PHYTOTHERAPY RESEARCH, VOL. 7, 111–115 (1993)

Antiprotozoal Activity of Dehydrozaluzanin C, a Sesquiterpene Lactone Isolated from *Munnozia maronii* (Asteraceae)

Alain Fournet*
Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM), Département Santé, 213, rue La Fayette, 75480 Paris, Cedex 10, France

Victoria Muñoz
Instituto Boliviano de Biologia de Altura (IBBA), CP 717, La Paz, Bolivia

François Roblot, Reynald Hocquemiller and André Cavé
Laboratoire de Pharmacognosie, associé au CNRS, Faculté de Pharmacie, Université Paris XI, 92296 Châtenay-Malabry, Cedex, France

Jean-Charles Gantier
Laboratoire de Parasitologie, Faculté de Pharmacie, Université Parix XI, 92296 Châtenay-Malabry, Cedex, France

The petroleum ether extract of the leaves of *Munnozia maronii* was found to inhibit *in vitro* the growth of promastigote forms of *Leishmania* and epimastigote forms of *Trypanosoma cruzi* with an IC_{90} of 25 µg/mL. Activity-guided fractionation of the extract by chromatography identified the sesquiterpene lactone 1 of the guaiane series. The complete structure of 1 was elucidated using ^{1}H and ^{13}C NMR experiments at high field. The isolated compound was shown to be a new natural guaianolid, dehydrozaluzanin C, previously known as synthetic oxidative derivative of zaluzanin C (Romo de Vivar *et al.*, 1967). This compound inhibited, *in vitro*, the growth of 12 strains of *Leishmania* and 15 strains of *T. cruzi* at concentrations between 50 and 2.5 µg/mL. The leishmanicidal activity of dehydrozaluzanin C was tested on BALB/*c* mice infected with amastigote forms of *Leishmania amazonensis*. Dehydrozaluzanin C reduced the severity of *L. amazonesis* lesions in BALB/c mice but was less active than the reference compound Glucantime.

Keywords: Munnozia maronii; Asteraceae; antiprotozoal; *Leishmania*; *Trypanosoma cruzi*; dehydrozaluzanin C; guaianolid.

INTRODUCTION

Leishmaniasis is a protozoan parasitic disease transmitted to human by phlebotomine sand flies. The estimated global prevalence is 12 million cases, with 400 000 to 2 000 000 new cases reported per year (Croft, 1988). The drugs of first choice are pentavalent antimonials such as *N*-methylglucamine antimonate (Glucantime) or sodium stilbogluconate (Pentostam) but require long-term therapy and often induce toxic effects (Croft, 1988). Moreover, visceral leishmaniasis clinically resistant to antimony has been reported (Berman, 1988).

In Bolivia, the Instituto Boliviano de Biologia de Altura (IBBA) and ORSTOM (French Institute of Scientific Research for the Development in Cooperation) have been searching for Bolivian plants containing new natural compounds active against leishmaniasis and Chagas disease. We have investigated the leaves of *Munnozia maronii* (Asteraceae), an abundant herbaceous plant which grows at an altitude of 1500 m to 3000 m in the sub-Andean tropical region. *Munnozia maronii* is not used in traditional medicine. In a preliminary screening, a petroleum ether extract of the leaves displayed activity *in vitro* (25 µg/mL) against promastigotes of three strains of *Leishmania* ssp, *L. braziliensis*, *L. amazonensis* and *L. donovani* and epimastigote forms of three strains of *Trypanosoma cruzi* (Tulahuen, C8Cl1 and 27R27 Cl1). The present paper describes the *in vitro* and *in vivo* antiprotozoal effects of the isolated guaianolid responsible for the biological activity of the extract.

* Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Isolation and chemistry

General experimental procedures. The UV spectrum was recorded on a Unicam SP 1800 spectrophotometer, and the IR spectrum on a Perkin Elmer 257. All the ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker AC 200 P spectrometer in $CDCl_3$ (δ ppm) operating at 200 and 50 MHz respectively. Eims spectrum was obtained from Nermag R 1010 C mass spectrometer operating at 70 eV. Optical rotation was measured in $CHCl_3$ using a Schmidt–Haensch polarimeter type Polartronic I. Modelization experiments were performed using a Tipos Alchemy II logicial.

