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Preliminary *in vivo* antitrypanosomal activity and cytotoxicity of *Entada abyssinica, Securinega virosa* and *Ehretia amoena*

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***Entada abyssinica, Securinega virosa* and *Ehretia amoena* are traditionally used in Southern Uganda to treat Human African Trypanosomiasis (HAT). Extracts from the roots and root barks of *E. abyssinica, S. virosa* and *E. amoena* were investigated for *in vivo* antitrypanosomal activity against *Trypanosoma brucei brucei* in mice. The extracts were also tested *in vitro* for potential cytotoxicity against a 3-cell line panel consisting of TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells using the highly sensitive Sulforhodamine B assay. The ethanolic extract from the roots of *E. abyssinica* showed a reduction of *T. b. brucei* parasitaemia at a single dose of 400 mg/kg. The petroleum ether extract from the root bark of *E. abyssinica* showed the highest cytotoxicity with GI_{50}, total growth inhibition, LC_{50} and LC_{100} values of less than 6.25 µg/ml. Our findings confirm earlier *in vitro* antitrypanosomal studies and lend credence to the traditional use of these plants against trypanosomiasis.**

Key words: Medicinal plants, antitrypanosomal activity, anticancer activity, *in vivo* assay, SRB assay, bioactive components

INTRODUCTION

Human African trypanosomiasis (HAT) is caused by the haemoflagellates *Trypanosoma brucei gambiense* in West and Central Africa, and *Trypanosoma brucei rhodesiense* in Eastern Africa [1–3]. HAT is a major public health problem in 36 sub-Saharan African countries which threatens over 60 million lives on a daily basis [1,3]. The current treatment of the disease relies on chemotherapy using suramin, pentamidine, melarsoprol and eflornithine [3]. Most of these drugs display undesirable toxic adverse effects and studies have demonstrated the emergence of drug-resistant trypanosomes against the drugs [4,5]. Therefore, the development of safe and cost-effective new drugs for the treatment of African trypanosomiasis is urgently required in order to control the disease.

Recent discoveries of active antitrypanosomal plant extracts have highlighted the potential for plants to provide lead compounds for the development of new drugs effective against trypanosomiasis [4,6].

Entada abyssinica (Leguminosae), *Securinega virosa* (Euphorbiaceae) and *Ehretia amoena* (Boraginaceae) are plant species used traditionally in southern Uganda to treat human trypanosomiasis [7]. The aqueous concoctions made from the roots of these plant species are used in the management of HAT. To the best of our knowledge, the *in vivo* activities of these concoctions have so far not been reported. In a study by Freiburghaus and co-workers, water, methanolic and lipophilic extracts of *E. abyssinica, S. virosa* and *E. amoena* collected in Uganda and Tanzania were tested *in vitro* against *Trypanosoma brucei rhodesiense* and only the lipophilic extracts exhibited promising *in vitro* activity against *T. b. rhodesiense* (IC_{50} < 1 µg/ml) [7]. Both *in vitro* and *in vivo* studies are important for the determination of efficacy in the development of novel, safe and environmentally friendly antitrypanosomal products.

The objective of the current study was to explore the *in vivo* antitrypanosomal activity of the petroleum ether and ethanolic extracts of *E.*

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abyssinica, S. virosa and *E. amoena* against *T. b. brucei* and to determine the cytotoxicity of various extracts from the three plant species.

MATERIALS AND METHODS

Plant materials

Leaves and root barks *E. abyssinica* were collected from Chalinze (Coast region) whereas *S. virosa* and *E. amoena* leaves, stem barks, roots and rootbarks were collected from Pugu forest in Dar es Salaam region, Tanzania, in April 2013. The plants were identified and authenticated by Haji Selemani, a taxonomist at the Herbarium in the Department of Botany, University of Dar es Salaam. Voucher specimens were deposited at the same place.

Extraction: The leaves, stem/stem barks and roots/root barks of *E. abyssinica, S. virosa* and *E. amoena* were air-dried in the shade, ground and separately soaked at room temperature for two days in petroleum ether and ethanol. The crude extracts were concentrated *in vacuo* using a rotary evaporator and stored in the refrigerator at 4 °C for further bioassays.

