African Journal of Biotechnology Vol. 4 (2), pp. 177-182, February 2005
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2005 Academic Journals

Full Length Research Paper

Comparative *in vitro* trypanocidal activities of petroleum ether, chloroform, methanol and aqueous extracts of some Nigerian savannah plants

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Accepted 27 December, 2004

Using *Trypanosoma brucei* as test organism, about two hundred extracts of varying polarities obtained from different parts of about forty tropical plants harvested from the savannah vegetational belt of Nigeria were evaluated for their *in vitro* trypanocidal activities at concentrations of 2 and 4 mg/ml. The proportion of petroleum ether, chloroform, methanol and aqueous extracts that eliminated motility within 60 min at the highest concentration tested were 77, 67, 50 and 47%, respectively, while 10, 11, 19 and 14% of these extracts were completely non-active under the test condition. Among the plants studied, extracts of *Adenium obesum* (stem bark), *Afrormosia laxiflora* (leaves and stem bark), *Cochlospermum planchonii* (stem bark), *Prosopis africana* (stem and root barks), *Striga* spp (leaves), *Terminalia avicennioides* (root and stem bark) and *Swartzia madagascariensis* (fruit pulp) exhibited the highest trypanocidal activity. These results suggest that tropical plants could be a very promising source of new generations of trypanocidal agents.

Key words: Medicinal plants, trypanocidal effects, in vitro assay, trypanosomiasis, Nigeria.

INTRODUCTION

Trypanosomiasis is a potentially fatal disease of humans and domestic animals in tropical Africa and South America (Fairlamb, 1982). The disease has undergone a dramatic and devastating resurgence in recent years (Smith et al., 1998) especially in Sub-saharan Africa (Welburn et al., 2001). Some 50 million people in 36 African countries are at the risk of acquiring the infection (Kuzoe, 1993). Recently, it was estimated that 300,000 to 500,000, people are currently infected and 100 deaths are caused each year by the disease.

Human African trypanosomiasis (HAT) is caused by the haemoflagellate, *Trypanosoma brucei gambiense* in West and Central Africa, and *Trypanosoma brucei rhodisiense* in Eastern Africa. In regions where HAT prevails, several other trypanosome species, including *T. vivax* and *T. congolense,* are prevalent which affect health of cattle and other livestock (Picozzi et al., 2002). Thus, the significance of trypanosomiasis to human health, nutrition and economy is enormous.

Unfortunately, existing treatment for trypanosomiasis are either old, toxic and / or expensive (Atouguia and

Abbreviations: DMSO, Dimethylsulfoxide; EDTA, ethyldiaminetetraacetic acid; PBS, phosphate buffered saline; WHO, World Health Organization.

Costa,1999). Incidences of therapeutic failures with these drugs are not uncommon. Besides, there are other problems associated with chemotherapy including drug availability, especially in rural areas, distribution and pharmacological properties of drugs, differences in the epidemiology of the disease response to therapy, and relapses (Gutteridge 1985; Fairlamb, 1985, 1990; Aldous, 1994; Onyelili and Egwu, 1995; Atouguia and Costa, 1999). Therefore, the need to search for cheaper, more effective, easily available and less toxic drugs cannot be over-emphasized.

In the immediate past, the possibility of sourcing for new generations of trypanocidal agent has been receiving some consideration (Igweh and Onabanjo, 1989; Owolabi et al., 1990; Wosu and Ibe, 1989). Freiburghaus et al. (1996, 1997, 1998) evaluated several medicinal plants of Tanzanian and Ugandan origin for their *in vitro* trypanocidal activity. Their results revealed that plants could in deed be a good source of trypanocidal drugs.

Based partly on a recent survey (Atawodi et al., 2002), we assessed methanol extracts of some Nigerian savannah plants for their in vitro trypanocidal activity. Our results showed that extracts of *Khaya senegalensis, Piliostigma reticulatum, Securidaca longepedunculata and Terminalia avicenniodes* were strongly trypanocidal to both *Trypanosoma brucei* and *T. congolense,* while extracts of *Anchomanes difformis, Cassytha spp, Lannea kerstingi, Parkia clappertoniana, Striga spp, Adansonia digitata and Prosopis africana* were trypanocidal either only to *T. brucei* or *T. congolense* (Atawodi et al., 2003). The trypanocidal activity of other extracts from these and other Nigerian plants are not known. Hence, the aim of this work is to compare the trypanocidal effects of petroleum ether, chloroform, methanol and aqueous extracts of some Nigerian savannah plants under *in vitro* condition. The result should throw more light on the therapeutic relevance of different parts of some Nigerian savannah plants.

