Acetogenins and Other Compounds from *Rollinia emarginata* and Their Antiprotozoal Activities

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Abstract: Bioactivity-directed fractionation of the MeOH extract of the stem barks of *Rollinia emarginata* resulted in the isolation of six compounds, four acetogenins, rolliniastatin-1, sylvaticin, squamocin, and rollidecin B, one lignan, lirioresinol B, and an oxoaporphine, liriodenin. Their structures were determined by spectroscopic analysis and their *in vitro* leishmanicidal and trypanocidal properties are reported.

Key words: *Rollinia emarginata* Schldl., Annonaceae, acetogenins, *Leishmania*, *Trypanosoma cruzi*, Leishmanicidal, trypanocidal.

Introduction

Diseases caused by trypanosomatid parasites, human African diseases, leishmaniasis, and Chagas' disease afflict millions of people in the World. These three major diseases have no effective common cures (1). Currently, no treatment is available for these diseases. Seeking new chemotherapeutic compounds relevant to leishmaniasis and Chagas' disease, natural products (2) represent an original alternative to find new active compounds.

Rollinia emarginata Schldl. (Annonaceae) is a 15–18 m tall tree growing in the tropical South America (Paraguay, Bolivia, Argentine and South of Brasil) (3). In Paraguay this tree is called *arituki î* by the Guarani Indians which means "aratiku" = fruit of sky and "î" = small or low. Stem barks of *R. emarginata* are used with the hierba maté, *Ilex paraguayensis* St Hilaire (Aquifoliaceae) to treat migraine and as relaxant. From the aerial parts of *R. emarginata*, the isolation of three alkaloids (anonaine, asimilobine and reticuline) has been reported (4).

In a preliminary screening, the crude extracts of stem barks of *R. emarginata* displayed activity *in vitro* at a concentration of 100 μg/ml against three strains of promastigote forms of *Leishmania* species, *L. braziliensis*, *L. amazonensis*, and *L. donovani* and against the bloodstream forms of another Trypanosomatidae, *Trypanosoma cruzi*, responsible for the Chagas' disease.

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Materials and Methods

General experimental procedures

Optical rotations were determined on a Schmidt-Haensch Polartronic I polarimeter. UV spectra were obtained on a Philips PU 8720 spectrometer. IR spectra were measured on a Perkin-Elmer 257 spectrometer. The ^{1}H-NMR and ^{13}C-NMR spectra ($CDCl_3$) were obtained with Bruker AC-200 or AC-400 instruments at 200 and 50 MHz or at 400 and 100 MHz, respectively. EIMS and CIMS (methane) were performed on a Nermag R10-10C spectrometer. HPLC analyses were performed with a Waters 501 pump, a Waters 991 spectrophotometer (214 nm) and a Waters WISP automatic injector on a μBondapak C_{18} prepacked column (10 μm, 8 × 100 mm), elution with MeOH-H_2O at various mixtures and at flow rate 1 ml/min. Preparative HPLC was carried out with a Millipore-Waters (Milford MA, USA) system equipped with a 590 pump, a SSV injector, and a 484 UV detector (214 nm), and a μBondapak C_{18} prepacked column (10 μm, 25 × 100 mm), elution with MeOH-H_2O at various grad at flow rate 10 ml/min.

Plant material

The stem barks of *Rollinia emarginata* Schldl. were collected by A. Fournet in September 1995, in Paraguay near Piribebuy, Department of Cordillera and identified by N. Soria (Department of Botany, National University of Asuncion, Paraguay). A voucher specimen (AF 925) has been deposited at the Herbarium of Chemical Sciences Faculty, Asuncion, Paraguay.

Extraction and isolation

The dried pulverized stem barks (960 g) were macerated with MeOH. The MeOH extract (60 g) was diluted with 8% vol. of water and submitted to liquid-liquid partition with hexane, leading to 9 g of a concentrated extract. The hydromethanolic phase was extracted with CH_2Cl_2 to yield 15 g of extract, of which it was submitted to extraction liquid-liquid by CH_2Cl_2 (1.2 g), then the mixture CH_2Cl_2/MeOH 90 : 10 (6.5 g) and by MeOH (7.5 g). The CH_2Cl_2 extract (1.2 g) was chromatographed on a silica gel 60 H Merck column (40 g) and successively eluted with CH_2Cl_2/MeOH, 99 : 1, and AcOEt/MeOH, 50 : 50, to yield 74 fractions (30–40 ml each fraction). Fractions, 21–23, 56–60, and 67–74 were combined into pools according to their similar TLC patterns. The fractions 21–23 were subjected to preparative TLC using CH_2Cl_2/AcOEt/MeOH, 90 : 9 : 1,

