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In vitro and *in vivo* leishmanicidal studies of *Peperomia galioides* (Piperaceae)

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Summary

Petroleum ether and methylene chloride extracts of *Peperomia galioides* and three prenylated diphenols, grifolic acid, grifolin and piperogalin exhibited *in vitro* antileishmanial activity. During the course of infection of BALB/c mice with *Leishmania amazonensis,* the treatments with each of these compounds did not influence the progression of the disease.

Key words: *Peperomia galioides,* prenylated diphenols, *in vitro* and in vivo leishmanicidal studies.

Introduction

Leishmaniasis is a parasitic disease that affects 12 million people in the world and 350 million at risk, of whom some 1.5 to 2 million will be infected each year (World Health Organization, 1995). Pentavalent antimonials in the form of sodium stibogluconate (Pentostam®) or meglumine antimonate (Glucantime®) are the drugs of choice for treatment of all forms of leishmaniasis but require long- term therapy and often induce toxic effects.

In Bolivia, we have developed a searching program for Bolivian plants containing new natural active compounds against leishmaniasis (Fournet et al., 1994) In the *in vitro* preliminar screening, the petroleum ether and chloroformic extracts of whole plant, *Peperomia galioides* H. B. K. (Piperaceae) displayed activity at 25 µg/ml against three strains of *Leishmania* ssp., *L. amazonensis, L. braziliensis* and *L. donovani. P. galioides* is an herbaceous plant which grows at an altitude of 3500–2500 m in the Andean mountains. To our knowledge, this plant is not used in traditional medicine. In a previous study, we have isolated and identified by fractionation and purification monitored by bioassays, three prenylated-diphenols compounds, grifolic acid, grifolin and piperogalin (Mahiou et al., 1995). The present paper describes the *in vitro* and *in vivo* antileishmanicidal effects of these compounds as well as the activity of the initial crude extracts of *P. galioides.*

Materials and Methods

Chemicals

The prenylated-diphenols, grifolin, piperogalin and grifolic acid were isolated from *Peperomia galioides* H. B. K. The plant was collected in Unduavi (Bolivia) in April 1986 by A. Fournet and identified by D. H. Valdebenito (Ohio State University, Columbus, Ohio, USA). Voucher specimens are deposited (AF 615) in the National Herbarium of Bolivia (La Paz). The isolation was performed by fractionation and purification monitored by bioassays as previously described (Mahiou et al., 1995). Physical and spectral data (proton and ^{13}carbon nuclear magnetic resonance and mass spectrometry) were used to control the purity of terpenoids. The structures of terpenoids are shown in Fig. 1. N-Methyl-

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glucamine antimonate (Glucantime®) equivalent to 0.28 mg Sb^v/ml was purchased from Rhône-Poulenc, Paris, France.

Biological assays

Culture and maintenance of the promastigote forms of Leishmania *ssp.* The maintenance, cultivation, and isolation of promastigote-stage parasites have been described in detail elsewhere (Fournet et. al., 1994). The IC_{90S}, which represent the drug concentration required to inhibit parasite growth by 90%, were evaluated from the plot of log dose versus parasite growth expressed as a percentage of the value for the control ans are the means of at least two independent experiments performed in triplicate with different stock drug solutions.

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.

Infection

L. amazonensis MHOM/IFLA/BR/67/PH8 was used and maintained by passage every 6 to 8 weeks in hamsters. BALB/c (n = 8) were inoculated in the right hind footpad with $2x10^6$ 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 one day prior to the inoculation of amastigotes and were continued between 8 to 12 weeks.