Plant material. The leaves of *Munnozia maronii* were collected by A. Fournet (A.F. 434) in Yungas (Department of La Paz, altitude 2500 m, Bolivia) in July 1987.

0951–418X/93/020111–05 $07.50

Accepted (revised) 1 June 1992

The plant material was identified by H. Robinson of the National Museum of Natural History, Smithsonian Institution, Washington, USA. Voucher specimens were deposited in the National Herbarium of Bolivia (La Paz) and in the Smithsonian Institution.

Extraction and isolation. The air-dried leaves (720 g) were powdered and extracted with petroleum ether. The solvent was evaporated under reduced pressure to yield a greenish extract (61 g). This extract was fractionated on a silica gel column (Kieselgel H, Merck), eluted with petroleum ether and an increasing amount of EtOAc. The active fraction (6.7 g), eluted with pure EtOAc, was further purified by silicagel chromatography using petroleum ether–EtOAc (7–3) as eluant, affording 164 mg of dehydrozaluzanin C.

Spectrometrical and chemical characterization of dehydrozaluzanin C. Amorphous, $C_{15}H_{16}O_3$: 244; UV, λ max (EtOH) nm 217; IR (KBr) ν cm^{-1}: 1761 (γ-lactone), 1723, 1660, 1635, 1405, 1258, 1145, 1103, 995, 900, 890; $(\alpha)_D + 100°$ (c = 0,15, $CHCl_3$); MS: *m/z* (%): 244 (14), 215 (28), 173 (12), 150 (100); 122 (M^{++}, 14), 117 (20), 91 (72), 81 (33), 79 (49), 77 (48), 53 (57), 41 (42), 39 (54). ^1H and ^{13}C NMR (Table 1).

Biological assays

Parasites. Cultures of *Leishmania* and *Trypanosoma cruzi* were obtained from IBBA (Instituto Bolivano de Biologia de Altura, La Paz) and identified by isoenzyme analysis. Twelve strains of *Leishmania* were used during these investigations: *L. braziliensis* (MHOM/BR/75/M 2904), *L. guyanensis* (MHOM/BR/75/M4147), *L. panamensis* (4039), *L. peruviana* (MHOM/PE/00/SL2), *L. amazonensis* (IFLA/BR/67/PH8), *L. mexicana* (MHOM/BZ/82/BEL 21), *L. pifanoi* (MHOM/VE/57/LL1), *L. venezuelensis* (VE/74/PM-H3), *L. donovani* (MHOM/IN/83/HS-70), *L. donovani* (MHOM/IN/80/DD8), *L. infantum* (LH-4), *L. infantum* (MHOM/BR/00/M 2682).

Fifteen strains of *Trypanosoma cruzi* were used: Z2Cl4 (Bolivian strain), 133 79 Cl9 (isolated from man in Brazil), C8 Cl1 (Brazilian strain), 31 R16 Cl1 (isolated from man in Bolivia), SC 43 Cl2 (Bolivian strain), A98 Cl5 (Original from French Guyana), MIL 4 Cl10 (isolated from man in Bolivia), M62241 Cl6 (isolated from man in Brazil), 9280 Cl1 (isolated from man in Bolivia), CL (Brazilian strain), 27R 27 Cl1 (isolated from *Aotus* in Bolivia), Y (isolated from man in Brazil), Tulahuen (isolated from *Triatoma infestans* in Brazil), A 97 Cl1 and A 99 Cl7 (isolated from *Didelphis marsupialis* in French Guyana) and PB 3 Cl7 (isolated from *Rhodnius pictipes* in Bolivia).