to yield lirioresinol B (11 mg), R_f 0.42. HPLC purification of 140 mg from the fractions 56–60 (265 mg) using a μBondapak C_{18} prepacked column (10 μm, 25 × 100 mm) eluted with MeOH/H_2O (85 : 15) (flow rate 10 ml/min, UV detection at 214 nm) afforded rolliniastatin (67 mg, t_R = 30 min). 100 mg of fractions 61–66 (172 mg) were submitted to semipreparative HPLC using MeOH/H_2O (82 : 18) (flow rate 10 ml/min, UV detection at 214 nm) afforded sylvaticin (17 mg, t_R = 26 min) and squamocin (17 mg, t_R = 37 min). The fractions 67–74 (55 mg) were submitted twice to HPLC purification using MeOH / H_2O (82 : 18) and MeOH/H_2O (72 : 28) (flow rate 10 ml/min, UV detection at 214 nm) to obtain rollidecin (3 mg, t_R = 111 min).

The extract CH_2Cl_2/MeOH, 90 : 10 (6.5 g) was treated with HCl (0.1 N), and the acidic solution was basified with NH_4OH to pH 9–10 and extracted with CH_2Cl_2. Evaporation of the organic solvent under reduced pressure led to the alkaloid extract. This extract was chromatographed on a silica gel column (30 g) (Kieselgel H, Merck), eluted with $CHCl_3$ and with $CHCl_3$/MeOH 90 : 10 (150 ml) to provide liriodenine (12 mg).

Rolliniastatin-1 (**1**): $C_{37}H_{66}O_7$; $[\alpha]_D$: +17° (*c* 0.20, MeOH); UV (MeOH): λ_{max} nm (log ε) = 215 (5.03); IR data (3); CIMS: *m/z* = 623 $[MH]^+$ (100), 605 $[MH - H_2O]^+$, 587 $[MH - 2H_2O]^+$, 569 $[MH - 3H_2O]^+$, 451, 433, 415, 381, 363, 345, 311, 293, 275, 241, 171, 153, 141, 111, 97; SMIE: *m/z* = 415, 381, 363, 345, 311 (100), 293. For ^{1}H- and ^{13}C-NMR data (5).

Sylvaticin (**2**): $C_{37}H_{66}O_8$; $[\alpha]_D$: +5° (*c* 0.21, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε) = 207 (3.96); IR data (4), (12); CIMS: *m/z* = 639 $[MH]^+$ (100), 621 $[MH - H_2O]^+$, 603 $[MH - 2H_2O]^+$, 585 $[MH - 3H_2O]^+$, 567 $[MH - 4H_2O]^+$, 449, 431, 413, 379, 361, 343, 309, 291, 267, 171, 153, 141, 123, 111, 97; EIMS: *m/z* = 361, 309, 267 (100), 141, 111, 97, 69, 43; ^{1}H- and ^{13}C-NMR data (6).

Squamocin (**3**): $C_{37}H_{66}O_7$; $[\alpha]_D$: +19° (*c* 0.23, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε) = 210 (3.88); IR data (5); CIMS: *m/z* = 623 $[MH]^+$, 605 $[MH - H_2O]^+$, 587 $[MH - 2H_2O]^+$, 569 $[MH - 3H_2O]^+$, 519, 501, 483, 435, 417, 399, 365, 347, 329, 295 (100), 267, 239, 169, 111, 97; EIMS: *m/z* = 417, 399, 347, 329, 295 (100), 267, 239, 169, 111, 97, 69; ^{1}H- and ^{13}C-NMR (7).

Rollidecin B (**4**): $C_{37}H_{66}O_8$; $[\alpha]_D$, UV and IR data see (8); CIMS: *m/z* = 639 $[MH]^+$ (100), 621 $[MH - H_2O]^+$, 603 $[MH - 2H_2O]^+$, 585 $[MH - 3H_2O]^+$, 567 $[MH - 4H_2O]^+$, 449, 431, 413, 379, 361, 309, 299, 291, 281, 263, 247, 229, 211, 171, 153, 141, 123, 111, 97; EIMS: *m/z* = 449, 379, 309 (100), 211, 141, 97, 43; ^{1}H- and ^{13}C-NMR data (8).

