 167 μg/ml.

The 23 active extracts were further tested to measure the IC_{50} value. Table 3 shows the IC_{50} values for the 23 most active extracts. Of the 9 plants investigated 8 showed at least one active

Table 1 Plants used by healers to treat sleeping sickness in Uganda and their traditional way of preparing extracts

Family	Species	Plant parts	Way of preparing extracts, application and dose
Boraginaceae	*Ehretia amoena* Klotsch.	leaf	not known, oral
Capparidaceae	*Capparis elaeagnoides* Gilg.	root	cold water infusion, one spoonful at a time orally according to the condition
Compositae	*Vernonia auriculifera* Hiern.	root	cold water infusion, oral administration when the patient is feverish
	Vernonia subuligera O. Hoffm.	root	not known, oral
Clusiaceae	*Garcinia huillensis* Oliv.	root and bark	boiled water extract, oral administration when the disease is in the advanced stage
Euphorbiaceae	*Securinega virosa* Baill.	root	powder from pounded root, one spoonful at a time orally according to the condition
Fabaceae	*Entada abyssinica* Stud. ex A. Rich.	root	boiled water extract, oral administration when the patient has lost appetite, has little energy, is feverish and finds it difficult to urinate
Mimosaceae	*Albizia gummifera* C. A. Smith	root	not known, oral
Verbenaceae	*Lantana camara* L.	leaf	topical application immediately after the bite around the area of the tsetse bite

extract with an IC_{50} value below 10 μg/ml. For *Capparis elaeagnoides* no activity could be determined. Highest activities with IC_{50} values below 1 μg/ml were found in 8 of the 23 active extracts. The lowest IC_{50} value was assessed for the dichloromethane rootbark extract of *Albizia gummifera*, with 70 ng/ml.

MTC values were obtained for the active extracts and used for the calculation of selectivity indices (SI) (Table 3). The best index was that for the petroleum ether root extract of *Securinega virosa* with more than 20-fold, followed by the petroleum ether rootbark extract of *Vernonia subuligera* with 17.3-fold and the petroleum ether root extract of *Entada abyssinica* with 13.6-fold. All other extracts had SI values equal to or below tenfold and have to be considered more or less cytotoxic. For comparison, commercially available drugs have significantly higher SI values (e.g. 1920-fold for suramin).

Discussion

The results of the present investigation provided evidence for the importance of ethnobotany as a guide for the selection of biologically active plant material and confirmed the efficacy of plants traditionally used to treat sleeping sickness in Uganda. Among the 9 plants investigated, only one plant lacked *in vitro* antitrypanosomal activity, namely *Capparis elaeagnoides*, whereas from all other plants investigated at least one extract revealed activity below 10 μg/ml. Significant activity with IC_{50} values below 10 μg/ml was found in one-third of all extracts tested, and 8 extracts even showed highest activity with IC_{50} values below 1 μg/ml.

The most active plant in our investigation was *Albizia gummifera* with an IC_{50} value of 70 ng/ml for the dichloromethane rootbark extract. So far, neither chemical nor toxicological investigations of this plant have been undertaken nor have extracts been tested for activity against trypanosomes. The marked activity and the lack of chemical data call for further phytochemical studies on this plant.

Another interesting plant was *Entada abyssinica*. Several extracts of different polarities and various parts of this plant showed antitrypanosomal activity. Interpretation of this finding is difficult and requires bioassay-guided fractionation and chemical investigation.

Lantana camara showed good antitrypanosomal activity with an IC_{50} value of 1.4 μg/ml. Interestingly, this plant is used only topically in traditional medicine. This might be explained by the highly toxic principles found in this chemically well investigated plant (Sharma *et al.* 1988; Sharma & Sharma 1989).

Table 2 Antitrypanosomal activity of plant extracts

Plant	Origin[1], month of collection	Part[2]	Extract[3] PE	CH_2Cl_2	MeOH	H_2O	H_2Otra.
Albizia gummifera	T, December	RB	+/−[4]	+	+	+	nd
Capparis elaeagnoides	U, December	RT	−	−	−	−	−
Ehretia amoena	T, December	RB	nd	+	+/−	+/−	+/−
		SB	nd	−	+	+/−	−
		LF	−	+	+/−	−	nd
	T, March	LF	−	+	nd	nd	nd
Entada abyssiniaca	U, December	RT	nd	−	+	−	+/−
	U, August	RT	+	+	+	−	+/−
	T, December	RB	+	+	+/−	−	+/−
		SB	−	+/−	+	+/−	+
Garcinia huillensis	U, December	RT	nd	+	−	−	−
Lantana camara	U, August	LF	+	+/−	+/−	−	nd
Securinega virosa	U, August	RT	+	+	+	−	nd
Vernonia auriculifera	U, December	RT	nd	+	+/−	−	−
Vernonia subuligera	T, December	RB	+	−	−	−	−
		SB	−	−	−	−	−
		LF	+/−	+	+/−	+/−	nd
Suramin			MIC: 111 ng/ml				
Pentamidine isethionate			MIC: 2 ng/ml				

[1] Origin: T, Tanzania; U, Uganda.

