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Short communication

Antileishmanial activity of furoquinolines and coumarins from *Helietta apiculata*

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ABSTRACT

The bark infusion of *H. apiculata* are used to treat wound healing related to cutaneous leishmaniasis and as anti-inflammatory.
Aim of the study: To isolate, purify active constituents of *H. apiculata* stem bark, and evaluate their *in vitro* and *in vivo* antileishmanial activities.
Materials and methods: Isolation by chromatographic methods and chemical identification of furoquinoline alkaloids and coumarins, then evaluation of the *in vitro* leishmanicidal activity of these compounds against three strains of *Leishmania* sp. promastigotes and *in vivo* against *Leishmania amazonensis* in Balb/c mice.
Results: Furoquinoline alkaloids and coumarins presented a moderate *in vitro* activity against promastigote forms of *Leishmania* sp. with IC_{50} values in the range between 17 and $>50\,\mu g/ml$. Balb/c mice infected with *Leishmania amazonensis* were treated with γ-fagarine by oral route, or with 3-(1'-dimethylallyl)-decursinol or (-)-heliettin by subscutaneous route for 14 days at 10 mg/kg daily. In these conditions, γ-fagarine, 3-(1'-dimethylallyl)-decursinol and (-)-heliettin showed the same efficacy as the reference drug reducing by 97.4, 95.6 and 98.6% the parasite loads in the lesion, respectively.
Conclusion: These compounds showed significant efficacy in *L. amazonensis* infected mice, providing important knowledge to improve its potential role for a future use in the treatment of cutaneous leishmaniasis.

Introduction

Helietta apicultata Benth. (Rutaceae) is a South American tree or shrub growing in Paraguay, Brazil and Argentina commonly called « yvyra ovi » in Paraguay or « canela-de-veado » in Brazil. The infusion of bark of *H. apiculata* are used in Paraguay as wound healing that could be related to leishmanicidal ulcers, and also as anti-inflammatory when are associated with the hierba mate, *Ilex paraguayensis* St Hillaire (Aquafoliaceae). Following the evaluation of the leishmanicidal activity of the quinolines (Fournet et al., 1993) we have found a common plant growing in Paraguay and containing analogs of these compounds, the furoquinoline alkaloids. In various studies (Goloubkova et al. 1998) from *Helietta apiculata* collected in Argentina and Brazil, furoquinoline alkaloids and coumarins were isolated and identified.

In a preliminary screening, alkaloidal extract of the stem bark of *H. apiculata* displayed an *in vitro* activity at a concentration of 50 μg/ml against three strains of promastigote forms of *Leishmania* species, *L. braziliensis*, *L. amazonensis* and *L. donovani*. Activity-directed fractionation and purification of the alkaloidal and coumarins constituents of the stem bark gave five furoquinoline alkaloids and four coumarins identified by their physical and spectral data.

In the present study, we describe the *in vitro* antileishmanial activity of the isolated compounds, and the efficacy of γ-fagarine and two coumarins, (+)-3-(1'-dimethyllallyl)-decursinol and (-)-heliettin when they are administered by the oral route (γ – fagarine) or by subcutaneous (coumarins) via in the *Leishmania amazonensis* infected Balb/c mice.

Materials and methods

General experimental procedures

UV spectra were measured on a Shimadzu UV 1601 spectrometer in MeOH. The ^{1}H-NMR and ^{13}C-NMR spectra were obtained

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with JEOL GSX 270 MHz spectrometer in $CDCl_3$. Mass spectra were recorded on a Nermag R30-10 Mass spectrometer (EIMS).

Plant material

Stem barks of *H. apiculata* Benth were collected by A. Fournet in March 1995 near Piribebuy, Department of Cordillera, Paraguay, and identified by N. Soria (Department of Botany, National University of Asuncion, Paraguay). A voucher specimen (AF 912) has been deposited at the Herbarium of Chemical Sciences Faculty, San Lorenzo, Paraguay.

