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In vitro and *in vivo* trypanocidal effect of lipophilic extracts of medicinal plants from Mali and Burkina Faso

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ABSTRACT

Aim of the study: To determine the *in vitro* and *in vivo* antitrypanosomal activity of extracts of traditionally used plants.
Materials and methods: 47 dichloromethane extracts were tested *in vitro* in the Long-term Viability Assay (LtVA) on *Trypanosoma brucei brucei*. The most active ones were also tested *in vivo* using a standardised mouse test.
Results: 13 extracts (28%) were active *in vitro* with MIC-values ≤ 100 μg/ml, 6 extracts showed MIC-values ≤ 50 μg/ml. The root extract of *Securidaca longepedunculata Fresen*. (Polygalaceae) and the leaf extract of *Guiera senegalensis* J. F. Gmel. (Combretaceae) were able to reduce parasitaemia in mice, experimentally infected with *Trypanosoma brucei brucei* by 48 and 42% at the dose of 150 mg/kg b.w. intraperitoneally, two times daily for 3 days. The extract of *Acacia nilotica Delile* (Mimosaceae) stem bark showed immunosuppressive effect *in vivo*.
Conclusion: The results confirm an effect of the ethnobotanically used plants. Further investigation is needed to optimize the effectiveness of the extracts.

1. Introduction

The German colonial encyclopedia (1920) refers to the African Animal Trypanosomosis as follows: It is a disease that has already been recognised as disastrous epizootic in 1857 by Livingstone on his journey to the area of Sambesi. It is transmitted by tsetse flies. The causative organism of the disease is a trypanosome that has been discovered by Bruce in 1894 (Von Ostertag, 1920). Until now African Animal Trypanosomosis is a major problem for the region of Sub-Saharan Africa. Ten million square kilometres of land that could be lush and fertile is not in production because of the tsetse-transmitted disease. This region includes land in 32 of the world's poorest countries. The consequence of a successful elimination of the pathogen can be seen on the isle of Zanzibar. The small and delimited region made it possible to eradicate the tsetse flies. About 6 years after the eradication cattle was freed from trypanosomes. The result was that milk production has tripled, local beef production has doubled and the use of animal manure for crop farming has increased five-fold (Okhoya, 2003).

African Animal Trypanosomosis (AAT) is mainly caused by *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei brucei* furthermore simultaneous infection is seen. The disease is most important for cattle but also pigs, camels, goats and sheep are affected. The infection of cattle leads in subacute, acute or chronic disease and often ends in death. Disease-control bases on the decimation of tsetse flies, the breeding of trypanotolerant species and the prophylactic and therapeutic use of trypanocides. So far the first two methods do not lead to sufficient control. Therefore, control is based on a small number of compounds on the market. Their intensive use over decades leads to drug resistance which grows to a major problem (Peregrine, 1994). New and cheap trypanocides are urgently needed. Up to 80% of the African population still relies on traditional medicine as primary health care. Within the traditional medicine the use of medicinal plants plays an important role and has a tradition lasting for millenniums. Twenty-five percent of modern medicines are made from plants that were first used traditionally (WHO, 2003). Ethnobotanically used plants in African medicine can be a good source for new drugs in the treatment of trypanosomosis.

In the present work, the dichloromethane extracts of 47 plant parts from 37 traditionally used plant species were examined for *in vitro* and partly also for *in vivo* trypanocidal activity. So far, only few

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of these plants have been evaluated for their trypanocidal activity (Asuzu and Chimene, 1990; Freiburghaus et al., 1996, 1997; Youan et al., 1997; Adewunmi et al., 2001).

2. Materials and methods

2.1. Plant material

The investigated plants (Table 1) have been used in the traditional medicine against sleeping sickness and/or nagana for a long time and still they play an important role. The selection was made according to D. Diallo using the pre-existent investigations of the Département de Médecine Traditionnelle in Bamako, Mali (institutional report, Diallo, 1996 (Bizimana et al., 2006)). They have been collected in the southern part of Mali and Burkina Faso in November 2001 by N. Bizimana. C. Djibil and D. Diallo did the authentification of the plants. Voucher specimens are preserved at the Herbaria of the Département de Médicine Traditionnelle in Bamako, Mali and the Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Germany.

2.2. Preparation of crude plant extracts

20–40 g of the dried and powdered plant parts were extracted with a ten-fold quantity of dichloromethane under reflux for 30 min. The extracts were filtered and evaporated to dryness under reduced pressure at 40 °C.

