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Research Paper

Antitrypanosomal and cytotoxic activities of pyrrolizidine alkaloid-producing plants of Ethiopia

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Abstract

Objectives The objective was to determine the in-vitro effect of extracts from 19 Ethiopian plant species and four pure pyrrolizidine alkaloids on bloodstream forms of *Trypanosoma brucei brucei* and human leukaemia HL-60 cells.

Methods Crude plant extracts were prepared using methanol and dichloromethane. The alkaloidal extracts from *Solanecio angulatus* flowers were prepared with and without zinc reduction using the acid–base extraction method. Cell proliferation inhibitory activity of the extracts and compounds was assessed using Alamarblue.

Key findings The most active extract was the dichloromethane extract of *Solanecio angulatus* flowers, with an IC50 value of 12.17 μg/ml. The best selectivity index (SI > 41.08) was obtained for the same extract determined with HL-60 cells. The reduced alkaloidal extract prepared from *S. angulatus* flowers and after acid–base extraction showed more antitrypanosomal activity than unreduced alkaloidal extract with an IC50 value of 14.35 μg/ml and with a selectivity index of 12.23.

The second most active extract was the dichloromethane extract of *Crotalaria phillipsiae* twigs with an IC50 value of 12.67 μg/ml and a selectivity index of 34.35. Most of the other extracts tested showed moderate antitrypanosomal activities to variable extents. Among the four pure pyrrolizidine alkaloids tested, senecionine showed moderate antitrypanosomal activity with an IC50 value of 41.78 μg/ml.

Conclusions *Solanecio angulatus* (flowers) and *Crotalaria phillipsiae* (twigs) could serve as sources of novel trypanocidal compounds for the treatment of trypanosomiasis.

Keywords Ethiopia; *in vitro*; HL-60 cells; medicinal plants; pyrrolizidine alkaloids; *Trypanosoma brucei brucei*

Introduction

The African trypanosomiases are fatal diseases, commonly known as sleeping sickness in humans and nagana in domestic livestock. The causative agents are the protozoan parasites of the genus *Trypanosoma,* which are transmitted by bites of tsetse flies (*Glossina morsitans*).[1] Ethiopia, situated near the northeast limit of the tsetse fly belt area, is affected by African animal trypanosomiasis. Trypanosomiasis in Ethiopian cattle, locally referred to as 'Ghendi', is a serious constraint on livestock farming in areas of southwestern Ethiopia.[2]

The common drugs isometamidium chloride, homidium and diminazene have been on the market for more than 40 years.[3] Given their use over a long period of time, almost all of these trypanocides are gradually losing their efficacy.[4]

Because of the relatively limited market in Africa and the high cost of developing and licensing new drugs, there is little interest in the development of new trypanocides for use in either animals or humans.[3] Due to this and the development of drug resistance by trypanosomes, there is an urgent need to develop alternative and efficient drugs, either synthetically or from plant origins. To this end, screening of large number of plants should bring in potential new lead compounds for the treatment of various ailments, including trypanosomiasis.

Most of the plants that were included in our study belong to five plant genera, namely *Crotalaria*, *Cynoglossum*, *Heliotropium*, *Senecio* and *Solanecio*. These are widely used for

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the treatment of various ailments in Ethiopia and are known to contain pyrrolizidine alkaloids. The profiles of these alkaloids in most of the plants studied were documented by Asres *et al*.[5–7] Pyrrolizidine alkaloids have hepatotoxic activities.[8,9] They are metabolically activated in the liver to pyrrols with DNA-alkylating properties, therefore they can induce mutations or even cancer (reviewed by Fu *et al*.[10]). Some of them, however, have shown antitumor activities through inhibition of cell division. For instance, monocrotaline isolated from *Crotalaria sessiliflora* has been used effectively for the treatment of skin cancer in humans.[11] Indicine-*N*-oxide isolated from *Heliotropium indicum* has also been used for the treatment of tumours in rats and mice, and as a result it was selected for human clinical trials by the National Cancer Institute.[12]