Extraction and isolation

The dried pulverized stem barks (1.1 kg) were alkalinized with 6% NH_4OH (approximately 250 ml) and extracted with $CHCl_3$ in a Soxhlet apparatus. The extract was concentrated under reduced pressure to yield a residue (24.3 g). This extract was fractionated first by vacuum liquid chromatography on Merck silica gel 60 H and successively eluted with $CHCl_3$ and gradient of $CHCl_3$/MeOH, 18 fractions of 200 ml were collected. Repetition of the chromatographic separations (silica gel) and preparative TLC led to the isolation of nine known compounds. By comparison with the reported data, the known compounds were identified as dictamine (12 mg) (Ito et al. 2000), γ-fagarine (98 mg), skimmianine (17 mg) (Dreyer 1969), flindersiamine (13 mg) (Vaquette et al. 1976), maculine (31 mg) (Robertson 1963), scopoletin (13 mg), scoparone (8 mg) (Sankar et al. 1982), (+)-3-(1'-dimethylallyl)-decursinol (90 mg) (Galan et al. 1988) and (-)-heliettin (123 mg) (Massanet et al. 1987; Pozzi et al. 1967).

Parasites

In vitro anti-leishmania activity (promastigote forms)

L. amazonensis (MHOM/BR/PH8), *L. infantum* (MHOM/FR/91/LEM 2259), and *L. braziliensis* (MHOM/BR/75/M-2903) were grown in 96-well microtiter plates at 27 °C in a 5% CO_2 atmosphere in the dark, in M199 medium containing 10% fetal calf serum (FCS) and supplemented with 40 mM HEPES, 100 μM adenosine, 0.5 mg hemin per liter, and 50 μg gentamycin per ml. Two hundred microliters of culture medium were placed in the well containing the maximum concentration of extract, and 100 μl in the following (C_2 to C_7 and controls); 2 μl of an extract solution of 20 mg/ml in DMSO were added in C_1 and a serial dilution in the wells was performed. After 1 h at 27 °C under a 5% CO_2 atmosphere, 100 μl of culture medium complemented with 1.75×10^6 parasites/ml, from a logarithmic phase culture, were added. The final volume in the well was 200 μl. After a 72 h incubation period, the viability of parasites was evaluated by the tetrazolium-dye (MTT) colorimetric method. The results are expressed as the concentration inhibiting parasite growth by 50% (IC_{50}). Amphotericin B was the reference drug.