Culture and maintenance of *Leishmania*. Promastigote forms of *Leishmania* were grown at 28°C in USMARU medium (Evans, 1987) containing 10% heat inactivated (56°C for 30 min) fetal bovine serum. Promastigote cultures in the logarithmic phase of growth were maintained by transferring 10^6 cells per mL. The extracts and the fractions of *Munnozia maronii* were dissolved in 50 µL of DMSO (dimethyl sulphoxide) and added to the medium, from which aliquots were drawn. Parasites were counted every day in a haemocytometer and the results were compared with those of controls grown without drug. The 90% inhibitory concentrations (IC_{90}) were chosen for the comparison of susceptibilities of the strains to drugs tested. Pentamidine^R (Aldrich Chemical and May & Baker, England) and ketoconazole (Janssen Pharmaceutica Co, Belgium) were used as reference drugs for measuring the efficacy of the extracts of *Munnozia maronii*. Each assay was performed in triplicate.

Culture and maintenance of *Trypanosoma cruzi*. Epimastigotes of *T. cruzi* were maintained in continuous exponential growth in liver infusion tryptose medium (LIT, Bacto) supplemented with 10% fetal calf serum at 28°C with an inoculum of 10^6 cells per mL. Aliquots were taken every day and the parasites were counted in a haemocytometer; the counts were compared with those of controls grown without drug. Nifurtimox (Bayer, Germany) and benznidazole (Roche, USA) were used for comparison.

***In vivo* studies.** Female BALB/*c* mice were obtained from the Charles River Breeding and then were bred at IBBA (La Paz, Bolivia). Mice were 8 weeks old and weighed 18–20 g when experiments were initiated. *Leishmania amazonensis* (IFLA/BR/67/PH8) was used. Female BALB/*c* mice ($n = 8$) were infected subcutaneously in the right rear foot pad with 0.2 mL phosphate buffer saline (PBS) containing 10^6 amastigotes obtained from donor hamsters. The growth of infection was calculated weekly by measuring the diameter of both rear feet with a direct reading vernier caliper (Ref: Kroelin 10DI 00T6). The size of the lesion in mm was determined by subtracting the measurements obtained for the uninfected foot from that of the infected foot. Measurements began 1 day before the inoculation of *L. amazonensis* and were performed for 9 weeks.

Glucantime was given at 200 mg/kg/d and dehydrozaluzanin C at 100 mg/kg/d. Preliminary studies had shown that these doses were the most effective limiting the mortality induced by the development of *L. amazonensis* lesions in BALB/*c* mice (Coleman *et al.*, 1989). Drug treatment was initiated 1 day after the inoculation of parasites and was carried on once daily for 14 days. The dehydrozaluzanin C was dissolved in 50 µL of polysorbate (Tween 80, Prolabo). Control mice ($n = 8$) received 0.2 mL PBS for 14 days. For each experiment the mean and standard error of the mean (SEM) were calculated.

RESULTS

Structure determination of the guaianolide 1

The chemical structure of **1** was established according to its spectral data. The UV and IR spectra indicate the presence of a lactone ring ($\lambda_{max} = 217$ nm; $\nu_{max} = 1761\ cm^{-1}$), of an isolated ketone ($\nu_{max} = 1723\ cm^{-1}$) and of several isolated or conjugated double bonds. The molecular weight of **1** (*m/z* = 244) and its ^1H and ^{13}C-NMR spectra indicate a molecular formula of $C_{15}H_{16}O_3$, consistent with a sesquiterpene lactone structure. The sequence of the protonated carbon atoms was established using ^1H-^{13}C-NMR (COSY)

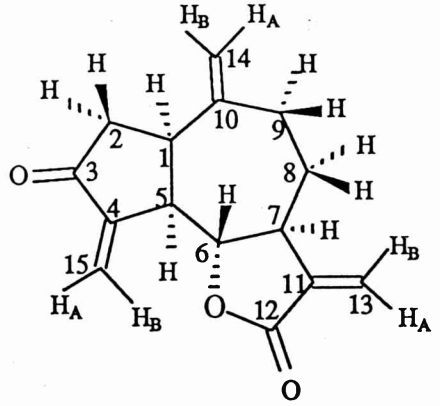

Figure 1. Long-range correlations observed on the ^{1}H-NMR COSY-45° spectrum of dehydrozaluzanin C.