Crude extracts from pyrrolizidine alkaloid-bearing plants with antineoplastic activity[13] or pyrrolizidine alkaloids with significant tumour inhibitory[14] and/or antiulcerogenic properties[15] could also be good sources of lead compounds against trypanosomes, as these parasites are similar to some types of cancer cells in their rapid growth within mammals.[16] Most other types of alkaloid that have been tested against trypanosomes were found to be inactive, except for a number of isoquinoline, indole, and quinoline alkaloids.[17] All these observations and the wide utilization of plants containing pyrrolizidine alkaloids for treatment of disease in Ethiopia prompted us to investigate further for antitrypanosomal and cytotoxic activities *in vitro*.

Materials and Methods

Reagents

Fetal bovine serum, MEM and RPMI 1640 media were purchased from Invitrogen, Karlsruhe, Germany. Pyrrolizidine alkaloids (retronecine, heliotrine, monocrotaline and senecionine; Figure 1) were purchased from Roth, Karlsruhe, Germany. AlamarBlue was purchased from Biosource International, Hamburg, Germany. Resazurin and diminazene aceturate were purchased from Sigma-Aldrich, Germany.

Plant materials

The plants were collected from their natural habitats in different parts of Ethiopia at different times between 23 October 1996 and 21 July 2006 by Ato Melaku Wondafrash and Dr Kaleab Asres and were identified by Mr Melaku Wondafrash, Addis Ababa University. The specimens were given voucher specimen numbers and were deposited at the National Herbarium, Addis Ababa University, Ethiopia for further reference.

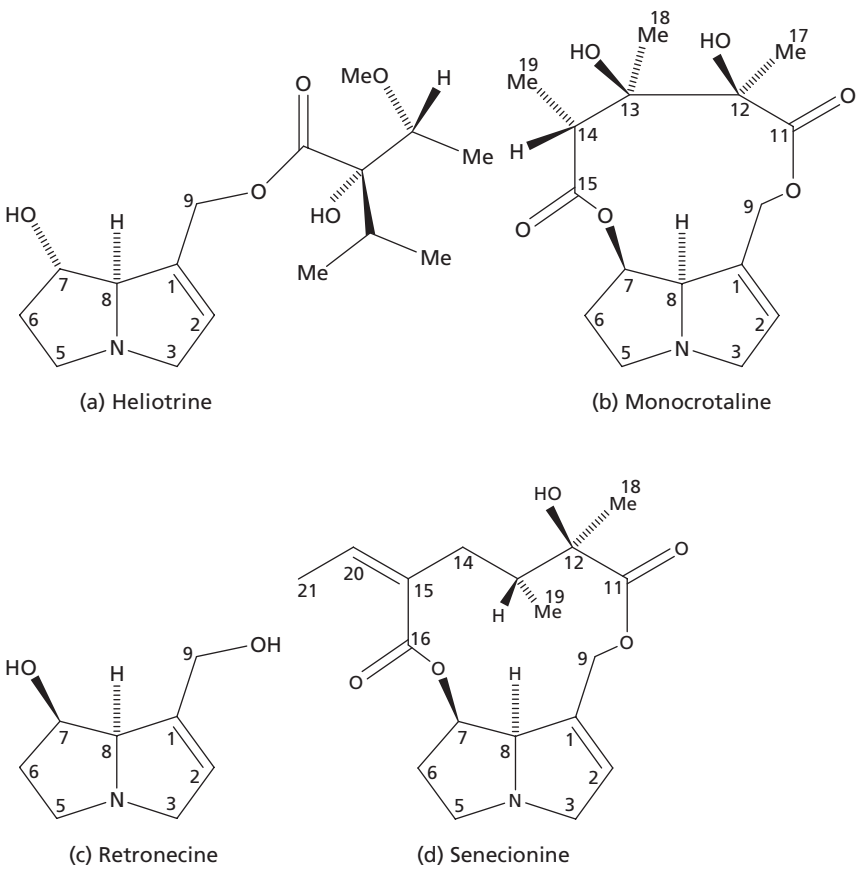

Figure 1 Four pyrrolizidine alkaloids included in the study. (a) Heliotrine. (b) Monocrotaline. (c) Retronecine. (d) Senecionine.