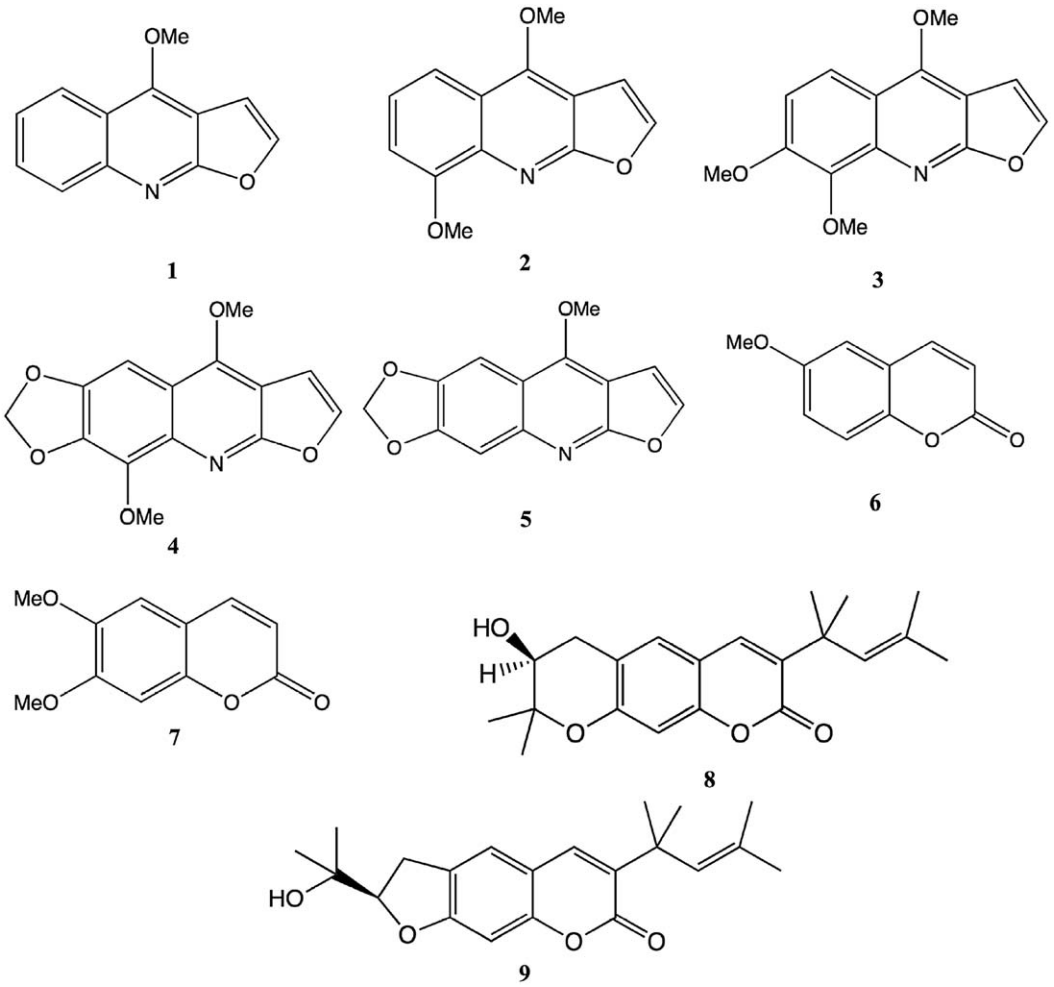

Fig. 1. The structure of furoquinolines and coumarins from the stem bark of *Helietta apiculata*: dictamine (**1**), γ-fagarine (**2**), skimmianine (**3**), flindersiamine (**4**), maculine (**5**), scopoletin (**6**), scoparone (**7**), (+)-3-(1'-dimethylallyl)-decursinol (**8**) and (-)-heliettin (**9**).

In vivo activity against Leishmania amazonensis

Chemicals. The structures of γ-fagarine, 3-(1'-dimethylallyl)-decursinol (**8**) and (-)-heliettine (**9**) are shown in Fig. 1 and N-methylglucamine antimonate (Glucantime®) equivalent to 0.28 mg Sb^v/ml was purchased from Rhône-Poulenc, Paris, France.

Animals: Female and male BALB/c were purchased by the Faculty of Veterinary Sciences, National University, La Plata, Argentina and JICA (Japan International Cooperation Agency) and bred at the Instituto de Investigaciones en Ciencias de la Salud (Asuncion, Paraguay) and were 6 to 8 weeks of age when used. Golden hamsters (*Mesocritus auratus*) were used to maintain the parasites. The animals were sacrificed by cervical dislocation following international recommandations (AVMA 2007).

Infection: *L. amazonensis* MHOM/IFLA/BR/67/PH8 is used and identified by isoenzyme analysis. The strain was maintained by 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). Disease progression was monitored by the measurement of lesion diameters weekly for 10 weeks.

Drug treatment: the treatment was initiated six weeks after inoculation when the infection was well established, and lesions were obvious. Two days before administration of drug, the mice were ramdomly divided into groups of eight. N-methylglucamine antimonate was dissolved in 50 µl of PBS and administered to BALB/c mice in regimens of 100 mg per kg of body weight daily for 15 days by subcutaneous route. γ-fagarine, 3-(1'-dimethylallyl)-decursinol and (-)-heliettin were tested at a dose level of 10 mg/kg body weight and were made up in 50 µl PBS and 5 µl of polysorbate (Tween 80, OSI, France). 3-(1'-dimethylallyl)-decursinol and (-)-heliettin were administered by subcutaneous route and γ-fagarine by oral route for 15 days. The untreated group received daily 50 µl of PBS and 5 µl of Tween 80.

