Trypanocidal and Cytotoxic Effects of 30 Ethiopian Medicinal Plants

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Trypanocidal and cytotoxic effects of traditionally used medicinal plants of Ethiopia were evaluated. A total of 60 crude plant extracts were prepared from 30 plant species using CH_2Cl_2 and MeOH. Effect upon cell proliferation by the extracts, for both bloodstream forms of *Trypanosoma brucei brucei* and human leukaemia HL-60 cells, was assessed using resazurin as vital stain. Of all CH_2Cl_2 and MeOH extracts evaluated against the trypanosomes, the CH_2Cl_2 extracts from five plants showed trypanocidal activity with an IC_{50} value below 20 μg/mL: *Dovyalis abyssinica* (Flacourtiaceae), IC_{50} = 1.4 μg/mL; *Albizia schimperiana* (Fabaceae), IC_{50} = 7.2 μg/mL; *Ocimum urticifolium* (Lamiaceae), IC_{50} = 14.0 μg/mL; *Acokanthera schimperi* (Apocynaceae), IC_{50} = 16.6 μg/mL; and *Chenopodium ambrosioides* (Chenopodiaceae), IC_{50} = 17.1 μg/mL. A pronounced and selective killing of trypanosomes with minimal toxic effect on human cells was exhibited by *Dovyalis abyssinica* (CH_2Cl_2 extract, SI = 125.0; MeOH extract, SI = 57.7) followed by *Albizia schimperiana* (CH_2Cl_2 extract, SI = 31.3) and *Ocimum urticifolium* (MeOH extract, SI = 16.0). In conclusion, the screening of 30 Ethiopian medicinal plants identified three species with good antitrypanosomal activities and low toxicity towards human cells. *Dovyalis abyssinica* might be a promising candidate for phytotherapy of trypanosomiasis.

Key words: In vitro Trypanocidal Activity, *Trypanosoma brucei brucei*, HL-60 Cells, Ethiopian Medicinal Plants

Introduction

Ethiopia is characterized by great physiogeographic variation that accentuated the diversity of plant and animal life (Abebe and Ayehu, 1993). The Ethiopian flora comprises more than 7000 species of higher plants, of which about 12% are endemic (Gebre Egziabher, 1991); about 715 species of plants have been documented to be used as medicinal drugs (Abebe *et al*., 2003). Medicinal plants are quite often employed in cultural and health care systems of many Ethiopians. Therefore, plants remain indispensable sources of preventive and curative traditional medicines for both human beings and livestock. Most of the time, medicinal plants comprise 87% of traditional medical preparations (Abebe and Ayehu, 1993; Abebe *et al*., 2003). Indeed, many controlled clinical studies have shown the therapeutic values of plant extracts used in phytotherapy (Wink, 2008). In modern medicine, however, a single compound is usually preferred for treatment of a particular disease. Nevertheless, there are times when a complex mixture of plant secondary metabolites is preferred to a single compound especially when therapeutic effect is additive or synergistic (Wink, 2008).

The African trypanosomes are protozoan blood parasites and mainly transmitted by tsetse flies of the genus *Glossina*. The diseases caused by these pathogenic protozoans are fatal and are called sleeping sickness in humans and nagana in domestic livestock (Brun *et al*., 2010). Human sleeping sickness is caused by two subspecies of *Trypanosoma brucei* namely: *T. b. gambiense* and *T. b. rhodesiense* (Brun *et al*., 2010). African animal trypanosomiasis is a disease complex caused by *T. congolense*, *T. vivax*, or *T. brucei brucei*, or a simultaneous infection with one or more of these trypanosomes. Both, human and animal trypanosomiases, negatively affect the whole economy of Africa by weakening both the health of humans and their domestic animals. Currently only seven trypanocidal drugs are available for the

treatment of both types of diseases. Except the recently (1990) introduced drug eflornithine (difluoromethylornithine), the other six drugs have been in use for more than 50 years. The appearance of drug-resistant trypanosomes, the toxicity of trypanocidal drugs to patients, unaffordability of the drugs, and lack of either their sustainable production or development of new trypanocidal drugs by pharmaceutical companies call for an urgent search for new, less or non-toxic, and affordable drugs.

In Africa and elsewhere in the world, natural products of plant origin are culturally accepted as alternative medicine and/or complementary medicine to the modern drugs. However, the problem with this type of traditional medicine in Africa and particularly in Ethiopia is that the traditional healers (herbalists) do not tell the composition or even the dose of the putative therapeutic preparation. Moreover, they pass their knowledge secretively to the most trusted and knowledgeable person of their own families. It is highly likely that indigenous knowledge of folk medicine might be lost. To mitigate the aforementioned problem, over the last three decades various scholars have documented the traditional medicinal uses of Ethiopian plants. But, only a few comprehensive pharmacological or biological studies have been carried out to validate the putative medicinal uses of these traditional medicines. Sometimes, the application of traditional medicinal plants involves a magico-religious approach which is not totally related to the causative agent or specific symptoms of diseases like trypanosomiasis. There is also little or no information on the toxicity of medicinal plants being used in the traditional medical practices.