experiments and selective irradiations. The positions of the three exocyclic double bonds were established by the observation of long-range correlations on the ^{1}H-^{13}C-NMR COSY-45° spectrum (Fig. 1). The observed couplings between the aliphatic protons clearly establish the relative configuration of **1** by comparison with the corresponding Dreiding model and literature data (Bohlmann *et al.*, 1980; Bohlmann *et al.*, 1981; Hazim *et al.*, 1980). Modelization experiments indicate that any modification in the stereochemistry, in agreement with the NMR data, increases the internal energy of the molecule. The structure and relative configuration of the sesquiterpene lactone **1** is thus established as the guaianolide represented in Fig. 1. This structure seems to be identical with dehydrozaluzanin C, obtained by Romo de Vivar *et al.* (1967) by oxidation of natural zaluzanin C. The positive specific rotation indicates the same absolute configuration. Dehydrozaluzanin C is isolated for the first time as a natural product. Its complete ^{1}H and ^{13}C-NMR data, never reported before, are given in Table 1. The ^{13}C-NMR attributions were made from the ^{1}H NMR data, by the way of heteronuclear ^{1}H-^{13}C correlations.

Table 1. NMR data of dehydrozaluzanin C in $CDCl_3$ (δ ppm, $CHCl_3$=7,27)

	^{1}H-NMR (200 MHz)		^{13}C-NMR (50 MHz)
H-1α	3.12 ddd[a]	C-1	39.6
H-2α	2.68 dd[a]	C-2	44.6
H-2β	2.56 dd[a]	C-3	204.4
H-5α	3.27 tdd[a]	C-4	144.4
H-6β	4.01 t[a]	C-5	48.6
H-7α	3.03 m	C-6	86.8
H-8α	≈2.30 m	C-7	44.0
H-8β	1.46 m	C-8	31.6
H-9α	≈2.20 m	C-9	38.2
H-9β	≈2.60 m	C-10	148.2
H-13$_A$	6.30 d[a]	C-11	138.6
H-13$_B$	5.58 d[a]	C-12	not detected
H-14$_A$	4.94 s	C-13	121.4
H-14$_B$	4.60 s	C-14	113.6
H-15$_A$	6.25 dd[a]	C-15	122.1
H-15$_B$	05.87 dd[a]		

[a] Coupling constants (*J* Hz): 1α, 2α(7.9); 1α, 2β (2.6); 1α, 5α (9); 2α, 2β (18.5); 5α, 6β (9); 5α, 15$_A$ (3); 5α, 15$_B$ (2.7); 6β, 7α (9); 7α, 8α (<0.5); 7α, 8β (≈12); 7α, 13$_A$ (3.5); 7α, 13$_B$ (3.1); 8α, 8β (≈14); 8α, 9α (≈6); 8α, 9β(≈3); 8β, 9α (≈13); 8β, 9β (≈4); 9α, 9β (≈13); 13$_A$, 13$_B$ (<0.5); 14$_A$, 15$_B$ (<0.5); 15$_A$, 15$_B$ (0.7).

In vitro and *in vivo* effects on *Leishmania*

After 24 h incubation with dehydrozaluzanin C, the IC_{90} for ten strains of promastigote forms of *Leishmania* was 5 μg/mL. The IC_{90} for *Leishmania panamensis* (40390) and *L. donovani* (DD8) were 50 μg/mL and 25 μg/mL respectively. After 72 h of contact with dehydrozaluzanin C, the IC_{90} was 5 μg/mL for *L. panamensis*, 10 μg/mL for *L. donovani* (DD8), and 2.5 μg/mL for the others strains. For comparative purposes, results obtained in the presence of dehydrozaluzanin C, pentamidine and ketoconazole in the culture medium are presented together in Table 2.

The effect of Glucantime and dehydrozaluzanin C on the development of *L. amazonensis* lesions in BALB/*c* mice is presented in Fig. 2. As previously demonstrated, the use of either Glucantime or dehydrozaluzanin C never prevented the development of lesions but reduced their severity. The progression of the *L. amazonensis* infection was slower in mice treated with Glucantime than in mice treated with dehydrozaluzanin C, but the amount of reference compound was higher (200 mg/kg instead of 100 mg/kg).