MATERIALS AND METHODS

Plants

Plants were collected from different Northern Nigerian States within the savannah vegetation belt. The states include Kaduna, Bauchi, Kogi, Plateau and Adamawa. The Department of Biological Sciences, Ahmadu Bello University, Zaria or the Department of Botany, University of Jos, Nigeria confirmed the identities of the plants.

Sample preparation and extraction

Appropriate parts of plants were harvested, dried under the shade or in open air in the laboratory (to avoid heat destruction of the active components). Dried materials were pounded in laboratory mortar into small particles. Fifty grams (50 g) of the pounded dried plants materials were weighed and sequentially extracted by shaking for 2 h on Wrist Action Shaker after overnight soaking in 150 ml of relevant solvent. After filtration, samples were rinsed with additional 3 x 60 ml portions of the solvent. Combined filtrates were dried at room temperature under electric fan. Water extracts were however dried on water bath at 45°C. In some cases however, the sequential extraction was through reflux with 300 ml of the solvent beginning with petroleum ether and followed by chloroform, methanol and water in that order. The extracts were stored in the refrigerator at 4°c until required.

Test organism

Trypanosoma brucei brucei was the test organism used. It was obtained from stabilates maintained at the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Plateau State, Nigeria. The parasite was maintained in the laboratory by continuous passage in rats and mice until required. Passage was considered necessary when parasitamia was in the range of 16 – 32 parasites per field (usually 3 - 5 days post infection in rats and 10-12 days in mice). In passaging, 1 x 10^3 parasites were introduced intraperitoneally or intramuscularly into rats in 0.1 - 0.2 ml blood/PBS solution. For several passages, approximately 90% blood solution (v/v) was obtained by cardiac puncture into 1 ml syringe containing 0.1 ml EDTA (1% w/v). About 0.1 - 0.2 ml of the blood collected as described above or blood (diluted with PBS to contain approximately 1 x 10^3 parasite/ml) was injected into clean animals acclimatized under laboratory condition for at least one week.

Determination of parasitamia

Parasitaemia was monitored in blood obtained from the tail, pre-sterilized with methylated spirit. The number of parasites was determined microscopically at X 400 magnification using the "Rapid Matching" method of Herbert and Lumsden (1976). Briefly, the method involves microscopic counting of parasites per field in pure blood or blood appropriately diluted with buffered phosphate saline (PBS, pH 7.2). Logarithm values of these counts obtained by matching with the table of Herbert and Lumsden (1976) is converted to antilog to provide absolute number of trypanosomes per ml of blood.

In vitro test for trypanocidal activity

Exactly 10 mg of the different plant extracts were weighed into Eppendorf tubes and first dissolved in 100 µl of 10% dimethylsulfoxide (DMSO) in PBS. Phosphate buffered saline (400 ml) was then added to produce extract solutions of 20.0 mg/ml (stock). Another extract concentration (10.0 mg/ml) was prepared from the first extract solution by appropriate dilution with PBS. Aqeous extracts were dissolved directly in 500 µl PBS. Extract solutions were prepared just before use.

Assessment of *in vitro* trypanocidal activity was performed in triplicates in 96 well microtiter plates. In wells of microtiter plates, (Flow laboratories Inc., Mclean, Virginia 22101,USA), 20 µl of blood containing about 20-25 parasites per field obtained as described under "determination of parasitaemia" was mixed with 5 µl of extract solution of 20.0 mg/ml and 10.0 mg/ml to produce effective test concentrations of 4 and 2mg/ml, respectively. To ensure that the effect monitored was that of the extract alone, a set

Table 1. Effect of different concentrations of various plant extracts of some Nigerian Savannah plants on motility of *Trypanosoma brucei.*