Animals

Male and female albino mice ranging between 17 – 24 g in weight were obtained from the animal house at Muhimbili University of Health and Allied Sciences (MUHAS). The mice were maintained in metal cages (38 × 25 × 17 cm) containing at most 5 mice per cage in a well ventilated room. The animals were supplied with broiler finisher feed and treated to tap water *ad libitum*. They were acclimatized to laboratory conditions for three days before they were used in the experiments.

Ethical considerations

The study was conducted according to the Muhimbili University of Health and Allied Sciences (MUHAS) Research Policy Guidelines of August, 2011 and internationally accepted principles for laboratory animal use and care including the 2003 AAALAC International guidelines and the 2011 Guide for the Care and Use of Laboratory Animals guidelines [13,14]. Ethical clearance was obtained from the Senate Research and Publications Committee of the MUHAS.

Parasites

The population of trypanosomes used in this study were *T.b. brucei* IL4159 (I1tat1.1) blood stabilates (non resistant lab strains) initially derived from *T.b. brucei* IL3303 (I1tat1.1) from the International Livestock Research Institute (ILRI), Kenya, in January 1999. The trypanosomes were kept under liquid nitrogen at Temeke Veterinary Center in Dar es Salaam.

Parasites resuscitation: A sample of *T.b. brucei* IL4159 (I1tat1.1) blood stabilate was resuscitated by mixing with 0.4 ml of phosphate-buffered saline-glucose (PSG) solution (pH 8, ionic strength 0.145, Batch No. 401 ILRI) in a 1 ml syringe. The presence of viable *T.b. brucei* was ascertained under a light microscope at ×250 magnification. The trypanosome population for the experiment was obtained from 15 mice infected by injecting intraperitoneally (i.p) the resuscitated parasites in 0.2 mL of PSG and maintained by passaging. Passaging was performed in six mice (three passages sequentially using two mice each time) prior to the start of the experiment.

In vivo antitrypanosomal activity of plant extracts

In vivo antitrypanosomal activities of the crude extracts from *E. abyssinica, S. virosa* and *E. amoena* were performed as reported elsewhere [4,8]. *T.b. brucei* parasites were collected from the tail blood of infected donor mice. Dilutions were made using normal saline until 1-10 trypanosomes (estimated to be 10^3–10^4 trypanosomes per millilitre of blood) were seen under the microscope (×40 objective) (Table 1). About 0.1 ml of diluted blood was injected intraperitoneally into experimental mice. A single experiment included a total of 15 mice, divided equally into 5 groups. The five groups, each containing mice labelled M1, M2 and M3, were housed in different metal cages. Treatment was carried out immediately after parasitaemia detection on day 3 post infection. Groups 1 and 2 were infected and administered orally with single doses of 300 mg/kg and 400 mg/kg body weight of extract, respectively [4,8].

Group 3 was infected but not treated and acted as the negative control. Group 4 was infected and administered with normal saline (the solvent used to reconstitute the extract) and acted as the solvent control. Group 5 was infected and administered intramascularly with the conventional drug, diminazene aceturate (3.5 mg/kg) and acted as the positive control. The dose volume of extracts used was 5 ml/kg body weight. Parasitaemia was checked every 24 hours by taking a drop of tail blood on a microscope slide and examining it at ×10 objective. Once parasitaemia was detected, parasites were counted at ×40 objective. Interpretation of the results was based on established procedures [8] as shown in Table 1.

Table 1. Estimation of number of trypanosomes per millilitre of blood from microscopic field counts

Trypanosomes per field	Estimated parasitaemia (Trypanosomes/ml)
>100	$>5 \times 10^6$
>10	$>5 \times 10^5$
1 – 10	$10^4 – 5 \times 10^5$
1 per 2 fields – 1 per 10 fields	$5 \times 10^3 – 5 \times 10^4$
1 – 10 per preparation	$10^3 – 10^4$
1 per preparation	$10^2 - 10^3$