Preparation of plant extracts

Crude extract preparation

The parts of each plant investigated in this study were ground and macerated in methanol and dichloromethane solvents, respectively, and left on a shaker for two consecutive days. They were then filtered and evaporated to dryness under reduced pressure using a Rotavapor at 45°C.

Alkaloid extraction

Alkaloidal extracts were prepared only from *Solanecio angulatus* flowers, which showed the best antitrypanosomal activity. The extraction of alkaloids was performed according to the methods of Asres *et al*.[7] Briefly, 20 g of the powdered plant part was extracted with 80% methanol and evaporated to dryness. Two methanol extracts of the same kind were prepared and dissolved separately in 20 ml of 1 M HCl. One of the two acidic extracts was treated with zinc dust (0.2 g) and left on a shaker for 24 h to free pyrrolizidine alkaloids from their N-oxides. After 24 h of shaking, the two extracts were filtered. The filtrates (with and without zinc reduction) were made more alkaline (pH 9) with concentrated ammonia and applied onto columns packed with Chem Tube Hydromatrix (Varian, Inc., Palo Alto, CA, USA). The alkaloids were then eluted with dichloromethane. The organic solvent was evaporated under reduced pressure to yield alkaloid extract. The patterns of various alkaloids within the total alkaloid extract have already been described by Asres *et al*.[7]

Cell cultures

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

Bloodstream forms of *T. b. brucei* TC221 cells were grown in Baltz medium[18] supplemented with 20% inactivated fetal bovine serum and 1% penicillin–streptomycin. HL-60 cells (human myeloid cell line) were grown in RPMI 1640 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 cytotoxic activities

Both assays were performed as described in Merschjohann *et al*.[17] The extracts and compounds were dissolved in dimethyl sulfoxide (DMSO). The extracts were further serially diluted with the medium in two-fold fashion into seven different concentrations so as to attain final concentrations ranging from 500 to 3.91 μg/ml, in 96-well plates. The extract which was diluted in the medium was dispensed into each well in 100 μl. Each different concentration of the test drug was created in triplicate and repeated twice. The concentration of the solvent, DMSO, did not exceed 1.25% in the medium that contained the highest concentration of extract or compound tested. Wells containing the solvent and wells without the solvent were included in the experiment.

Both *T. b. brucei* and HL-60 cells were seeded into 96 wells at a density of 1×10^4 cells per 100 μl. The cells were incubated with the test drugs for a total of 48 h and the antitrypanosomal activity and cytotoxicity of extracts were evaluated using AlamarBlue.[19] In most cases, resazurin was used as the cell proliferation indicator dye with some modifications from the method that was used by Rolón *et al*.[20] Briefly, 10 μl and 6 μl of resazurin, respectively, were added to trypanosome and HL-60 cell cultures, and the cultures were incubated with the resazurin 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 at dual wavelengths of 492 nm and 595 nm. The concentration at which 50% of the growth of cells was inhibited was calculated by linear interpolation, taking two concentrations above and below 50%.[21]

Results

A total of 74 extracts from 19 plant species, including alkaloidal extracts from *Solanecio angulatus,* and four pure pyrrolizidine alkaloids were tested against *T. b. brucei* and HL-60 cells. The IC50 values are presented in Table 1 and Table 2. For most plant parts, the dichloromethane extracts were found to be more active on *T. brucei brucei* than their corresponding methanol extracts (Table 1). The dichloromethane extract of *S. angulatus* flowers was found to be the most potent extract against *T. b. brucei,* with an IC50 value of 12.17 μg/ml. The total alkaloidal extract of flowers of the same plant was compared with some of the alkaloids in their pure form (Table 2). It was found that the alkaloidal extract that was obtained without zinc reduction and termed 'alkaloid extract A' was less active than its crude dichloromethane or crude methanol extract or even than pure pyrrolizidine alkaloids (Table 2). On the other hand, the alkaloid extract that was obtained after zinc reduction and termed 'alkaloid extract B' showed greater antitrypanosomal activity than each pure alkaloid (Figure 1). From the four pyrrolizidine alkaloids tested, senecionine showed moderate antitrypanosomal activity, with an IC50 value of 41.78 μg/ml. The other active extracts were the crude dichloromethane extracts of *Crotalaria phillipsiae* twigs (IC50 = 12.67 μg/ml) and *Solanecio manni* leaves (IC50 = 24.89 μg/ml). Most of the other extracts tested showed moderate antitrypanosomal activities to variable extents. Those extracts with IC50 values greater than 100 μg/ml were considered to be inactive.