S/No	Botanical name	Vernacular name[§]	Part	Time (min) after which motility ceased, reduced drastically (*) or reduced slightly (**) with different effective concentrations of extracts (mg/ml).							
				Pet. Ether		**Chloroform**		**Aqueous**		**Methanol[§§]**	
				4 mg/ml	**2 mg/ml**	**4 mg/ml**	**2 mg/ml**	**4 mg/ml**	**2 mg/ml**	**4 mg/ml**	**2 mg/ml**
1	Adansonia digitata	Baobab	Root bark	NT	NT	20**	45**	40*	10**	55*	NA
			Leaves	40	35*	40*	45**	40	25*	NA	NA
2	*Adenium obesum*	Karya	Stem bark	40	50	30/25	30	45	60	45	NA
			Roots	35	NA	25	35	30	30**	35	40**
3	*Afrormosia laxiflora*	Makarfo	Leaves	20	25	25	30	40	45	20	30
			Stem bark	35	45	20	50	50	NA	25	35
			Root bark	30	50	40	45	45	55	30	50**
4	*Afzelia africana*	Kawo	Leaves	30	40	45	60*	45*	55*	60	25*
			Stembark	30	25*	40	40*	NT	NT	NT	NT
5	*Albuca spp*	Gadali	Bulb	20*	50**	NA	NA	55	NA	NT	NT
6	*Anchomanes difformis*	Chakara		NT	NT	NT	NT	40*	45*	45	50**
7	*Annona senegalensis*	Gwanda daji	Roots	30	35	40	50	NA	NA	NA	NA
			Stem bark	40*	55*	NT	NT	NT	NT	40*	50*
8	*Boswellia dalzielli*	Hano, Ararrabi	Leaves	NA	NA	NA	NA	50*	50*	35	50
			Stem bark	40*	40*	NA	NA	35*	NA	20	30
			Root	NA	NA	40	60*	55*	NA	20	20
9	*Canarium schweinfurthii*	Atile	Stem bark	45	50*	45	35*	NA	NA	45*	NA
10	*Cassytha filiformis*	Rimfa adua	Leaves/stem	40	55	45	50**	40*	40*	25	55
11	*Cochlospermum Planchonii*	Rawaya	Leaves	20	30*	20	35	NA	NA	40	50**
			Root	40	55	40	40**	45	45**	30	40
12	Diospyros mespiliformis	Kanya	Leaves	40**	NA	45	25*	45*	45*	NT	NT
13	*Erythrophleum suaveolus*		Stem bark	40	50*	40	45	NA	NA	45	55*
14	*Ficus sycomorus*	Baure	Stem bark	NT	NT	NT	NT	55*	NA	60	NA
15	*Guiera senegalensis*	Sabera	Leaves	NT	NT	NA	NA	30*	55*	NT	NT
16	*Khaya senegalensis*	Madaci	Stem bark	25	30	35*	NA	35*	55**	35	NA
			Root bark	NT	NT	NT	NT	50	30**	40	40**
17	*Lannea kerstingii*	Faru	Leaf	NT	NT	NT	NT	55**	NA	40	40*
			Stem	NT	NT	NT	NT	35	40	50	55*
			Root	NT	NT	45	55*	20	30	40	40
18	*Lawsonia inermis*	Lallai (Ganye)	Roots	55	45*	20**	50**	45	55	NT	NT
			Leaves	30	35	55	60*	NA	NA	NA	NA
19	*Lonchocarpus laxiflorus*	Shunin biri	Stem bark	45*	NA	35*	NA	NA	NA	30	45*
20	*Magnifera indica*	Mango	Root	45	50	NA	NA	45	55*	30**	NA
21	*Momordica balsamina*	Garahuri	Whole plant	35	35*	35	55	40*	40*	55*	NA
22	*Moringa oleifera*	Zogale	Leaves	NA	NA	30	40*	30	50	30	40*
			Stem bark	40*	50**	20	40	30	45	30	50
			Root bark	25	40	45	55*	30	40	35*	35*
23	*Nauclea latifolia*	Tapashiya	Leaves	25*	NA	45*	NA	25*	35*	30	35*
			Stem bark	25	35**	45*	55*	NA	NA	30	45
			Root	15	35	40*	40**	40*	50**	30	50*

Table 1. contd.