Drug treatment

The treatments were initiated four 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 six. N-Methylglucamine antimonate was dissolved in 50 µl of PBS and administered to BALB/c mice in regimen of 100 mg per kg of body weight daily for 15 days by subcutaneous route. The terpenoids and petroleum ether or CH_2Cl_2 extracts of *P. galioides* were tested at dose level of 25 mg/kg and were dissolved in 40 µl PBS, 5 µl of polysorbate (Tween 80, OSI, France) and 5 µl of dimethylsulfoxide (DMSO). Drugs were administered orally once daily for 15 days or by subcutaneous route for 10 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 sacrified ten days 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 Potter 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 x 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 given means ± standard deviations, unless indicated otherwise. Comparison of parasite suppression in the infected footpad of the untreated control group and drug-treated group was analyzed by two-way analysis of variance (ANOVA) and by Student's t test. Data were considered statistically significant at $P<0.05$. Analyses were performed on a personal computer with Excel 5.0a.

Results and discussion

In vitro studies

The antileishmanial activity of *P. galioides* and three prenylated diphenols, grifolin, piperogalin and grifolic acid is presented in Table 1, and also for comparison, results obtained with two leishmanicidal reference drugs, meglumine antimonate and pentamidine. The crude extracts of *P. galioides* showed activity against three strains of *Leishmania* at 100 µg/ml. The fractionation and purification, monitored by bioassays, led to isolation of three compounds, identi-

Table 1. In vitro antileishmanial activity (IC90 (mg/ml) of total extracts of *P. galioides* and prenylated diphenols.

Compounds	IC_{90} (µg/ml)			
	L.b.[2] (2903)	L.a.[3] (PH 8)	L.a.[3] (H–142)	L.d.[4] (HS 70)
Petroleum ether extract	100	100	100	100
Methylene chloride extract	100	100	100	100
Piperogalin	15	15	15	15
Grifolin	20	20	20	20
Grifolic acid	100	100	100	100
Pentamidine[1]	1	1	1	1
N-methylglucamine antimonate[1]	>100	>100	>100	>100

[1] Reference drugs
[2] *Leishmania braziliensis,* [3]*Leishmania amazonensis,* [4]*Leishmania donovani*

fied as piperogalin, grifolin and grifolic acid. The IC_{90} values of piperogalin, grifolin and grifolic acid were 15 µg/ml, 20 µg/ml and 100 µg/ml respectively. These compounds were described by Mahiou et al., 1995. The chemical structures of the products are presented in Fig. 1.

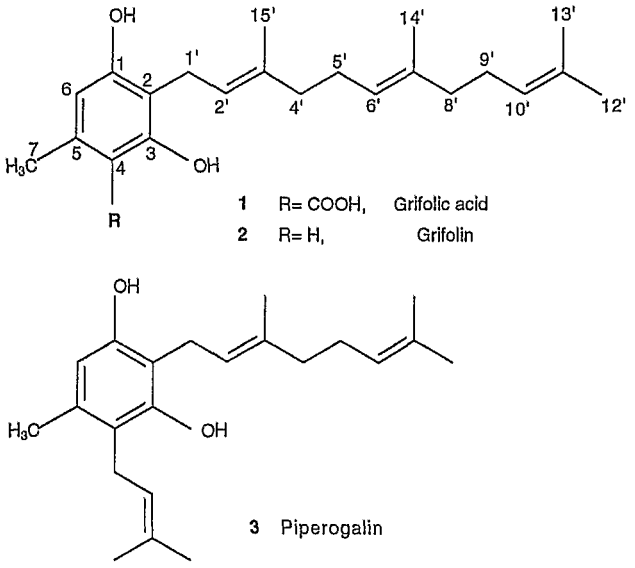

Fig. 1. The chemical structures of prenylated diphenols.