In vitro effects on *Trypanosoma cruzi*

The dehydrozaluzanin C was tested on 15 strains of *Trypanosoma cruzi*. The results obtained in the presence of dehydrozaluzanin C, nifurtimox and benznida-

Table 2. Comparison of *in vitro* inhibitory effects (IC_{90} (μg/mL) on 12 strains of promastigote forms of *Leishmania* at different times after addition of dehydrozaluzanin C, pentamidine and ketoconazole

	Dehydrozaluzanin C			Pentamidine			Ketoconazole		
Time	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Strains									
L. braziliensis (M2904)	5	2.5	2.5	5	1	1	>100	>100	50
L. guyanensis (M4147)	5	2.5	2.5	5	2.5	2.5	>100	>100	100
L. panamensis (4039)	50	10	5	2.5	1	1	>100	>100	100
L. peruviana (SL2)	5	2.5	2.5	5	1	0.5	>100	>100	50
L. amazonensis (PH8)	5	2.5	2.5	2.5	1	0.5	>100	>100	50
L. mexicana (BEL21)	5	2.5	2.5	2.5	1	0.5	>100	>100	>100
L. pifanoi (LL1)	5	2.5	2.5	2.5	1	0.5	>100	>100	100
L. venezuelensis (PM-H3)	5	2.5	2.5	5	2.5	1	>100	>100	>100
L. donovani (HS-70)	5	2.5	2.5	5	2.5	0.5	>100	>100	100
L. donovani (DD8)	25	10	10	5	2.5	0.5	>100	>100	>100
L. chagasi (M2682)	5	2.5	2.5	5	2.5	0.5	>100	>100	>100
L. infantum (LH 4)	5	2.5	2.5	5	2.5	0.5	>100	>100	>100

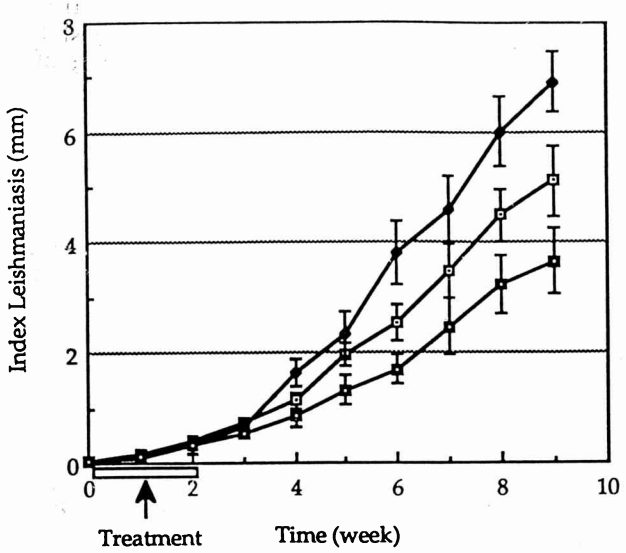

Figure 2. Effect of dehydrozaluzanin C (100 mg/kg/d) and Glucantime^{R} (200 mg/kg/d) on the development of *L. amazonensis* (PH 8) in BALB/*c* (±SEM). Drugs were given for a 14 d period commencing 1 d after inoculation of *L. amazonensis*. □, Dehydrozaluzanin C; ■, Glucantime; ◆, control.

zole are presented in Table 3. After 24 h the dehydrozaluzanin C showed an inhibitory activity on all strains. The IC_{90} varied widely, from 5 µg/mL to 50 µg/mL. Finally, after 72 h of contact with the sesquiterpene lactone, six strains were inhibited at a concentration of 2.5 µg/mL, one at 5 µg/mL (M6241 Cl4), seven at 10 µg/mL and only one at 25 µg/mL (13379 Cl9). Nifurtimox and benznidazole showed no inhibitory activity against the multiplication of epimastigote forms of *Trypanosoma cruzi* below 25 µg/mL.