24	*Parkia clappertoniana*	Dorowa	Stem bark	45	50	50*	55*	50	50*	55	60*
			Root bark	NT	NT	NT	NT	55	60*	55	NA
25	*Piliostigma reticulatum*	Kalgo	Leaf	NT	NT	NT	NT	25	30	45**	60**
			Stem	NT	NT	NT	NT	50	45*	60*	NA
			Root	NT	NT	NT	NT	50	60	NA	NA
26	Prosopis africana	Kirya	Leaves	35	45*	25	35	30	35	40**	NA
			Stem bark	35	NA	25	30	35	40	35	50
			Root bark	30	45	50	30**	30	40	30	50*
27	*Saba florida*	Ciyo Gamye	Whole plant	25	40	50*	50*	55	55*	NA	NA
28	*Securidaca longepedunculata*	Sanya	Stem bark	35	55**	NT	NT	20	20	45	45*
			Root bark	NT	NT	NT	NT	NA	NA	NA	NA
29	*Sterculia setigera*	Kukuki	Root bark	25	25	20	30*	35**	45**	60**	NA
30	*Striga spp*	Wuta wuta	Leaves	30	35	50	40*	25	35	40	NA
31	*Syzygium guinense*	Malmo	Stem bark	25	35	30	20*	55*	55**	NT	NT
32	*Swartzia madagascariensis*	Bayama	Leaves	NT	NT	35	40	40*	NA	40	40
			Stem bark	30	35	30	50	NA	NA	NA	NA
			Roots	25	35	25	50	40	NA	25	50
			Pulb	40	40*	40	50*	35	45	25	40
33	*Terminalia avicenioides*	Baushe	Roots	25	35	35	45	30	55**	30	50*
			Stembark	40**	NA	NA	NA	30	45*	35	45*
			Leaves	NT	NT	NT	NT	50**	NA	50**	NA
34	*Vernonia spp*	Shuwakan daji	Leaves	NA	NA	40	45	30*	NA	35**	NA
35	*Vitex doniana*	Dinya, Tinya, Tunci, (Fulani)	Stem bark	45	25*	40*	50*	50**	NA	NT	NT
36	*Bridelia ferruginea*	Kirni, Kisni	Stem bark	50	40**	25*	40*	40*	NA	35	50
37	*Standard Trypanocidal drug*	Diminal® (Diminazene diaceturate)	-	NT	NT	NT	NT	20	30	NT	NT

NT = Not Tested; NA = Not Active; § = Vernacular names are in Hausa except where otherwise stated.

§§ = Some of these data are taken from Atawodi et al., 2003.

of control was included which contained the parasite suspended in 2% DMSO only. For reference, tests were also performed with the same concentrations of Diminal[R] (445 mg diminazene diaceurate+ 555 mg phenazone/g, Eagle Chemical Company LTD, Ikeja, Nigeria), a commercial trypanocidal drug.

After 5 min incubation in closed Eppendorf tubes maintained at 37°C, about 2 µl of test mixtures were placed on separate microscope slides and covered with cover slips. The parasites were observed microscopically every 5 min for a total duration of sixty minutes. It should be noted that under this *in vitro* system adopted, parasites survived for about 4 hours when no extract was present. Cessation or drop in motility of the parasites in extract-treated blood compared to that of parasite-loaded control blood without extract was taken as a measure of trypanocidal activity. The shorter the time of cessation of motility of the parasite, the more active the extract was considered to be (Atawodi et al., 2003)

RESULTS

The most active petroleum ether extracts were that of *Afrormosia laxiflora* leaves and root bark, *Afzelia africana* leaves, *Annona senegalensis* root bark, *Khaya senegalensis* stem bark, *Lawsonia inermis* leaves, *Moringa oleifera* roots, *Nauclea latifolia* roots, *Prosopis africa*na leaves and root bark, *Sterculia setigera* root bark, *Striga* leaves, *Syzygium guinense* stem bark and *Terminalia avicennioides* root bark, while the most active chloroform extracts were that of *Adenium obesum* (roots and stem bark), *Afrormosia laxiflora* leaves and stem bark, *Swartzia madagascariensis* leaves and roots, and *Terminalia avicennioides* root bark (Table 1).

Among the aqueous extracts, that of *Cochlospermum planchonii* (stem bark), barks of *Lannea kerstingii* (stem and root barks), *Moringa oleifera* (leaves, stem and root bark) and *Piliostigma reticulatum* (leaves), *Prosopis africana* (leaves, stem and root barks), *Securidaca longepedunculata* (leaves), *Striga* spp (leaves) and *Swartzia madagascariensis* (fruit pulp).

Afrormosia laxiflora (leaves and stem bark), *Boswellia dalzielli* (leaves, stem and root barks), *Bridelia ferruginea*

(stem bark), *Cassytha filiformis* (aerial part), *Moringa oleifera* (stem bark), *Nauclea latifolia* (stem bark) and *Swartzia madagascariensis* (fruit pulp and roots) were the most active methanol extracts (Table 1).

Of all the parts of the plants investigated, it was only in *Adenium obesum* (stem bark and roots), *Afrormosia laxiflora* (leaves and stem bark), *Cochlospermum planchonii* (stem bark), *Parkia clappertoniana* (stem bark), *Prosopis africana* (stem and root barks), *Striga* spp (leaves), *Terminalia avicennioides* (root bark) and *Swartzia madagascariensis* (fruit pulp) that trypanocidal activity was observed with all four extracts (petroleum ether, chloroform, methanol and water extracts) at the highest concentration tested.