SRB cytotoxicity assay

The SRB assay is a colorimetric assay developed to measure drug-induced cytotoxicity and cell proliferation of various human tumour cell lines *in vitro* [9,10]. The assay is similar to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay but comes at a lower cost, gives better linearity and higher sensitivity and has a stable end point that does not require time sensitive measurement [11,12]. The SRB assay was performed at the Council for Scientific and Industrial Research (CSIR), Pretoria, South Africa in accordance with the protocol of the Drug Evaluation Branch of the National Cancer Institute (NCI). The human cell lines TK10 (renal), UACC62 (melanoma) and MCF7 (breast) were obtained from NCI in the framework of a collaborative research program between CSIR and NCI. Cell lines were routinely maintained as a monolayer cell culture at 37 ºC, 5% CO_2, 95% air and 100% relative humidity in a general purpose growth media, RPMI, containing 5% fetal bovine serum, 2 mM L-glutamine and 50 µg/ml gentamicin. For the cytotoxicity assay, cells (3-19 passages) were inoculated in 96-well microtitre plates at plating densities of 7×10^3 - 10×10^3 cells/well and incubated for 24 h. One plate was fixed with TCA to represent the measurement of the cell population for each cell line at the time of extract addition (T_0). The rest of the plates containing cells were treated with the plant extracts previously dissolved in dimethyl sulfoxide (DMSO) and diluted in medium to produce 5 concentrations (6.25-100 µg/mL or 0.01-100 µM).

Wells with no extracts added served as negative controls (C) while wells containing the medium but no cells acted as blanks. Etoposide was used as a reference standard. Plates were incubated for 48 h after the addition of the extracts or reference drug. Thereafter, viable cells were fixed to the bottom of each well with cold 50% TCA, washed, dried and dyed with SRB. Unbound dye was removed and protein-bound dye was extracted with 10 mM Tris base for optical density determination at 540 nm using a multi-well spectrophotometer. Optical density measurements were used to calculate the percentage cell growth according to Equations 1 and 2.

$$\left[\frac{(T_i - T_0)}{(C - T_0)}\right] \times 100 \text{ for } T_i \geq T_0 \qquad \text{Equation 1}$$

$$\left[\frac{(T_i - T_0)}{T_0}\right] \times 100 \text{ for } T_i < T_0 \qquad \text{Equation 2}$$

Where T_i is the optical density of the test well after 48 h period of exposure to extracts, T_0 is the optical density at time zero and C is the control optical density.

The results were reported as total growth inhibition (TGI), the concentration of plant extracts at which the solutions to the two equations have a value of 0 and signifies a cytostatic effect. The biological activities of the extracts were grouped into 4 categories: inactive (TGI >50 µg/ml), weak activity (15 µg/ml < TGI <50 µg/ml), moderate activity (6.25 µg/ml < TGI <15 µg/ml) and potent activity (TGI <6.25 µg/ml).

RESULTS AND DISCUSSION

Antitrypanosomal activities of *E. abyssinica, S. virosa* and *E. amoena*

The ethanolic extract from the roots of *E. abyssinica* showed reduction in parasitaemia from day 4 to day 7 post treatment at a single 400 mg/kg body weight dose (Group 2). This was followed by an increase in parasitaemia resulting in the death of experimental mice by day 15 post treatment (Table 2). Mice in group 1 showed increase in parasitaemia from day 3 post infection and died by day 8. Similarly, mice in the control groups 3 (infected but not treated) and 4 (solvent control) showed consistent increase in parasitaemia killing the mice by day 8. Ethanolic extracts from the root barks of *E. amoena* and the roots of *S. virosa* as well as the petroleum ether extracts from the roots of all the three plant species had no antitrypanosomal activity.

Table 2. Antitrypanosomal activity of the *E. abyssinica* ethanolic root extract against *T. b. brucei*; Counts of swarming trypanosomes on a microscope slide.