All the extracts and compounds tested against trypanosomes also showed some activities against HL-60 cells, but with higher IC50 values. The selectivity index (SI), which is the ratio of cytotoxicity against HL-60 to activity against *T. b. brucei*, was calculated and presented for each test drug in Tables 1 and 2.

Discussion

Generally, among the extracts tested, the crude dichloromethane extracts were more active against both types of cell than their corresponding methanol extracts. The trypanocidal

Table 1 Antitrypanosomal and cytotoxic activities of Ethiopian medicinal plants

Plant species	Plant part	Extract type	IC50 (μg/ml)		Selectivity index (SI)
			T. b. brucei	HL-60	
Crotalaria agatiflora (Fabaceae)	seeds	MeOH	186.01	>500	>2.69
		CH_2Cl_2	156.49	>500	>3.20
Crotalaria axillaries	seeds	MeOH	126.36	196.84	1.56
		CH_2Cl_2	NT	NT	–
	leaves	MeOH	137.10	489.77	3.57
		CH_2Cl_2	57.59	191.16	3.32
Crotalaria emarginella	leaves	MeOH	137.62	266.69	1.94
		CH_2Cl_2	48.55	380.69	7.84
Crotalaria fascicularis	twigs	MeOH	150.01	>500	>3.33
		CH_2Cl_2	56.89	>500	>8.79
Crotalaria incana	pods	MeOH	68.35	>500	>7.32
		CH_2Cl_2	52.67	>500	>9.49
	twigs	MeOH	61.97	404.61	6.53
		CH_2Cl_2	28.55	>500	>17.51
	leaves	MeOH	46.29	232.22	5.01
		CH_2Cl_2	48.27	>500	>10.35
	seeds	MeOH	164.99	>500	>3.03
		CH_2Cl_2	144.73	>500	>3.45
Crotalaria gillettii	aerial part	MeOH	111.89	>500	>4.47
		CH_2Cl_2	104.06	>500	>4.80
	leaves	MeOH	112.97	>500	>4.43
		CH_2Cl_2	NT	NT	–
Crotalaria laburnifolia	leaves	MeOH	53.80	>500	>9.29
		CH_2Cl_2	51.44	332.39	6.46
	pods	MeOH	82.68	468.75	5.67
		CH_2Cl_2	93.84	>500	>5.32
	seeds	MeOH	182.23	>500	>2.74
		CH_2Cl_2	162.80	>500	>3.07
	twigs	MeOH	98.62	401.58	4.01
		CH_2Cl_2	59.05	173.70	2.94
Crotalaria mildbraedii	twigs	MeOH	94.41	>500	>5.30
		CH_2Cl_2	51.49	>500	>9.72
	leaves	MeOH	106.50	>500	>4.69
		CH_2Cl_2	25.32	>500	>19.74
Crotalaria phillipsiae	twigs	MeOH	70.78	407.37	5.76
		CH_2Cl_2	12.67	435.22	34.35
	leaves	MeOH	113.63	>500	>4.40
		CH_2Cl_2	49.19	>500	>10.16
Crotalaria pycnostachya	pods	MeOH	70.38	390.40	5.54
		CH_2Cl_2	67.00	290.84	4.34
	leaves	MeOH	56.32	188.77	3.35
		CH_2Cl_2	50.15	356.06	7.10
	seeds	MeOH	165.81	313.87	1.89
		CH_2Cl_2	164.80	>500	>3.03
Crotalaria spinosa	pods	MeOH	111.64	463.68	4.15
		CH_2Cl_2	45.58	280.13	6.15
Cynoglossum geometricum (Boraginaceae)	leaves	MeOH	101.36	183.95	1.81
		CH_2Cl_2	64.01	312.62	4.88
	flowers	MeOH	161.98	360.20	2.22
		CH_2Cl_2	48.75	>500	>10.26
Heliotropium cinerascens (Boraginaceae)	twigs	MeOH	134.19	247.91	1.84
		CH_2Cl_2	222.92	161.31	0.72
Heliotropium steudneri	leaves	MeOH	99.94	>500	>5.00
		CH_2Cl_2	48.85	>500	>10.24
	twigs	MeOH	125.61	>500	>3.98
		CH_2Cl_2	98.01	441.48	4.50
Heliotropium somalense	leaves	MeOH	135.35	>500	>3.69
		CH_2Cl_2	49.06	>500	>10.19
	twigs	MeOH	107.97	>500	>4.63
		CH_2Cl_2	27.09	192.93	7.12