[2] Plant part: LF, leaf; RB, rootbark; RT, root; SB, stembark.

[3] Extract solvent: PE, petroleum ether; CH_2Cl_2, dichloromethane; MeOH, methanol; H_2O, distilled water; H_2Otra., decoction half an hour; nd: not determined.

[4] Activity: MIC $\geq$ 167 µg/ml: −; MIC = 56 µg/ml: +/−; MIC $\leq$ 19 µg/ml: +.

It was surprising to note that aqueous extracts prepared in the traditional way used by the healers showed no activity in our *in vitro* assays, whereas extracts prepared with organic solvents did show antitrypanosomal activity. This discrepancy may be due to a number of reasons. First, we cannot be certain that our preparation of the aqueous extracts was identical to the traditional preparation procedure; for example, the containers used by the traditional healer might contain traces of oil and, therefore, lipophilic compounds might also be extracted. Second, it has to be kept in mind that *in vitro* assays cannot detect active metabolites which may be responsible for efficacy in the *in vivo* situation. Extracts which lack *in vitro* activity may show antitrypanosomal activity after oral administration in an animal model where inactive precursor molecules may be broken down.

The ratio of cytotoxic/antitrypanosomal activity (selectivity index, SI) was found to be in a modest range for all crude plant extracts. The best SI, 21-fold, was determined for the petroleum ether root extract of *Securinega virosa*. Commercially available drugs have SIs over 1000. Since there is a lack of SI data of similar studies, the validity of these values remains unclear. However, bioassay-guided fractionation of plant extracts including cytotoxicity testing of fractions may provide further information and, therefore, might allow interpretation of previously given crude extract SI values.

Few reports exist on the plants investigated in the present study. Concerning other uses in traditional medicine, a limited number of reports exist on *Albizia gummifera*, *Ehretia amoena*, *Entada abyssinica*, *Garcinia huillensis*, *Lantana camara*, *Securinega virosa* and *Vernonia auriculifera* (Haerdi 1964; Watt & Breyer-Brandwijk 1962; Kokwaro 1976; Oliver-Bever 1986); however, sleeping sickness has not been among these documented

Table 3 Mean IC_{50} values of crude plant extracts with activity below 10 μg/ml, MTC values for WI-38 cells and the corresponding selectivity indices (SI)

Plant species	Part, extract[1]	IC_{50} value mean ± 1 SD (μg/ml)	MTC (μg/ml)	SI[2]
Albizia gummifera	RB, CH_2Cl_2	≤0.07 ± 0.03[3]	2	10
Albizia gummifera	RB, MeOH	0.2 ± 0.1	2	2.9
Albizia gummifera	RB, H_2O	1.7 ± 0.2	19	3.2
Ehretia amoena	RB, CH_2Cl_2	≤4.1 ± 2.4	19	1.4
Ehretia amoena	SB, MeOH	9.6 ± 3.0	112	7
Ehretia amoena (T, December)	LF, CH_2Cl_2	≤0.9 ± 0.2	56	2.1
Ehretia amoena (T, March)	LF, CH_2Cl_2	≤3.3 ± 1.1	56	0.6
Entada abyssinica (U, August)	RT, MeOH	3.3 ± 1.3	6	0.8
Entada abyssinica (U, December)	RT, MeOH	6.8 ± 4.4	4.7	0.2
Entada abyssinica (U, August)	RT, PE	≤0.4 ± 0.2	19	13.6
Entada abyssinica (U, August)	RT, CH_2Cl_2	≤0.5 ± 0.3	19	9.5
Entada abyssinica (T, December)	RB, PE	≤1.4 ± 1.2	12.5	2.6
Entada abyssinica (T, December)	RB, CH_2Cl_2	≤2.3 ± 1.6	6	0.6
Entada abyssinica (T, December)	SB, MeOH	1.3 ± 0.5	19	3.8
Entada abyssinica (T, December)	SB, H_2Otra	8.4 ± 6.2	19	0.6
Garcinia huillensis	RT, CH_2Cl_2	≤4.4 ± 2.5	6	0.1
Lantana camara	LF, PE	≤1.4 ± 0.3	19	9.5
Securinega virosa	RT, PE	≤0.5 ± 0.1	19	21.1
Securinega virosa	RT, CH_2Cl_2	≤2.1 ± 1.4	19	2.2
Securinega virosa	RT, MeOH	5.9 ± 4.6	19	0.8
Vernonia auriculifera	RT, CH_2Cl_2	9.4 ± 2.8	19	2.7
Vernonia subuligera	LF, CH_2Cl_2	≤0.6 ± 0.1	2	0.7
Vernonia subuligera	RB, PE	0.8 ± 0.4	19	17.3
Suramin		10.7 ± 0.2 ng/ml	220	1920
Pentamidine isethionate		0.4 ± 0.1 ng/ml		n.d.

