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Leishmanicidal and Trypanocidal Activity of Extracts and Secondary Metabolites from Basidiomycetes

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Seventeen extracts and seven secondary metabolites isolated from basidiomycetes were tested in medium culture against promastigote forms of *Leishmania* spp. and bloodstream forms of *Trypanosoma cruzi*. Extracts from the culture filtrate or mycelium were generally inactive against the parasites except the *Zucoagaricus* genus mycelium extract which reduced by 47% the number of bloodstream forms. Striatin A, striatin B and podoscyphic acid exhibited *in vitro* activity at 10, 5 and 100 μg/mL, respectively. One compound showed activity against bloodstream forms of *T. cruzi*, the sesquiterpenoid naematolin, lysing the parasites by 79%. BALB/c mice infected with *L. amazonensis* were treated 3 weeks post-infection with striatin A and striatin B by subcutaneous route for 15 days at 10 mg/kg daily. The reference drug, N-methylglucamine antimonate, administered by subcutaneous injections at 28 mg Sbv/kg/day for 15 days reduced the parasite burden by 71.2% ($p<0.05$). Subcutaneous administration of straitin A at 10 mg/kg produced a weak decrease of the parasite burdens in the footpad by 17.6%. The treatment with striatin B had no effect and showed higher toxicity than striatin A. © 1997 by John Wiley & Sons, Ltd. Phytother. Res. **11**, 193–197, 1997

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INTRODUCTION

Diseases caused by protozoa, such as *Leishmania* sp. and *Trypanosoma cruzi*, are responsible for considerable mortality and morbidity throughout the world, especially in the tropics of South America.

In Paraguay, we established a programme to identify new active drugs, principally natural compounds for the treatment of cutaneous leishmaniasis and Chagas' disease. The Instituto de Investigaciones en Ciencias de la Salud (IICS), the French Institute of Scientific Research for the Development in Cooperation and the Laboratory of Biotechnology of the University of Kaiserslautern in Germany have initiated a study to evaluate the protozoal activity of secondary metabolites isolated from basidiomycetes. The basidiomycetes (mushrooms) constitute a class of fungi with an estimated number of 30 000 species whose secondary metabolism and biologically active compounds have scarcely been investigated (Anke, 1989). The present work, the *in vitro* leishmanicidal (promastigote forms) and *in vitro* trypanocidal (bloodstream forms) effect of 14 species of basidiomycetes and seven secondary metabolites was evaluated by described methods (Fournet *et al.*, 1994). The test against *T. cruzi* was selected because of the excellent results obtained in experimental prophylactic additions stimulating blood (Rojas de Arias *et al.*, 1994). Also, we tested two diterpenoids isolated from *Cyathus striatus* (Hecht *et al.*, 1978) on *Leishmania amazonensis* infected BALB/c mice comparing their efficacy with a reference drug, N-methylglucamine antimonate (Glucantime®).

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MATERIALS AND METHODS

Isolation and chemistry

Compounds tested. The extracts of basidiomycetes were supplied by Professor T. Anke, LB-Biotechnologie der Universität Kaiserslautern, Germany. The preparation of culture filtrate and mycelium from basidiomyctes was described by Anke (1989). The strains are deposited in the culture collection of the LB Biotechnologie, University of Kaiserslautern, Germany.

The secondary metabolites from basidiomycetes (see Fig. 1 and Table 2) were isolated as described; aleurodiscal from *Aleurodiscus mirabilis* (Berk. & Curt.) Höhn) (Lauer *et al.*, 1989), mniopetal E from *Mniopetalum* sp. 87256 (Kuschel *et al.*, 1994; Velten *et al.*, 1994), naematolin from *Hypholoma* species (Backens *et al.*, 1984), oosporein from *Cordyceps* A66-89 (Anke, 1989), podoscyphic acid from *Podoscypha* species (Erkel *et al.*, 1991), striatin A and striatin B from *Cyathus striatus* (Hecht *et al.*, 1978).

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Biological assays

In vitro studies. Cultures of *Leishmania* ssp. and *Trypanosoma cruzi* were obtained from IICS (Instituto de Investigaciones en Ciencias de la Salud, Asuncion, Paraguay) and identified by isoenzyme analysis.

Promastigote growth inhibition studies were performed on *L. amazonensis* (IFLA/BR/67/PH8), *L. braziliensis* (MHOM/BR/75/M 2903) and *L. donovani* (MHOM/BR/74/PP75) grown at 22°C in Schneider's drosophila medium containing 20% fetal bovine serum. Compounds were dissolved in 5 μL of dimethyl sulphoxide (DMSO), then in medium and placed in microtitre plates in triplicate. The activity of compounds was evaluated after 48 h by optical observation of a drop of each cell culture with a microscope by comparison with control cells and with reference drugs (N-methylglucamine antimonate and pentamidine).