In vivo studies

The results obtained with Glucantime®, *P. galioides* extracts, grifolin, grifolic acid and piperogalin treatments on the development of *L. amazonensis* lesions in BALB/c are presented in Tab. 2. The subcutaneous treatment with antimonial agent at 100 mg/kg for fifteen days reduced significally the lesion weight by 78.89% ($P<0.001$) and the parasite loads in infected footpads by 87.61% ($P<0.001$) versus the untreated mice. Effect of treatments with *P. galioides* petroleum ether extract or with the active compound isolated by fractionation monitored by bioassays, grifolic acid, during the course of infection of BALB/c mice infected with *L. amazonensis* is presented in Fig. 2. No significant difference was observed between the untreated BALB/c mice and treated mice with these compounds. In contrast, we have observed in grifolic acid treated mice a significant increasing of the weight lesions by 131.47% (subcutaneous via) and 182.51% (oral route) and the parasite loads by 183.41% (subcutaneous route) and 305.51% (oral route). The effects of *P. galioides* methylene chloride extract and the *in vitro* active compounds isolated from this extract by bioassay guided fractionation, grifolin and piperogalin are presented in Fig. 3. Also, none of these compounds could

Table 2. Effect of treatments with N-methylglucamine antimonate by subcutaneous route, *P. galioides* extracts and prenylated phenols administered by oral or subcutaneous routes on *Leishmania amazonensis* infected BALB/c mice.

Compounds (dose)	Via adm.[1]	Lesion wt (g) (mean±SD)[2]	%Suppression of weight lesion	% Supression of Mean parasites	
				parasites load in the lesion	quantitation in the lesion
None(control)[b]		0.0660 ± 0.0239	–	–	5.1×10^6
Meglumine antimonate[c] (28 mg Sb^V/kg)x15	subcutaneous	0.0139 ± 0.0106	–78.89% ($P<0.001$)*	–87.61% ($P<0.001$)*	6.3×10^5
Petroleum ether extract (25 mg/kg)x10	subcutaneous	0.0955 ± 0.04477	+44.72%	+40.58%	7.14×10^6
Petroleum ether extract (25 mg/kg)x15	oral	0.0849 ± 0.0256	+28.53%	+16.37%	5.9×10^6
Methylene chloride extract – (25 mg/kg)x10	subcutaneous	0.1016 ± 0.0570	+53.63%	+21.73%	6.2×10^6
Methylene chloride extract – (25 mg/kg)x15	oral	0.0885 ± 0.0142	+34.02%	+99.96%	1.0×10^7
Piperogaline (25 mg/kg)x10	subcutaneous	0.2042 ± 0.0312 ($P<0.001$)*	+209.21 ($P<0.01$)*	+353.39%	2.3×10^7
Piperogalin (25 mg/kg)x15	oral	0.1818 ± 0.0434 ($P<0.001$)*	+175.32% ($P<0.05$)*	+362.59	2.4×10^7
Grifolin (25 mg/kg)x10	subcutaneous	0.1420 ± 0.0370 ($P<0.01$)*	+114,99%	+94,55%	9.9×10^6
Grifolin (25 mg/kg)x15	oral	0.1617 ± 0.0389 ($P<0.003$)*	+144.88% ($P<0.02$)*	+169.67%	1.4×10^7
Grifolic acid (25 mg/kg)x10	subcutaneous	0.1529 ± 0.0485 ($P<0.02$)*	+131.47% ($P<0.004$)*	+183.41%	1.5×10^7
Grifolic acid (25 mg/kg)x15	oral	0.1866 ± 0.0651 ($P<0.003$)*	+182.51% ($P<0.03$)*	+305.51%	2.1×10^7

[1] Via administration

[2] Values represent the means ± standard deviation, six mice per group were used.

* *P* values were also calculated by ANOVA.