(Continued)

Table 1 *(Continued)*

Plant species	Plant part	Extract type	IC50 (μg/ml)		Selectivity index
			T. b. brucei	HL-60	
Senecio hadensis (Asteraceae)	leaves	MeOH	57.43	>500	>8.71
		CH_2Cl_2	52.40	>500	>9.54
	flowers	MeOH	98.21	217.65	2.21
		CH_2Cl_2	104.62	>500	>4.78
Solanecio angulatus (Asteraceae)	leaves	MeOH	58.66	130.77	2.23
		CH_2Cl_2	41.23	>500	>12.13
	flowers	MeOH	12.47	27.39	2.20
		CH_2Cl_2	12.17	>500	>41.08
Solanecio gigas	flowers	MeOH	71.55	412.58	5.77
		CH_2Cl_2	48.79	>500	>10.25
Solanecio manni	leaves	MeOH	50.58	>500	>9.88
		CH_2Cl_2	24.89	220.13	8.84
Diminazene aceturate (standard drug)	–	–	0.088	>128.88	>1464.00

NT, not tested; MeOH, crude extract obtained with methanol; CH_2C_{12}, crude extract obtained with dichloromethane.

Table 2 Antitrypanosomal and cytotoxicity of *S. angulatus* flowers alkaloidal extracts in comparison with other pure compounds

Extract/compound	IC50 (μg/ml)		Selectivity index
	T. b. brucei	HL-60	
Alkaloid extract-A[a]	94.68	>250	>2.64
Alkaloid extract-B[a]	14.35	175.50	12.23
Senecionine	41.78 (124.57 μM)[b]	>250	>5.98
Retronecine	50.95 (328.30 μM)	>250	>4.91
Heliotrine	55.85 (178.21 μM)	>250	>4.48
Monocrotaline	83.60 (256.95 μM)	133.72	1.60
Diminazene aceturate (standard drug)	0.088 (0.17 μM)	>128.88	>1464.00

[a]Alkaloid extract-A was obtained without zinc reduction whereas alkaloid extract-B was obtained after the acidic extract had been reduced with zinc dust, as described in Materials and Methods. [b]indicates the equivalent IC50 value of each compound in micromolar.

and cytotoxic activities of crude dichloromethane extracts can be explained in part by their lipophilic nature, which renders the cells leaky and thereby results in cell death. The best plant extract against *T. b. brucei* was the dichloromethane extract of *S. angulatus* flowers. The plant's flowers were subjected to further investigation to determine whether its alkaloids were responsible for its trypanocidal and cytotoxic activities. The effects of the crude dichloromethane extract and alkaloid extracts of *S. angulatus* are explained below.