PTD	Group 1			Group 2			Group 3			Group 4			Group 5		
	M1	M2	M3	M1	M2	M3	M1	M2	M3	M1	M2	M3	M1	M2	M3
3	5/p	18/p	1	5/p	2/p	1/p	13/p	5/p	18/p	15/p	5/p	2/p	15/p	6/p	5/p
4	18/p	2	3	12/p	13/p	18/p	1	15/p	6	1	15/p	1	18/p	0	0
5	1	48	1	10/p	0	10/p	471	3	98	28	1	33	0	0	0
6	45	89	0	0	0	0	00	68	D	78	55	61	0	0	0
	>10	>10						>10		>10	>10	>1			
7	0	0	1	0	0	0	D	0		0	0	00	0	0	0
8	D	D	63	0	1/p	7/p		D		D	D	D	0	0	0
			>10												
9			0	13/p	15/p	1							0	0	0
10			D	1	2/p	37							0	0	0
						>10									
11				32	13/p	0							0	0	0
				>10											
12				0	1	D							0	0	0
13				D	67								0	0	0
					>10										
14					0								0	0	0
15					D								0	0	0

PTD = post treatment days; p = preparation (whole sample on a microscope slide); D = Death

Group 1: Infected and administered orally with single doses of 300 mg/kg body weight of extract.

Group 2: Infected and administered orally with single doses of 400 mg/Kg body weight of extract.

Group 3: Infected with parasites but was not treated and this acted as a negative control.

Group 4: Infected and administered with normal saline and acted as a solvent control.

Group 5: Infected and administered intramascularly with the conventional drug, diminazine aceturate (3.5 mg/Kg) and acted as a positive control.

Cytotoxic activities of the extracts of *E. abyssinica, S. virosa* and *E. amoena*

The growth inhibitory effects of the extracts from different parts of *E. abyssinica*, *S. virosa* and *E. amoena* plant species were tested against the 3-cell line panel consisting of TK10, UACC62 and MCF7 cancer cells in the SRB assay. The results for four parameters, TGI, GI_{50}, LC_{50} and LC_{100} calculated for the three cell lines at different concentrations of crude extracts are summarised in Tables 3 and 4. According to the CSIR criteria [15], the samples are considered as inactive if TGI values for at least two cell lines are higher than 50 µg/ml and potent if the values are less than 6.25 µg/ml. The petroleum ether extract from the root bark of *E. abyssinica* (EAbPR) was the most cytotoxic against the three cell lines with a TGI value of <6.25 µg/ml for all cells (Table 3). The EAbPR was more potent than etoposide, the positive control (Figure 1). The calculated GI_{50}, LC_{50} and LC_{100} values for this extract against the three cell lines were all <6.25 µg/ml (Table 4). The petroleum ether and ethanolic extracts from the stem bark of *S. virosa* exhibited weak cytotoxic activity (TGI = 21.91 - 74.84 µg/ml) against the cancer cell lines (Table 3). Equally weak activity was observed with the ethanolic extract from the roots of *S. virosa* and the ethanolic extract from the leaves of *E. amoena*. The petroleum ether and ethanolic extracts from the rootbark of *E. amoena* did not show any cytotoxic activity (Tables 3 and 4).

Table 3. Total growth inhibition of the extracts from different parts of *E. abyssinica, S. virosa and E. amoena*

Species	Plant tissue	Extract	TGI, µg/mL			Conclusion
			TK-10	UACC-62	MCF-7	
E. abyssinica	Leaf	Petroleum ether	>100	>100	82.97	Inactive
		Ethanol	77.30	72.20	>100	Inactive
	Rootbark	Petroleum ether	<6.25	<6.25	<6.25	Potent
		Ethanol	53.67	72.81	>100	Inactive
S. virosa	Leaf	Petroleum ether	>100	>100	>100	Inactive
		Ethanol	>100	>100	>100	Inactive
	Twigs	Petroleum ether	>100	82.72	>100	Inactive
		Ethanol	>100	65.52	55.03	Inactive
	Stem bark	Petroleum ether	45.56	60.82	21.91	Weak
		Ethanol	74.84	47.71	39.57	Weak
	Root	Petroleum ether	>100	>100	>100	Inactive
		Ethanol	63.98	45.05	30.23	Weak
E. amoena	Leaf	Petroleum ether	61.08	75.15	58.03	Inactive
		Ethanol	44.78	53.17	33.09	Weak
	Rootbark	Petroleum ether	>100	>100	>100	Inactive
		Ethanol	>100	>100	>100	Inactive
		Etoposide	**20.69**	**29.22**	**>100**	

Table 4. Cytotoxicity of *E. abyssinica, S. virosa* and *E. amoena* on the three cell lines