The present study was thus designed with the primary objective to identify and validate 30 medicinal plants of Ethiopia as potential candidates for the treatment of trypanosomiasis. In addition, the present study was carried out to document the cytotoxicity of these plants against human leukaemia (HL-60) cells as new anticancer substances are also in need. A comparison of IC_{50} values of extracts on *T. b. brucei* with respect to their corresponding cytotoxicity to HL-60 cells can serve as a guide to the initial selection of extracts for early drug discovery or phytotherapy of trypanosomiasis.

Material and Methods

Reagents

Fetal bovine serum, MEM, and RPMI 1640 media were purchased from Invitrogen (Karlsruhe, Germany). Resazurin and diminazene aceturate were purchased from Sigma-Aldrich (Steinheim, Germany).

Plant materials

The plants were collected from different parts of Ethiopia at different times between December 20, 2007 and February 5, 2008 by one of us (E. N.) from their natural habitats. Plant samples were identified by Mr. Melaku Wondafrash (Addis Ababa University, Ethiopia) and deposited at National Herbarium, Addis Ababa University, Ethiopia and at Department of Biology, Institute of Pharmacy and Molecular Biotechnology, Heidelberg, Germany for further reference.

Preparation of plant crude extracts

The parts of each plant investigated in this study were ground and macerated in MeOH and CH_2Cl_2, respectively, and left on a shaker for two consecutive days. Extracts were then filtered and evaporated to dryness under reduced pressure using a rotary evaporator (Büchi, Labortechnik, Essen, Germany) at 45 °C.

Cell cultures

The human myeloid cell line HL-60 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). *Trypanosoma brucei brucei* TC221 bloodstream form was initially obtained from Prof. Peter Overath (Max-Planck Institut für Biologie, Tübingen, Germany) and was continuously maintained in our laboratory.

Bloodstream forms of *T. b. brucei* TC221 cells were grown in Baltz medium (Baltz *et al*., 1985) supplemented with 20% inactivated fetal bovine serum and 1% penicillin-streptomycin whereas HL-60 cells (human myeloid cell line) were grown in RPMI 1640 medium supplemented with 0.2 mM L-glutamine, 1% penicillin-streptomycin, and 10% heat-inactivated fetal bovine serum. Both cell types were incubated in a humified atmosphere containing 5% CO_2 at 37 °C.

Trypanocidal and cytotoxicity assays

The extracts and compounds were dissolved in dimethyl sulfoxide (DMSO). The extracts were

further serially diluted with the medium in a two-fold fashion into seven different concentrations so as to attain final concentrations ranging from 250 to 3.91 μg/mL in 96-well plates. Each concentration of the drug was tested in triplicate, each test was repeated twice. The solvent DMSO did not exceed 1.25% in the medium that contained the highest concentration of extract tested. Wells containing DMSO as well as wells without DMSO were included in the experiment.

Both *T. b. brucei* and HL-60 cells were seeded into 96 wells at a density of $1 \cdot 10^4$ cells per 100 μL of medium. The cells were incubated with the plant extracts for a total of 48 h. The antitrypanosomal and cytotoxic activities of extracts were evaluated using resazurin as cell proliferation indicator dye with some modifications after Rolón *et al*. (2006). Briefly, 10 μL and 6 μL of resazurin, respectively, were added to trypanosome and HL-60 cell cultures, and the mixtures were incubated for 24 h and 6 h, respectively, before measuring the 96-well plates after 48 h of incubation. The absorbance of the plates was read using a Tecan plate reader (Männedorf, Switzerland) at dual wavelengths of 492 nm and 595 nm. The concentration of extracts or reference drug at which the growth of cells was inhibited by 50% was calculated from a dose-response curve by linear interpolation taking two contents, above and below 50% (Huber and Koella, 1993).

Results

All the extracts tested against trypanosomes showed trypanocidal activities. It is remarkable that the extracts prepared from 19 plant species showed trypanocidal activity below an IC_{50} value of 50 μg/mL (Table I). Of all extracts evaluated against the trypanosomes, the CH_2Cl_2 extracts from five plants (*Acokanthera schimperi*, IC_{50} = 16.6 μg/mL; *Albizia schimperiana*, IC_{50} = 7.2 μg/mL; *Chenopodium ambrosioides*, IC_{50} = 17.1 μg/mL; *Dovyalis abyssinica*, IC_{50} = 1.4 μg/mL; *Ocimum urticifolium,* IC_{50} = 14.0 μg/mL) showed potent trypanocidal activity below an IC_{50} value of 20 μg/mL.