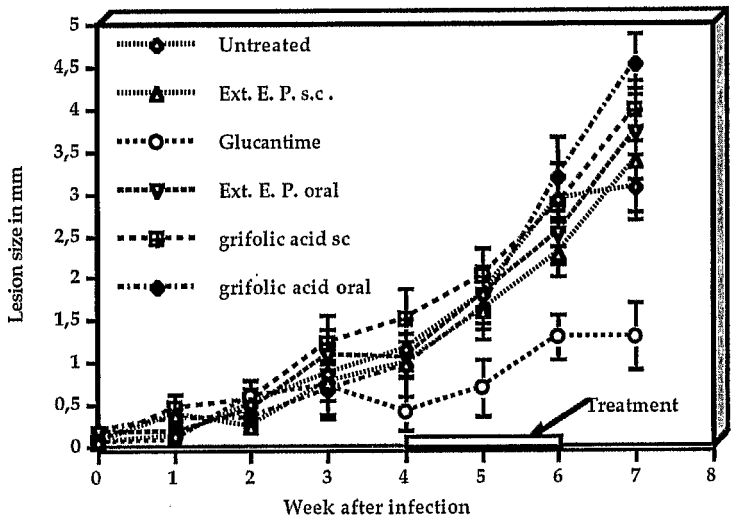

Fig. 2. Effect of treatments with meglumine antimonate (Glucantime®) (100 mg per kg per days for 15 days), petroleum ether extract of *Peperomia galioides,* grifolic acid administered orally at 25 mg/kg once daily for 15 days or subcutaneously at 25 mg/kg for 10 days, during the course of infection of BALB/c mice with *L. amazonensis.* Each point represents the mean difference in size ± standard deviation of the mean between infected and uninfected footpads ($n = 6$).

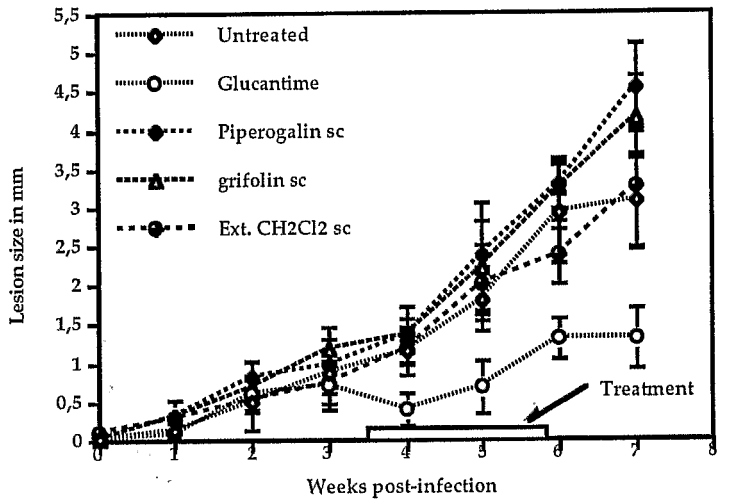

Fig. 3. Effect of treatments with meglumine antimonate (Glucantime®) (100 mg per kg per days for 15 days), methylene chloride extract of *P. galioides,* grifolin and piperogalin administered by subcutaneous route at 25 mg/kg for 10 days, during the course of infection of BALB/c mice with *L. amazonensis.* Each point represents the mean difference in size ± standard deviation of the mean between infected and uninfected footpads ($n = 6$).

reduce the progression of the *L. amazonensis* infection in the infected footpads. In contrast, it seemed that they favoured a significant growth of parasites.

The petroleum ether and methylene chloride extracts of *P. galioides* and the active prenylated-diphenols obviously exhibited an *in vitro* leishmanicidal properties. The confirmation of the *in vivo* activity of these compounds on the course of infection BALB/c mice with *L. amazonensis* did not give the expected results.

In conclusion, the data presented in this study indicate that a direct comparison between the efficacy of extracts or compounds against various strains of *Leishmania* is not always evident (Croft, 1994; Hudson, 1994). It is not easy to specify wether the *in vitro* drug activity will correlate with its *in vivo* activity or not (Bell et all., 1990; Fournet et al., 1993; Hocquemiller et al., 1991; Iwu et al., 1994). The *in vitro* activities of extracts and prenylated-diphenols on extracellular forms of *Leishmania* suggest that its activity is coupled with cytotoxicity.

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