DISCUSSION

The petroleum ether extract of the leaves of *Munnozia maronii* showed a significant activity on the promastigote forms of *Leishmania* and epimastigote forms of *Trypanosoma cruzi*. The bioassay guided fractionation produced the active compound, identified as the sesquiterpene lactone, dehydrozaluzanin C, isolated for the first time as a natural product. This compound was previously obtained by oxidation of zaluzanin C isolated from a Mexican Asteraceae, *Zaluzania augusta* (Romo de Vivar *et al.*, 1967). In a phytochemical study of *Munnozia maronii* (Bohlman and Grenz, 1979), the isolation and structure determination of a sesquiterpene lactone was reported but no mention was made about dehydrozaluzanin C. A sesquiterpene lactone with a similar guaianolide skeleton, dehydrocostuslacton, was isolated from another species of *Munnozia*, *M. gigantea* (Bohlmann and Grenz, 1979).

In our study, dehydrozaluzanin C exhibited an interesting activity *in vitro* against extracellular forms of the parasites; it was more efficient than standard leishmanicidal compounds, glucantime and ketoconazole. The *in vivo* studies with the BALB/*c* mice model of *Leishmania amazonensis* (PH 8) showed that dehydrozaluzanin C was less potent than Glucantime. In previous studies dehydrozaluzanin C did not exhibit direct activity against *L. amazonensis* in cell lines (Vero, U 937, and $HBK_{21}C_{13}$). The cytotoxicity (LD_{90}) on the mammalian cells was evaluated to 1 µg/mL (Muñoz, 1987).

The activity of dehydrozaluzanin C on the strains of epimastigote forms of *Trypanosoma cruzi* was more potent than the standard drugs, benznidazole and nifurtimox. *In vitro* biological assays on the bloodstream forms of *T. cruzi* (trypomastigotes) (Rojas de Arias *et al.*, 1990) did not show any activity on this form of the parasite.

The data presented in this study indicate that a direct comparison between the efficacy of drugs against various forms of parasites is not always evident. It is difficult to determine whether the *in vitro* drug activity will correlate with its activity *in vivo* or not (Bell *et al.*, 1990). The *in vitro* activities of dehydrozaluzanin C on *Leishmania* and *T. cruzi* suggest that its efficacy is coupled with high cytotoxicity. The mechanism of action of our compound is still unknown. Recently a sesquiterpene lactone of natural origin, 15-deoxygoyazensolide, has been described as an active compound against blood forms of *T. cruzi* (Chiari *et al.*, 1991).

Table 3. Comparison of *in vitro* inhibitory effects (IC_{90} (µg/mL) on 15 strains of epimastigote forms of *T. cruzi* at different times after addition of dehydrozaluzanin C, benznidazole and nifurtimox

	Dehydrozaluzanin C			Benznidazole			Nifurtimox		
Time	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Strains									
Z2Cl4	25	25	10	>100	>100	100	>100	50	25
133 79 Cl9	25	25	25	>100	>100	50	>100	>100	100
C8 Cl1	25	10	10	>100	100	50	>100	>100	100
31R 16 Cl1	25	25	10	>100	>100	>100	>100	100	50
A 98Cl5	25	10	10	>100	100	100	>100	>100	100
MIL 4 Cl10	25	2.5	2.5	>100	50	25	>100	100	50
M6241 Cl4	25	25	5	>100	>100	>100	>100	>100	50
92 80 Cl1	25	10	10	>100	100	50	>100	100	50
CL	5	2.5	2.5	>100	>100	100	>100	>100	100
27 R 27 Cl1	50	25	10	>100	>100	>100	>100	>100	>100
Y	10	5	2.5	100	25	25	>100	100	100
Tulahuen	10	5	2.5	>100	100	25	>100	100	25
A 97 Cl7	10	5	2.5	100	50	50	>100	100	50
A 99 Cl7	5	2.5	2.5	>100	>100	100	>100	100	25
PB 3 Cl2	50	50	10	>100	>100	50	>100	50	25