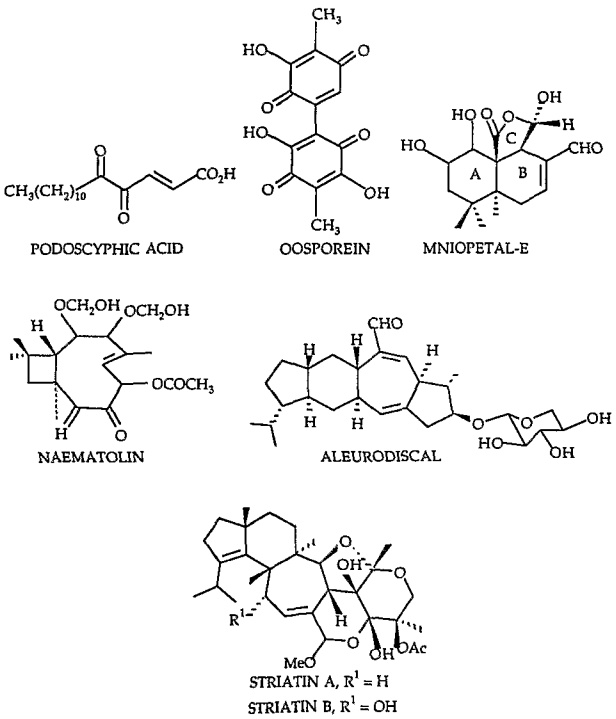

Figure 1. Structure of some secondary metabolites isolated from basidiomycetes.

Albino mice infected with *Trypanosoma cruzi* Y strain, 7 days after infection were used. Blood was obtained by cardiac puncture using 3.8% sodium citrate as anticoagulant in a 7:3 blood/anticoagulant ratio. The parasitaemia in infected mice ranged between 1×10^5 and 5×10^5 parasites per millilitre. Plant extracts were dissolved in cold DMSO to a final concentration of 250 μg/mL. Aliquots of 10 μL of each extract of different concentrations (4, 20, 40, 100 and 250 μg/mL) were mixed in microtitre plates with 100 μL of infected blood containing different parasite concentrations (1×10^5 and 10^6 parasites per mL). Infected blood and infected blood containing gentian violet at 250 μg/mL were used as controls. The plates were shaken for 10 min at room temperature and kept at 4°C for 24 h. Each solution was microscopically observed at 400x, placing a 5 μL-sample on a slide and covering it with a 22×22 mm coverglass for parasite counting (Rojas de Arias *et al.*, 1994; Schempler, 1978).

In vivo studies

Experimental animals. Female and male BALB/c mice were supplied by the IFFA-CREDO, France, and bred at the Instituto de Investigaciones en Ciencias de la Salud (IICS), Asuncion, Paraguay. Golden hamsters *(Mesocritus auratus)* were used to maintain the parasites. *L. amazonensis* MHOM/IFLA/BR/67/PH8 was used and maintained by

Table 1. ***In vitro*** **activities of crude extracts of basidiomycetes towards trypomastigote forms of** ***Trypanosoma cruzi*** **and three strains of promastigote forms of** ***Leishmania*** **spp.**

Strain[a]	Genus	Lysis of *Trypanosoma* at 250 μg/mL (%)		Activity against three strains of promastigote forms of *Leishmania*[b] strains at 100 μg/mL	
		CF	Mycelium extract	CF	Mycelium extract
92013	*Panaeolus*	0	0	–[d]	–
92061	*Mycena*	21	16	–	–
92012	*Propolis*	11	47	–	–
92023	*Formitopsis*	22	47	–	–
93018	*Phellinus*	22	9	–	–
93015	*Polyporus*	23	32	–	–
86125	*Oudemansiella*	0	42	–	–
92010	*Hyphodontia*	42	27	–	–
92014	*Galerina*	45	18	–	–
92019	*Zucoagaricus*	0	47	–	–
92021	*Agrocybe*	10	0	–	–
93017	*Cyathus*	31	0	–	–
93004	*Frametes*	0	6	–	–
93006	*Porling*	13	0	–	–
93021	*Pleurocybella*	3	13	–	–
92001	*Pleurotus*	27	13	–	–
93014	*Hirneola*	13	22	–	–

[a] Strain number refers to T. Anke's collection.
[b] *Leishmania amazonensis* (PH-8); *L. braziliensis* (2903); *L. donovani* (PP-75).
[c] Culture filtrate.
[d] Inactive at 100 μg/mL.