Plant Species	Activities	TK10, µg/mL	UACC62, µg/mL	MCF7, µg/mL
E. abyssinica – Root	**GI_{50}**	<6.25	<6.25	<6.25
	TGI	<6.25	<6.25	<6.25
	LC_{50}	<6.25	<6.25	<6.25
	LC_{100}	<6.25	<6.25	<6.25
S. virosa - Root	**GI_{50}**	27.31	21.79	17.96

	TGI	63.98	45.05	30.23
	LC_{50}	99.38	76.89	47.16
	LC_{100}	>100	>100	>100
E. amoena - *Rootbark*	**GI_{50}**	9.25	13.00	10.02
	TGI	22.32	26.46	23.40
	LC_{50}	53.94	40.33	86.07
	LC_{100}	>100	>100	>100
Etoposide (Standard)	**GI_{50}**	<6.25	<6.25	<6.25
	TGI	20.69	29.22	>100
	LC_{50}	87.83	>100	>100
	LC_{100}	>100	>100	>100

The observed reduction in parasitaemia of the *E. abyssinica* ethanolic root extract is in agreement with earlier *in vitro* studies that suggest the presence of active phytochemicals against trypanosomiasis [7]. Freiburghaus and coworkers reported the isolation of a diastereoisomer of the clerodane type diterpene (kolavenol) from the dichloromethane extract of the root bark of *E. abyssinica*. This compound showed trypanocidal activity against *T. b. rhodesiense* bloodstream trypomastigotes (IC_{50} 8.60 µM) [16,17]. Elsewhere, a kolavic acid derivative has been isolated from the stem bark of *E. abyssinica* and found to be active against the bloodstream form of *T. b. brucei* (IC_{50} 0.012 mM) [18]. Thus, the observed *in vivo* antitrypanosomal activity could be associated with the presence of kolavenol and/or kolavic acid derivatives in the roots.

The ethanolic crude extract from the *E. amoena* root barks and *S. virosa* roots had no *in vivo* antitrypanosomal activity contrary to the *in vitro* results reported previously [7]. This finding does not support the use of these plants for the management of trypanosomiasis by traditional practitioners. The observed lack of *in vivo* antitrypanosomal activity may be attributed to metabolic inactivation of the active phytoconstituents in the animal model or poor absorption.

Cytotoxicity assays of *E. abyssinica, S. virosa* and *E. amoena* crude extracts were previously investigated against WI-38 (human fibroblastoid) cells using the maximum tolerated concentration (MTC) [7]. This was defined as the highest concentration of the crude plant extract which did not affect the growth of WI-38 cells [7]. However, the results were interpreted in terms of selectivity indices (SI) calculated from MTC/MIC for *T. b. rhodesiense*. Based on the SI categorization, *S. virosa* had the highest SI followed by *E. abyssinica*. Extracts from *E. amoena* had the lowest SI [7]. The results remain unclear to date due to lack of SI data of similar studies.

The petroleum ether extract from the root of *E. abyssinica* indicated potent cytotoxic activity and this extract should be considered as a good candidate for further activity-guided fractionation to identify the bioactive components. Although extracts from *S. virosa* and *E. amoena* showed weak activity, further activity-guided fractionation is recommended.

CONCLUSION

This study has shown that *E. abyssinica* possesses in vivo antitrypanosomal activity. This finding supports the ethnopharmacological approach in the search for safe and novel antitrypanosomal agents as well as other biologically active compounds. Since traditional healers use aqueous decoctions, *in vivo* antitrypanosomal activities of aqueous extracts from these plant species should be carried out to determine the efficacy of the decoctions. Multi-dose administration and bioassay-guided fractionation and purification of the *E. abyssinica* extracts to obtain pure compounds will be carried out in the future experiments in the framework of the project. Antitrypanosomal activity study of the plant species collected during different seasons of the year is also planned for. Cytotoxicity studies of the crude extracts uisng the SRB assay revealed that the

petroleum ether extract from the root bark of *E. abyssinica* could contain potential anticancer compounds. Further activity-guided fractionation and isolation of pure compounds with anticancer activity from this extract and from the other crude extracts that showed appreciable cytotoxic activity is recommended.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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