The crude MeOH and CH_2Cl_2 extracts also exhibited variable cytotoxic activities against human HL-60 cells. The CH_2Cl_2 extracts that exhibited cytotoxic activity below an IC_{50} value of 100 μg/mL include *Acokanthera schimperi* (IC_{50} = 28.8 μg/mL), *Ferula communis* (IC_{50} = 99.9 μg/mL), *Guizotia scabra* (IC_{50} = 25.5 μg/mL), *Millettia ferruginea* (IC_{50} = 87.5 μg/mL), *Rosa abyssinica* (IC_{50} = 58.7 μg/mL), *Vernonia amygdalina* (IC_{50} = 22.4 μg/mL), *Leonotis ocymifolia* (IC_{50} = 61.0 μg/mL), *Hagenia abyssinica* (IC_{50} = 32.3 μg/mL), and *Solanum incanum* (IC_{50} = 82.0 μg/mL). The selectivity index (SI) which is the ratio of cytotoxicity in HL-60 (IC_{50}) to that of *T. b. brucei* (IC_{50}) cells is also shown in Table I.

Discussion

In Ethiopia a number of plants are used for the treatment of protozoan infections and several other diseases. The present study explored diverse plants belonging to different families with the aim of finding plant extracts that might be candidates for the treatment of trypanosomiasis. Some of the plants (*Calpurnia aurea, Clausena anisata*, *Leonotis ocymifolia*, and *Withania somnifera*) are used traditionally for the treatment of diseases caused by trypanosomes and leishmania (Kinetoplastidae). The CH_2Cl_2 extracts prepared from three out of the four plants showed trypanocidal activity below an IC_{50} value of 50 μg/mL. The most potent and promising plants as sources of trypanocidal agents (in decreasing order of trypanocidal activity) are *Dovyalis abyssinica*, *Albizia schimperiana*, *Ocimum urticifolium*, *Acokanthera schimperi*, and *Chenopodium ambrosioides*.

The CH_2Cl_2 extract of *Dovyalis abyssinica* leaves was the most active trypanocidal agent with a high selectivity index (SI = 125.0). *D. abyssinica* contains dovyalicin-type spermidine alkaloids (Rasmussen *et al*., 2006), which in part resemble the chemical structures of standard drugs (*e.g.* pentamidine), and therefore these compounds may be in part responsible for the biological activity of the plant. The second promising plant, *Albizia schimperiana*, contains spermine alkaloids as one of its active principles (Rukunga and Waterman, 1996). *Ocimum urticifolium* contains eugenol as one of main active compounds in its essential oil (Chogo and Crank, 1981). *Acokanthera schimperi* contains the cardiac glycoside ouabain as one of its active principles (Wink and van Wyk, 2008). *Chenopodium ambrosioides* is also a promising plant as a possible source of trypanocidal agents. The trypanocidal effect of *C. ambrosioides* may be due to the major and anthelminthic compound ascaridole (Kiuchi *et al*., 2002).

Looking at the cytotoxicity of extracts against human leukaemia cells (HL-60), MeOH and CH_2Cl_2 extracts from *Acokanthera schimperi*,

Table I. Antitrypanosomal and cytotoxic activities of Ethiopian medicinal plants.