Effect of treatment: The animals were sacrificed one week after cessation of treatments to assess parasitological loads in the infected footpad. Briefly, the mice were killed and the lesions of the infected footpad were excised, weighed and homogenized in a glass Teflon (Potter, OSI) homogenized in 5 ml of RPMI 1640 (Gibco, France) tissue culture medium supplemented with 10% fetal calf serum, 1 ml of glutamine (GIBCO, France) (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 or spleen 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

The program JMP 3.2.2 (version 3.2.2, ASA Insitute Inc., Cary, NC) for the Macintosh was used to calculate the means ± standard deviations (Sds). The difference between groups were determined by using Student's t test or the Kruskal–Wallis nonparametric analysis of variance test for comparing two groups. Significance was established for a P value of < 0.05.

Results and discussion

At a concentration of 100 µg/ml, the alkaloidal extract prepared from the stem bark of *H. apiculata* inhibited the growth of three strains of *Leishmania* sp. (Table 1). Bioassay-directed fractionation of this extract has led to the isolation, five known furoquinolines, dictamine (**1**), γ-fagarine (**2**), skimmianine (**3**), flindersiamine (4), maculine (**5**), and four known coumarins, scopoletin (**6**), scoparone (**7**), 3-(1'-dimethylallyl)-decursinol (**8**) and (-)-heliettin (**9**). Among the isolated compounds, only γ-fagarine, maculine, 3-(1'-dimethylallyl) decursinol and heliettine were active against promastigote forms of *Leishmania* spp. (IC50 between 17 and 30 µg/ml).

Table 1
Antileishmanial activity of the alkaloidal extract and compounds isolated from stem bark of *Helietta apiculata*.

Compound	IC_{50} (µM)		
	L. amazonensis[a]	*L. infantum*[a]	*L. braziliensis*[a]
$CHCl_3$ extract (µg/ml)	35.5 ± 1.5	28.5 ± 1.8	39.4 ± 2.9
Dictamine	> 50	> 50	> 50
γ-fagarine	17.3 ± 1.2	26.5 ± 2.5	22.2 ± 2.3
Skimmianine	> 50	> 50	> 50
Maculine	25.5 ± 2.5	29.0 ± 1.5	20.8 ± 1.9
Scopoletin	> 50	> 50	> 50
Scoparone	> 50	> 50	> 50
3-(1'-dimethylallyl)-decursinol	35.8 ± 4.5	27.5 ± 3.0	32.1 ± 2.8
(-)-Heliettin	18.5 ± 1.5	27.4 ± 1.8	21.5 ± 1.5
Miltefosine	9.4 ± 1.8	7.5 ± 1.5	10.4 ± 1.4
Amphotericin B	0.05 ± 0.01	0.02 ± 0.01	0.10 ± 0.03

[a] Results are the mean of three independent experiments with SD less than 10% in all cases.

The treatment with reference drug or γ-fagarine (**2**) produced a significantly reduction of the lesion weight by 66.9% ($p < 0.01$) and 90.5% ($p < 0.001$) respectively and a drastic reduction of the lesional parasites by 95.2% ($p < 0.01$) and 97.4% ($p < 0.005$) (Table 2). When we compare statistically the efficacy of the two drugs, we see that the results obtained with the oral treatment of γ-fagarine (**2**) were significantly more effective than those with the cutaneous N-methylglucamine treatment. It should be observed that the treatment with γ-fagarine was well tolerated by the mice.

In the mice treated with coumarins, the parasite burdens in the infected footpads were significantly reduced ($p < 0.001$) for 3-(1'-dimethylallyl)-decursinol (**8**) or (-)-heliettin (**9**) subcutaneous treatment by 95.6 and 98.6%, respectively (Table 2).