DISCUSSION

The observed trypanocidal activity of these plant extracts confirm earlier *in vivo* and *in vitro* studies that suggest that plant extracts could contain potent trypanocidal constituents (Igweh and Onabanjo, 1989; Owolabi et al., 1990; Asuzu and Chineme, 1990; Wosu and Ibe, 1989; Freiburghaus et al., 1996; 1997; 1998; Youan et al., 1997; Atawodi et al., 2003). However, it is not possible to compare many of our results with those of earlier reports because most plants investigated here were not previously studied for trypanocidal activity, although the use of some of the plants in the traditional management of trypanosomiasis have recently been reported (Atawodi et al., 2002). Nevertheless, our findings on *Afzelia africana*, *Annona senegalensis*, *Diospyros mepiliformis* and *Securidaca longepedunculata* are largely similar to that of Freiburghaus et al. (1996). Quantitative differences in activity may be due to known variation in chemical composition arising from differences in geographical location and time/season of collection.

That most plants showed differential activity between extracts and between parts are confirmation of our earlier assertion (Atawodi *et al,* 2003) that any statement on a plant's trypanocidal activity should be taken within the context of the plant part and the solvent extract tested. In some instances, where extracts obtained by reflux (hot) extraction were compared to those acquired through cold extraction (result not shown), it was observed that more activity was observed with cold extract, indicating that trypanocidal components of many plants are heat-labile. This may explain why some plants reported to be traditionally useful for treating trypanosomiasis are not active when scientifically evaluated in the past. It may therefore be advisable to use cold rather than hot extraction, where possible, when evaluating the trypanocidal activity, or indeed, other biological activities of medicinal plants.

The mechanism by which the extracts of these plants exert their trypanocidal activity is unknown since the active ingredient(s) were not isolated. However, Previous reports indicate that a number of tropical plants contain constituents that have been demonstrated to be clinically efficacious against many protozoal diseases. (Le Grand, 1989; Oliver-Bever, 1986; Etkin, 1981; Sepulveda-Boza and Cassels, 1996; Hopp et al., 1976; Bodley et al., 1995; Gbile and Adesina, 1987). Similarly, it is known that existing trypanocidal drugs exert their therapeutic action through a variety of mechanisms. Thus, while arsenic compounds poison the cell by action on glucose catabolism through glutathione, suramin target glycolysis in the glycosomes, while pentamidine and other diamidines disrupt the kinetoplast and may also interfere with polyamine synthesis. Yet others (e.g. eflornithine), are selective inhibitors of ornithine decarboxylase, depleting the biosynthesis of polyamines such as spermidine, a precursor of trypanothione. That the active extracts are of the different polarities (considering the physicochemical properties of solvents used) is an indication that the bioactive constituents of these plants belong to a variety of chemical classes that will no doubt exert their trypanocidal action by one or more of these mechanisms. This is consistent with earlier reports which attributed the trypanocidal activity of certain plant extracts (Oliver-Bever, 1986; Sepulveda-Boza and Cassels, 1996) to the highly aromatic planar quaternary alkaloids, berberine and harmaine (Hopp et al., 1976; Oliver-Bever, 1986) whose anti-protozoal action is through intercalation with DNA (Phillipson and O'Neil, 1989).

Considered as a whole, these results suggest that many tropical plants have potential to provide therapeutic agents for treatment of African trypanosomiasis. However, because the metabolic disposition of bioactive constituents may differ between *in vivo* and *in vitro* conditions, (Freiburghaus et al., 1996; Atawodi et al., 2003), we are currently investigating plants with demonstrated high trypanocidal activity *in vitro* for similar efficacy *in vivo*. The phytochemistry and the toxicology of these extracts are also being assessed with a view to establishing the possibility of developing these plant extracts into new generation of effective and safe trypanocidal agents for combating trypanosomiasis, a disease that has continued to be of immense economic and health importance in many tropical countries of the world, especially in Africa (WHO, 1975; 1986; Warren, 1988; Kuzoe, 1993; Smith et al., 1998; Welburn et al., 2001).

ACKNOWLEDGEMENTS

I thank Mr. Y.E.O. Apeh and Gabriel Idakwo for technical assistance and typing the manuscript, respectively.

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