In Ethiopia, *Crotalaria* species are used for various treatments. The juice of the leaves of *Crotalaria spinosa* is employed as a means of alleviating kwashiorkor (a type of malnutrition),[22] as a blood anticoagulant and for the treatment of burns.[23] Juice of the leaves of *Crotalaria laburnifolia* is used to alleviate fungal disease on the skin.[24] The present investigation indicated that the *Crotalaria* species could also be good sources of lead compounds against trypanosomes. Among *Crotalaria* species, the dichloromethane extract of *Crotalaria phillipsiae* twigs, with an IC50 value of 12.67 μg/ml and an SI value of 29.93, is a promising candidate as a source of bioactive compounds for the treatment of trypanosomiasis. The twigs of this plant are known to contain indolizidine and pyrrolizidine alkaloids.[5] In addition to these two substances, literature surveys indicate the presence of flavonoids, hydroquinone, phenolic carboxylic acids[25] and triterpenes[26] in *Crotalaria* species. The antitrypanosomal activity observed could be attributed to one of these classes of compounds or a combination of several.

The pure pyrrolizidine alkaloids, heliotrine, monocrotaline, retronecine and senecinonine, which were included in our study (Figure 1), showed some antitrypanosomal activities, with higher IC50 values compared to the most potent trypanocidal alkaloids from indole, quinoline and isoquinoline skeletons.[17] On a molar basis the necine, retronecine, exerted the lowest antitrypanosomal activity followed by, in increasing order of trypanocidal activity (see Table 2):

(1) the 11-membered cyclic diester alkaloid, monocrotaline
(2) the monoester alkaloid, heliotrine
(3) the 12-membered cyclic diester alkaloid, senecionine.

Unequivocal determination of structure–activity correlation is not apparent. However, it appears evident that the presence of an ester functional group with branched side chains

increases the lipophilicity of the three alkaloids and thereby increases their trypanocidal activity. One of the mechanisms by which the alkaloids exert their effect is to make the membrane more permeable and this is corroborated by haemolysis (albeit not significant) of erythrocytes by heliotrine and senecionine.[27] The four pure alkaloids inhibit protein biosynthesis (20% to 50%) at concentrations ranging from 1 mM to 5 mM. They also bind significantly to neuroreceptors but they are inactive against acetylcholine-related enzymes, DNA, DNA polymerase I and reverse transcriptase.[27,28]

In Ethiopia *Cynoglossum* species are used for the treatment of syphilis, ear infection,[29] inflammation[30] and animal diarrhoea.[31] *Cynoglossum geometricum,* which was included in our study, showed moderate antitrypanosomal activity (Table 1). A literature survey indicated the presence of a number of pyrrolizidine alkaloids from *Cynoglossum* species.[32] The recent work by Jin *et al*.[33] also showed the presence of flavonoids and sterols.

Heliotropium species are used for treatment of various human ailments in Ethiopia.Various species of this genus are used for the treatment of burns, scorpion bite,[22] dandruff,[30] inflammation and stomach ache.[24,30] The dichloromethane extract of *Heliotropium somalense* twigs was the best extract against trypanosomes from among the *Heliotropium* extracts tested in our study. The antitrypanosomal activity might be attributed to one or more of the pyrrolizidine alkaloids,[13] quinones,[34] flavonoids,[35] or triterpenoids[36] that have been reported to be present in *Heliotropium* species.

Senecio and *Solanecio* species, which are closely related species, are also used for the treatment of various ailments in Ethiopia. *Solanecio gigas* is used as anti-abortifacient and is also used for the treatment of colic and typhus.[22,23,29] *Senecio manni*, although its traditional use in Ethiopia is not documented, it is one of the most widely used plant species in cattle disease management in Kenya.[37] *Solanecio angulatus*, which showed the most trypanocidal activity, is traditionally used to alleviate tooth pain in humans.[7] It is also used for the treatment of hepatitis in animals.[31]

The crude dichloromethane extract of *S. angulatus* flowers was more active than the corresponding crude dichloromethane extract of the leaves (Table 1), and had the strongest antitrypanosomal activity and the best SI of all the extracts and compounds tested. It seems that the plant's secondary metabolites are stored in larger quantities in organs of the plant that are important for the reproduction and survival of the plant, in this case, in the flowers of *S. angulatus* rather than in its leaves.[38]