Plant species	Plant part	Extraction solvent	IC_{50} [μg/mL]		Selectivity index (SI)
			T. b. brucei	HL-60	
Acanthaceae					
Justicia schimperiana	Flowers	MeOH	147.5	219.8	1.5
		CH_2Cl_2	46.2	135.6	2.9
Apiaceae					
Ferula communis	Aerial part	MeOH	118.8	236.6	2.0
		CH_2Cl_2	74.0	99.9	1.4
Apocynaceae					
Acokanthera schimperi	Leaves	MeOH	52.1	7.1	0.1
		CH_2Cl_2	16.6	28.8	1.7
Asteraceae					
Guizotia scabra	Flowers	MeOH	54.0	246.8	4.6
		CH_2Cl_2	34.0	25.5	0.8
Vernonia amygdalina	Aerial part	MeOH	80.2	158.9	2.0
		CH_2Cl_2	105.0	22.4	0.2
Vernonia hochstetteri	Flowers	MeOH	103.1	230.2	2.2
		CH_2Cl_2	86.5	140.9	1.6
Boraginaceae					
Cordia monoica	Leaves	MeOH	116.3	53.2	0.5
		CH_2Cl_2	100.0	219.9	2.2
Cordia sinensis	Leaves	MeOH	81.3	169.3	2.1
		CH_2Cl_2	113.2	206.4	1.8
Chenopodiaceae					
Chenopodium ambrosioides	Aerial part	MeOH	38.5	44.8	1.2
		CH_2Cl_2	17.1	219.0	12.8
Combretaceae					
Combretum molle	Bark	MeOH	48.5	>250.0	> 5.2
		CH_2Cl_2	44.0	>250.0	> 5.7
Ebenaceae					
Euclea divinorum	Leaves	MeOH	88.7	>250.0	>2.8
		CH_2Cl_2	29.4	187.7	6.4
Euphorbiaceae					
Croton macrostachyu	Aerial part	MeOH	49.3	108.2	2.2
		CH_2Cl_2	50.8	150.8	3.0
Fabaceae					
Albizia schimperiana	Leaves	MeOH	19.6	184.1	9.4
		CH_2Cl_2	7.2	225.6	31.3
Calpurnia aurea	Leaves	MeOH	85.2	147.5	1.7
		CH_2Cl_2	96.0	244.3	2.5
Millettia ferruginea	Aerial part	MeOH	49.2	248.4	5.1
		CH_2Cl_2	44.0	87.5	2.0
Flacourtiaceae					
Dovyalis abyssinica	Leaves	MeOH	2.9	167.2	57.7
		CH_2Cl_2	1.4	174.9	125.0
Lamiaceae					
Leonotis ocymifolia	Aerial part	MeOH	94.7	207.9	2.2
		CH_2Cl_2	47.2	61.0	1.3
Ocimum urticifolium	Leaves	MeOH	14.5	231.6	16.0
		CH_2Cl_2	14.0	156.2	11.2
Meliaceae					
Ekebergia capensis	Leaves	MeOH	56.4	186.8	3.3
		CH_2Cl_2	69.6	179.5	2.6

Table I continued.

Plant species	Plant part	Extraction solvent	IC_{50} [μg/mL]		Selectivity index (SI)
			T. b. brucei	HL-60	
Myrtaceae					
Syzygium guineense	Leaves	MeOH	46.6	>250.0	>5.4
		CH_2Cl_2	23.5	119.8	5.2
Polygonaceae					
Rumex nepalensis	Leaves	MeOH	104.5	>250.0	>2.4
		CH_2Cl_2	87.1	222.0	2.6
Rosaceae					
Hagenia abyssinica	Female flowers	MeOH	52.3	196.6	3.8
		CH_2Cl_2	22.9	32.3	1.4
Rosa abyssinica	Leaves	MeOH	38.7	153.3	4.0
		CH_2Cl_2	38.7	58.7	1.5
Rubiaceae					
Pavetta gardeniifolia	Leaves	MeOH	96.7	>250.0	>2.6
		CH_2Cl_2	23.8	133.7	5.6
Rutaceae					
Clausena anisata	Aerial part	MeOH	92.2	118.5	1.3
		CH_2Cl_2	49.8	225.4	4.5
Solanaceae					
Datura stramonium	Leaves	MeOH	72.9	120.4	1.7
		CH_2Cl_2	45.4	106.4	2.3
Solanum incanum	Leaves	MeOH	70.1	227.2	3.2
		CH_2Cl_2	21.0	82.0	3.9
Withania somnifera	Aerial part	MeOH	38.7	221.5	5.7
		CH_2Cl_2	39.5	187.1	4.7
Verbenaceae					
Lippia adoensis	Aerial part	MeOH	85.7	>250.0	>2.9
		CH_2Cl_2	nt	nt	nt
Verbena officinalis	Whole part	MeOH	87.3	225.6	2.6
		CH_2Cl_2	22.6	175.8	7.8
Standard drug					
Diminazene aceturate			0.09	>128.9	>1432.0

nt, not tested.
Results represent the mean of values obtained in two independent experiments, which did not differ by more than 10%.

CH_2Cl_2 extracts from *Guizotia scabra* and *Vernonia amygdalina* are very potent as source of active agents or serve as crude drugs for the treatment of leukaemia. The extracts should, however, be further tested on a primary cell culture or in an experimental animal model to validate their efficacy.

In conclusion, it might be rewarding to study the most active plants (*Dovyalis abyssinica*, *Albizia schimperiana*, *Ocimum urticifolium*, *Acokanthera schimperi*, and *Chenopodium ambrosioides*) in more detail for their exploitation in *in vivo* treatment of trypanosomiasis. The active constituents of the plants should also be isolated and optimized if they could either serve as drugs or lead structures for the development of new and more effective trypanocidal drugs. In our laboratory, isolation of total alkaloid extracts from the most active plants such as *Dovyalis abyssinica* is being pursued for the treatment of trypanosomiasis.

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