Table 2. ***In vitro*** **activity of some secondary metabolites isolated from basidiomycetes towards trypomastigote forms of *Trypanosoma cruzi* and promastigote forms of *Leishmania* spp.**

Product	Activity against *L. amazonensis* (PH8) (μg/mL)	Activity against *L. braziliensis* (2903) (μg/mL)	Activity against *L. donovani* (PP-75) (μg/mL)	Activity against *T. cruzi* at 250 μl/mL (%)
Gentian violet[a]				100
N-methylglucamine antimonate[a]	>100	>100	>100	
Pentamidine[a]	5	5	5	
Aleurodiscal	>100	>100	>100	18
Striatin A	10	10	10	0
Striatin B	5	10	5	22
Mniopetal E	>100	>100	>100	0
Oosporein	>100	>100	>100	22
Naematolin	>100	>100	>100	79
Podoscyphic acid	100	100	100	25

[a] Reference drugs

passage every 6 to 8 weeks in hamsters. BALB/c (n=8) were inoculated in the right hind footpad with 2×10^6 amastigotes obtained from donor hamsters. The parasites were delivered in 100 μL of phosphate buffered saline (PBS). Lesion development was monitored by serial measurements of footpad thickness with a dial gauge caliper (OSI, France). Size was expressed as the difference in thickness between the infected footpad and contralateral uninfected footpad. Measurements commenced 1 day prior to the inoculation of amastigotes and continued for 6 weeks.

Drug treatment. The treatments were initiated 3 weeks after inoculation when infection was well established and lesions were obvious. Two days before administration of treatments the mice were randomly divided into groups of eight.

N-Methylglucamine antimonate (Glucantime ®) with a pentavalent antimony (Sb^v) content of 28% by weight was purchased from the Rhône-Poulenc, France and was used as a reference drug. Glucantime ® was dissolved in 50 μL of PBS and administered to BALB/c mice at 28 mg Sb^v/kg of body weight daily for 15 days by subcutaneous route. Striatin A and striatin B were tested at a dose level of 10 mg/kg and were dissolved in 40 μL PBS, 5 μL of polysorbate (Tween 80, OSI, France) and 5 μL of DMSO. Striatin A and striatin B were administered by subcutaneous route once daily for 15 days. The untreated group received daily 40 μL of PBS, 5 μL of Tween 80 and 5 μL of DMSO.

Effect of treatment. The animals were killed 1 week after cessation of treatment to assess parasitological loads in the infected footpad. Briefly, the mice were killed and the lesions of infected footpad were excised, weighed and homogenized with a tissue glass grinder in 5 mL of RPMI 1640 medium (Gibco, France) supplemented with 10% fetal calf serum, 1 mL of glutamine (Gibco, France) at 29.4 mg/L, penicillin (100 U/mL) and streptomycin (100 μg/mL). Plates were examined and the number of amastigotes per host lesion cell nucleus were counted. The number of amastigotes per lesion per nucleus × lesion weight in gram (10^7) is approximately equal to the total number of amastigotes per organ (Buffet *et al.*, 1995; Stauber *et al.*, 1958). Parasite suppression was calculated from the ratio of the mean lesion amastigote counts of drug-treated mice and the mean lesion amastigote counts of untreated mice multiplied by 100 to obtain the percentage of parasite suppression.

Statistical analysis. Data give mean ± standard deviation, unless indicated otherwise. Comparison of parasite suppression in the infected footpad of the untreated control group and drug-treated group was analysed by the paired Student's t-test. Data were considered statistically significant at $p<0.05$ (two tailed).

RESULTS AND DISCUSSION

In the present study, 17 species of basidiomycetes were tested *in vitro* on promastigote forms of *Leishmania* ssp. and bloodstream forms of *Trypanosoma cruzi* (see Table 1). Culture filtrate or mycelium extracts from basidiomycete did not show *in vitro* activity against the parasites except the *Zucoagaricus* mycelium extract which reduced by 47% the bloodstream forms of *T. cruzi*. In contrast, after 48 h incubation with striatin A, striatin B and podoscyphic acid, the IC_{90} of these compounds for three strains of *Leishmania* were 10 μg/mL, 5 μg/ml and 100 μg/ml, respectively. Naematolin, sesquiterpene-caryophyllane (Anke, 1989), showed interesting activity against *T. cruzi* when compared with gentian violet, reducing by 79% the number of bloodstream forms. However, secondary metabolites and the compounds striatin A and mniopetal E did not present any activity against bloodstream forms of *T. cruzi*.