[1] Abbreviations, see Table 2.
[2] SI, MTC for WI-38 cells/MIC for *T. b. rhodesiense*.
[3] ≤: extract not completely solved in culture medium.
n.d., not done.

indications. Moreover, no report exists on the highly trypanocidal plant *Vernonia subuligera*. Chemical constituents responsible for activity have not so far been determined. Therefore, phytochemical studies of some of the interesting plants identified in our study are needed.

From this brief study it seems clear that traditional healers do indeed possess a noteworthy knowledge of medicinal plants which should be acknowledged and considered in the search for novel compounds for the treatment of sleeping sickness. Further studies, including *in vivo* assays for the determination of efficacy and toxicity besides chemical investigation, are planned and will be carried out with some of the active plants.

Acknowledgements

We express our sincere thanks to L. B. Mwasumbi for the identification of Tanzanian plants, to H. Schneider for botanical support and C. Schmid and Y. Grether Bühler for technical support. Editorial suggestions and discussions by Jennifer Jenkins are highly appreciated. Financial support from the Rudolf-Geigy-Stiftung is gratefully acknowledged.

References

Anokbonggo WW (1992) The role of African traditional medicine in health-care delivery alongside modern medicine. In *Botany 2000: East and Central Africa.* (eds

S Edwards & Z Asfaw). NAPRECA Monograph Series No. 5, Addis Ababa, pp 25–35.

Baltz T, Baltz D, Giroud Ch & Crockett J (1985) Cultivation in semi-defined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense* and *T. gambiense*. *EMBO Journal* **4**, 1273–1277.

Brun R & Lun Z-R (1994) Drug sensitivity of Chinese *Trypanosoma evansi* and *T. equiperdum* isolates. *Veterinary Parasitology* **52**, 37–46.

Cox PA & Balick MJ (1994) The ethnobotanical approach to drug discovery. *Scientific American* **June**, 60–65.

Doua F & Yapo FB (1993) Human trypanosomiasis in the Ivory Coast—therapy and problems. *Acta Tropica* **54**, 163–168.

Farnsworth NR, Akerele O, Bingel AS, Soejarto DD & Guo Z (1985) Medicinal plants in therapy. *Bulletin of the World Health Organization* **63**, 965–981.

Haerdi F (1964) Die Eingeborenen-Heilpflanzen des Ulanga-Distriktes Tanganjikas (Ostafrika). In *Afrikanische Heilpflanzen* (eds A Bühler, R Geigy, A Gigon & F Meier). *Acta Tropica* (**Suppl. 8**).

Hills M, Hudson C & Smith PG (1986) Global monitoring of the resistance of malarial parasites to drugs: statistical treatment of micro-test data. Working paper No. 285 for informal consultation on the epidemiology of drug resistance of malaria parasites. World Health Organization, Geneva.

Kaminsky R, Schmid C & Brun R (1996) 'In vitro selectivity index' for evaluation of cytotoxicity of antitrypanosomal compounds. *In Vitro Toxicology* **9**, 315–324.

Kokwaro JO (1976) *Medicinal Plants of East Africa.* East African Literature Bureau, Kampala, Nairobi, Dar Es Salaam.

Kuzoe FAS (1993) Current situation of African trypanosomiasis. *Acta Tropica* **54**, 153–162.

Obexer W, Schmid C & Brun R (1995) A novel *in vitro* screening assay for trypanocidal activity using the fluorescent dye BCECF-AM. *Tropical Medicine and Parasitology* **46**, 45–48.

Oliver-Bever B (1986) *Medicinal Plants in Tropical West Africa.* Cambridge University Press, Cambridge, Massachusetts.

Sharma OP, Makkar HPS & Dawra RK (1988) A review of the noxious plant *Lantana camara*. *Toxicon* **26**, 975–987.

Sharma OP & Sharma PD (1989) Natural products of the lantana plant—the present and prospects. *Journal of Sci Ind Research* **48**, 471–478.

Watt JM & Breyer-Brandwijk MG (1962) *The Medicinal and Poisonous Plants of Southern and Eastern Africa.* Livingstone, Edinburgh.

WHO (1994) Control of tropical diseases: sleeping sickness. *Brochure of the World Health Organization*, Geneva.