To determine further whether the observed trypanocidal activity should be ascribed to the plant's alkaloids, two alkaloid extracts were prepared from the flowers, with and without zinc reduction, and tested on trypanosomes. Interestingly, the two types of extracts showed great differences in their antitrypanosomal activities (Figure 2). The alkaloid extract that was obtained after zinc reduction greatly contributed to the antitrypanosomal activity of the plant and this can clearly be observed in both Table 2 and Figure 2. This observation indirectly revealed the existence of most plant alkaloids as their N-oxides. The N-oxides are polar and are present in aqueous solution, and without prior reduction they will be excluded in acid–base extraction procedures.

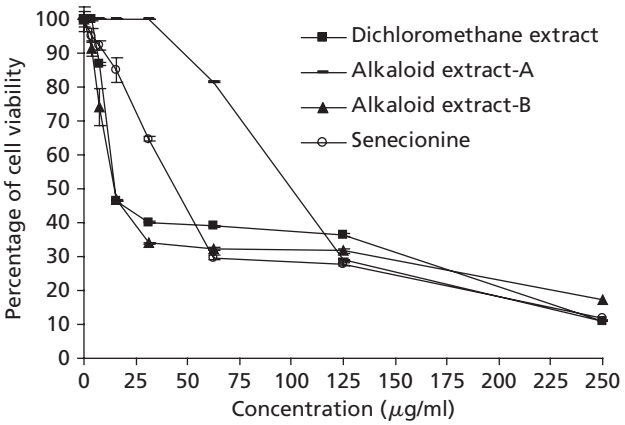

Figure 2 Anti-*T. b. brucei* effects of crude dichloromethane extract, alkaloid extract-A, alkaloid extract-B and senecionine from *Solanecio angulatus* flower. Each concentration of test drug was tested in triplicate and repeated twice. Results represent mean values of two independent experiments. The standard errors did not exceed 12% in all experiments.

The free bases that can be extracted with dichloromethane usually represent 8 to 10% of the total alkaloid extract, whereas pyrrolizidine N-oxides represent 92% of the total pyrrolizidine alkaloids and this was clearly observed in the alkaloid extract of *Solanecio gigas* flowers.[6] In our experiment, when N-oxides of *S. angulatus* flowers were reduced by zinc to their free pyrrolizidine alkaloids, they showed potent trypanocidal activity either by an additive or a synergistic mechanism. It is clearly seen from Figure 2 that the alkaloid extract-B, which was obtained by zinc reduction, is more active on *T. b. brucei* than its corresponding unreduced extract, alkaloid extract-A, or even than the pure alkaloid, senecionine. Nonetheless, the IC50 value of the reduced alkaloid extract against *T. b. brucei*, which is 14.35 μg/ml (Table 2), is higher than the IC50 value of the crude dichloromethane extract of the plant (IC50 = 12.17 μg/ml) (Table 1), suggesting that compounds other than alkaloids also contribute (albeit not significantly) to the trypanocidal activity of the plant. Compounds like monoterpenes[39] and sesquiterpenes, which have been observed in flowers of the related species, *Solanecio gigas,*[40] cannot be ruled out as contributing to the observed trypanocidal effect of the crude dichloromethane extract of *S. angulatus* flowers.

Conclusions

The two plants *Solanecio angulatus* (flowers) and *Crotalaria phillipsiae* (twigs) merit further consideration for the phytotherapy of trypanosomiasis as they showed good trypanocidal activities with good selectivity indices. Further bio-guided isolation of compounds from these two plants is, however, required to confirm which specific compounds are responsible for the observed trypanocidal activity. The observation that most of the other crude extracts were inactive against human leukaemia cells *in vitro* does not mean that they do not have activity on other cell types.

Unless the concentration of pure alkaloids in crude extracts is stated, care should be taken by the general population of Ethiopia in oral application of crude extracts, as these pyrrolizidine alkaloids, especially those having 1,2-unsaturated bonds in the necine base, are known to be converted to toxic metabolites by liver cells and subsequently cause liver cirrhosis.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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