The effect of the treatment with N-methylglucamine antimonate (Glucantime ®) at 28 mg/kg for 15 days, striatin A and striatin B at 10 mg/kg for 15 days and 3 days, respectively on the development of *L. amazonensis* lesions in BALB/c mice is presented in Fig. 2. We observed signs of high toxicity of striatin B after 3 days of treatment, and for this reason the administration of this drug was suspended. Nevertheless, the size of lesions on the striatin B treated mice decreased 2 weeks after the suspension of treatment. To verify that Glucantime, striatin A or striatin B therapies effectively reduced the parasite burdens during the treatment period, footpads of treated and untreated mice were analysed for the presence of parasites 1 week after the suspension of treatment. The footpad tissue from mice treated with antimony had 71.2% ($p<0.05$) fewer parasite burdens versus the footpad from the untreated mice.

Table 3. Effect of treatments with N-methylglucamine antimonate, striatin A or striatin B by subcutaneous route on *Leishmania amazonensis* infected BALB/c mice (n=8)

Drug (dose)	Lesion wt (g) (mean±SD)	Suppression of weight lesion (%)	Suppression of parasite load in the footpad lesion (%)	Mean parasites quantitation in the footpad lesion
None (control)	0.0789±0.0308	–	–	2.21×10^6
Meglumine antimonate 28 mg^v×15	0.0485±0.0321	−38.5	−71.2[a]	6.36×10^5
Striatin A 10 mg×15	0.1009±0.0227	+27.9	−17.6	1.82×10^6
Striatin B[b] 10 mg×3	0.1356±0.0746	+71.9	+38.9	3.07×10^6

[a] $p<0.05$ (treated mice versus control)
[b] Three mice died after three days of treatment with straitin B

Nevertheless, no significant difference was observed between the untreated BALB/c mice and the treated mice with striatin A and striatin B. However in these treated mice, the weights of lesions increased by 27.9% (with striatin A) and 71.9% (with striatin B), and parasite burden decreased weakly by 17.6% with striatin A and increased by 38.56% with striatin B treatment.

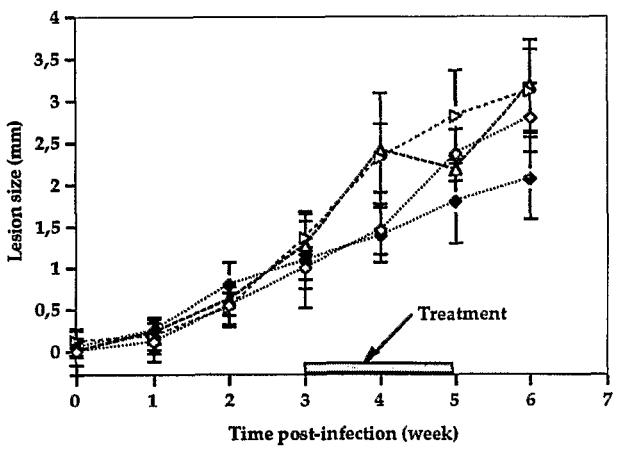

Figure 2. Effect of subcutaneous treatments with N-methyl meglumine antimonate (Glucantime®) (28 mg Sb^v/kg/day for 15 days), striatin A (10 mg/kg for 15 days) or striatin B (10 mg/kg for 3 days) during the course of infection of BALB/c mice (n=8) with *L. amazonensis*. Each point represents the mean difference in size±standard deviation of the mean between infected and uninfected footpaths. ◇, untreated; ◆, glucantime; ▷, striatin A; △, striatin B for 3 days.

To our knowledge, this work is the first time that secondary metabolites of basidiomycetes have been tested against *Leishmania* or *Trypanosoma cruzi*. All the compounds tested were described with antibiotic, antifungal properties (Anke, 1989) or with cytotoxic activity as podoscyphic acid inhibited an avian myetoblastosis virus and Moloney murine leukaemia reverse transcriptase (Erkel *et al.*, 1991; Kuschel *et al.*, 1994; Velten *et al.*, 1994).

In 1989, Anke indicated that studies of the mode of action of striatin A and striatin B revealed that the interference with the transport of essential precursors was the prime reason for the cytotoxicity of these compounds. It may be possible to link the toxicity of striatin B with its inhibitory activity against DNA synthesis in cells or with a mutagenic activity. It would be interesting to test striatin B in different experimental conditions, decreasing notably the concentration of drug, and to continue the evaluation of groups of secondary metabolites isolated from basidiomycetes, sesquiterpenoids, diterpenoids, acetylenes or quinones.

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