2.3. Trypanocidal drug

Diminazene aceturate (Berenil®, Hoechst AG, Germany, Batch No. 507W742) was used as positive control.

2.4. Preparation of stock solutions

Stock solutions of crude plant extracts, natural compounds, special extracts and fractions were prepared with 100% DMSO. Stock solution of diminazene aceturate was prepared with distilled water and filtered through a 0.2 μm membrane filter. All stock solutions were stored at −20 °C. The test extract solutions were prepared from the stock solutions by diluting with culture medium directly before the test. The highest concentration of DMSO was kept below 1%.

2.5. Trypanosome stock

For both *in vitro* and *in vivo* tests the trypanosome stock *Trypanosoma brucei brucei* STIB 345 was used (Brun et al., 1979). It has originally been isolated from an infected *Glossina pallidipes* in 1969.

2.6. Feeder layer cells

As feeder layer cells for the cultivation of blood stream trypanosomes, fibroblast-like cells have been used. They were originally isolated from 15-day-old embryos of *Microtus montanus*-mice (MEF-cells) and adapted for *in vitro* tests. The cell line was originated from the Swiss Tropical Institute in Basel.

2.7. Culture medium

The culture medium for the cultivation of bloodstream forms of *Trypanosoma brucei brucei* was prepared as described by Baltz et al. (1985) with some modifications. The stock medium consists of Minimal Essential Medium with 25 MM Hepes with Earlĭs salts without L-glutamine supplemented with 10 ml/l nonessential amino acids (100×), 0.292 g/l L-glutamine, 1.60 g glucose, 0.181 g/l L-cysteine, 0.110 g/l pyruvic acid-Na-salt, 0.039 g/l thymidine and 0.068 g/l hypoxanthine. In addition, this stock medium was mixed with 20% (v/v) horse serum, 5% (v/v) cattle serum and 1% anticontamination cocktail according to Maeser et al. (2002). The MEF-cells were cultivated in stock medium with 10% foetal bovine serum instead of horse and cattle serum.

2.8. Experimental animals

The experimental mice were originated from the breeding colony of the Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin. They were *Mastomys coucha*-mice of either sex from two different strains weighing 70–100 g. The animals received laboratory chow pellets and water ad libitum.

2.9. Long-term Viability Assay with Trypanosoma brucei brucei

The antitrypanosomal activity of plant extracts against *Trypanosoma brucei brucei* was assessed after 10 days of continuous culture as described by Kaminsky et al. (1989). The trypanosomes were cultivated in presence of feeder layer cells. Plant extracts were added to trypanosomes in the logarithmic growth phase to give a concentration of 50, 100, 200 and 500 μg/ml, respectively. Every consequent day half of the medium was replaced by medium containing plant extract at the test concentration. Diminazene aceturate (Berenil®, Hoechst) was used as positive control. DMSO at the highest concentration in the test was used as negative control. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of plant extract at which no trypanosome with normal morphology or motility could be determined on the tenth day of incubation.

All extracts were tested in triplicates in 96-well microtiter plates at 37 °C with 5% of CO_2 in a humidified incubator. For confirmation, extracts showing MIC-values ≤200 μg/ml were tested at least twice.

2.10. Cytotoxicity against mammalian cells

Fibroblast-like cells (MEF) served as feeder layer cells for the trypanosomes and were observed for cytotoxic effects compared to untreated controls during the test period of 10 days. The results are expressed as minimum toxic concentration (MTC) at which damage of the fibroblasts in form of modification of morphology or ablation was assessed by visual observation through a microscope.

The visual observation of the fibroblasts during the test was also important to assure constant conditions for the trypanosomes.

2.11. Dose-finding study

Extracts with high activity *in vitro* were administered to one uninfected mouse each in order to register side effects of the pure plant extracts. The extracts were dissolved in sweet almond oil and injected i.p. twice daily for 3 days at a concentration of 37.5; 75; and 150 mg/kg b.w., respectively. The initial concentration was chosen according to the *in vitro* cytotoxicity results. Side effects of the extracts in mice were evaluated by observing clinical signs and by pathological examination of liver, kidney, heart, lung, CNS and the lymphatic organs at the Department of Veterinary Pathology, Faculty of Veterinary Medicine, Freie Universität Berlin.

We declare that the animal experiments comply with the current German and European laws.