The furoquinoline alkaloids are biogenetically derived from the 2-substituted oxygenated 4-quinolones after a prenylation at C-3. The formation of the furan ring with the prenyl group at C-2 or C-4 gives linear or angular dihydrofuroquinolines or pyranoquinolines (Gray 1993; Mester 1983).

The weak *in vitro* activity against the culture of *Leishmania* species and good *in vivo* efficacy of γ-fagarine could be due to pharmacological activity of the metabolites. In previous studies, Desrivot et al. (2007) demonstrated that 2-substituted quinolines were rapidly metabolized by almost all cytochromes P450 (CYP450). In the course of drug discovery, parameters such as absorption, distribution, metabolism, excretion and toxicology (ADMET) are determinant parameters that should be studied at the earliest stages (Ansede and Thakker 2004). In mammals, (CYP450) are responsible for the oxidative metabolism of endogenous and exogenous products (Ortiz de Montellano and De Voss 2002). It would be quite possible that γ-fagarine (**2**) could be regarded as a prodrug. Therefore, it could be interesting to evaluate the *in vivo* antileishmanial activity of these metabolites.

The results obtained in this study shows that subcutaneous treatment with 3-(1'-dimethylallyl)-decursinol (**8**) or (-)-heliettin (**9**) at 10 mg/kg for 14 days produced the same effect as treatment with the reference drug (N-methylglucamine antimonate).

Table 2
Effects of treatments with N-methylglucamine antimonate (100 mg/kg per day) administered by subcutaneous route, γ-fagarine administered orally at 10 mg/kg, (+)-3-(1'-dimethyllallyl)-decursinol and (-)-heliettin administered by subcutaneous route at 10 mg/kg daily for 14 days on *L. amazonensis*-infected Balb/c mice (n=8).

Parameters	Untreated mice	N-methylglucamine antimonate	γ-fagarine	(+)-3-(1'-dimethyllallyl)-decursinol	(-)-heliettin
Route of administration	-	subcutaneous	oral	Subcutaneous	Subcutaneous
Lesion weight (g) (mean ± SD)	0.072 ± 0.044	0.024 ± 0.012	0.007 ± 0.009	0.033 ± 0.018	0.025 ± 0.020
% Supression of lesion weight	-	66.9[a]	90.5[b]	65.6[a]	73.9[a]
Mean number of parasites in lésion	$1.3 \times 10^7 \pm 0.2 \times 10^7$	$6.2 \times 10^5 \pm 1.1 \times 10^{5b}$	$3.4 \times 10^5 \pm 1.1 \times 10^{5b}$	$0.4 \times 10^5 \pm 0.3 \times 10^{5b}$	$0.10 \times 10^5 \pm 0.08 \times 10^{5b}$
% Supression of parasites burden in lesion	-	95.2	97.4	95.6	98.9

Values represent the means ± standard deviation (n = 8).

[a] $p < 0.01$ for treated versus control mice.
[b] $p < 0.001$ for treated versus control mice.

A number of hydroxy derivatives of cinnamic acid, the biogenetic precursor of coumarins, have been evaluated *in vitro* against *Leishmania* sp, (Tasdemir et al. 2006) but never in experimental infection by *Leishmania amazonensis*. Polyphenols, such as flavonoids and cinnamates play their role in the prevention of many degenerative diseases (Manach et al. 2004). Bioavailability studies have shown that the circulating form of most flavonoids is as a conjugate formed by deglycosylation, glucuronidation, sulfation, and methylation reactions mediated by a range of enzymes in the small intestine, liver, and colon, which is then excreted into bile and urine (Williamson et al. 2005).

These ethnopharmacological, chemical and biological studies showed that the stem bark of *H. apiculata* is effective against the cutaneous leishmaniasis. This work describes the first example of activity of coumarins against experimental cutaneous leishmaniasis. The present results show an interesting effect of this compound on cutaneous leishmaniasis by oral route, giving it a promising lead.

Uncited reference

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