Munnozia maronii often grows with other species of the *Munnozia* genus, *M. gigantea* (Rusby) and *M. senecioides* Benth. Different extracts of these plants were prepared and tested *in vitro* against promastigote forms of *Leishmania* and epimastigote forms of *Trypanosoma cruzi* but showed no inhibitory activity on these parasites. We have collected other samples of leaves of *Munnozia maronii* in the distant regions of the Department of La Paz, especially near the borders of Argentina. The petroleum ether extracts prepared from these samples did not present *in vitro* activity against the promastigote forms of *Leishmania* or epimastigote forms of *T. cruzi*. These results suggest the lack of dehydrozaluzanin C or only a weak concentration of this compound in these samples, and suggest a variation in the chemical composition of the leaves of *Munnozia maronii*.

Acknowledgments

We are grateful to Professor H. Moskowitz, Faculté de Pharmacie, Université Paris XI, for the modelization experiments with Alchemy II.

REFERENCES

Bell, C. A., Hall, J. E., Kyle, D. E., Grogl, M., Ohemeng, K. A., Allen, M. A. and Tidwell, R. R. (1990). Structure–activity relationship of analogs of pentamidine against *Plasmodium falciparum* and *Leishmania mexicana amazonensis*. *Antimicrob. Agents Chemother.* **34**, 1381–1386.

Berman, J. D. (1988). Chemotherapy of leishmaniasis: biochemical mechanisms clinical efficacy and future strategies. *Rev. Infect. Dis.* **10**, 560–586.

Bohlmann, F. and Grenz, M. (1979). Ein neues germacranolid aus *Munnozia maronii*. *Phytochemistry* **18**, 334–335.

Bohlmann, F., Jakupovic, J., King, R. M. and Robinson, H. (1981). New germacranolides, guaianolides and rearranged guaianolides from *Lasiolaena santosii*. *Phytochemistry* **20**, 1613–1622.

Bohlmann, F., Zdero, C. and Robinson, H. (1980). New sesquiterpene lactones and other constituents from *Fitchia speciosa*. *Phytochemistry* **19**, 1141–1143.

Chiari, E., Braga de Oliveira, A., Soares Raslan, D., Mesquita, A. L. and Tavares, K. G. (1991). Screening *in vitro* of natural products against blood forms of *Trypanosoma cruzi*. *Trans. R. Soc. Trop. Med. Hyg.* **85**, 372–374.

Coleman, R. E., Edman, J. D. and Semprevivo, L. H. (1989). The effect of pentostam and cimetidine on the development of leishmaniasis (*Leishmania mexicana amazonensis*) and concomitant malaria (*Plasmodium yoelii*). *Ann. Trop. Med. Parasitol.* **83**, 339–344.

Croft, S. L. (1988). Recent development in the chemotherapy of leishmaniasis. *Trends Pharmacol. Sci.* **9**, 376–381.

Evans, D. A. (1987). *Leishmania in vitro Methods for Parasite Cultivation*, ed. by A. E. R. Taylor and J. R. Baker, pp. 52–75. Academic Press, New York.

Hazim, A. F., Zaghloul, A. M. and Bohlmann, F. (1980). A further guaianolide from *Arctotis grandis*. *Phytochemistry* **19**, 2767–2768.

Muñoz, V. (1987). Obtención de la fracción activa y estudio de la actividad leishmanicida *in vitro* sobre promastigotes de la especie vegetal *Munnozia maronii*. Tesis de Farmacia y Bioquimica, Universidad de La Paz, Bolivia. Société Française de Parasitologie, Paris.

Rojas de Arias, A., Inchausti, A. and Fournet, A. (1990). *In vitro* trypanocidal activity of natural products against *Trypanosoma cruzi*, *7th International Congress of Parasitology, Paris*, (Abstract N° S 9 A).

Romo de Vivar, A., Cabrera, A., Ortega, A. and Romo, J. (1967). Constituents of *Zaluzania* species -II Structures of zaluzanin C and Zaluzanin D. *Tetrahedron* **23**, 3903.