Lirioresinol B (**5**): $C_{22}H_{26}O_8$; $[\alpha]_D$: (5); UV (EtOH) λ_{max} nm (log ε) = 212 (4.65); IR data (9); CISM: *m/z* = 419 (100), 235, 181; EIMS: *m/z* = 418, 387, 336, 251, 235, 226, 210, 193, 182, 181 (100), 167, 154; NMR ^{1}H-NMR (200 MHz, $CDCl_3$); ^{13}C-NMR (50 MHz, $CDCl_3$).

Liriodenine (**6**): $C_{17}H_9NO_3$; UV (EtOH) λ_{max} nm (log ε) = 204 (4.53), 248 (4.42), 268 (4.33), 311 (3.84), 415 (2.44); (EtOH + HCl): 257 (4.47), 278 (4.40), 394 (3.86); IR data see (10); CISM: *m/z* = 276 (100), 246; EIMS: *m/z* = 275 (100), 247, 246, 219, 217, 189, 188, 162; ^{1}H- and ^{13}C-NMR data (10).

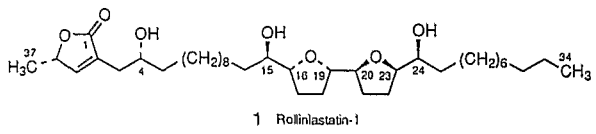
Leishmanicidal activity: Cultures of *Leishmania* ssp. were obtained from IICS (Instituto de Investigaciones en Ciencias de la Salud, Asuncion) and identified by isoenzyme analysis. Three strains of *Leishmania* were used during these investigations: *L. braziliensis* (MHOM/BR/75/M 2903), *L. amazonensis* (IFLA/BR/67/PH8), and *L. donovani* (MHOM/IN/83/HS-70) grown at 22 °C in Schneider's drosophila medium containing 20 % fetal bovine serum. Compounds were dissolved in 5 μl of dimethyl sulfoxide (DMSO), then in medium and placed in microtiter plates in triplicate. Minimal amount (μg) of compound to inhibit growth of *Leishmania* species was evaluated after 48 hours by optical observation on a drop of each cell culture with a microscope by comparison with control cells and with a reference drug (pentamidine). The maintenance, cultivation, and isolation of promastigote-stage parasites have been described in detail elsewhere (11).

Trypanocidal activity: Balb/c mice infected with *Trypanosoma cruzi* strain, seven 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 parasitemia in infected mice ranged between 1×10^5 to 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 microtiter 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 ten minutes at room temperature and kept at 4 °C for 24 h. Each solution was microscopically observed at 400×, placing a 5 μl-sample on a slide and covering it with a 22 × 22 mm coverglass for parasite counting (12–13).

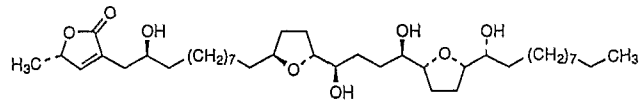
Results and Discussion

The dichloromethanic fraction of the stem bark of *R. emarginata* presented an activity against *Leishmania* sp. strains and the bloodstream forms of *T. cruzi*. The fractionation of this extract by HPLC using the *in vitro* leishmanicidal activity guide led to the isolation of five active compounds. As shown in Table **1**, rolliniastatin-1 (**1**), squamocin (**3**) and liriodenine (**6**) lysed the *Leishmania* strains by 5 μg/ml and sylvaticin (**2**) by 10 μg/ml. Whereas the fourth acetogenin isolated from *R. emarginata*, rollidecin B (**4**) was 10 times less active against the *Leishmania* strains.

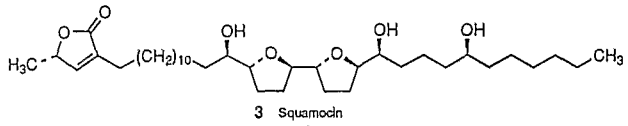
Annonaceous acetogenins have been described as antiprotozoal, insecticides, antimitotic, cytotoxic, fungicides and pesticides compounds (14, 15). Interestingly, the three most active compounds against *Leishmania* sp. showed, in the *in vitro* model, significant trypanocidal properties at a concentration of 250 μg/ml, as well. Rolliniastatin-1 (**1**), squamocin (**3**), and liriodenine (**6**) reduced the number of parasites in infected murine blood by 89, 67, and 53 %, respectively (Table **1**). In this study four acetogenins were identified but a structure-relationship was not found. Nevertheless, the leishmanicidal activity seems to be related to the number of hydroxy groups of these acetogenins. In fact, the maximum antiprotozoal activity was observed in acetogenins which present three hydroxy groups as in rolliniastatin-1 (**1**) and squamocin (**3**), while the activity was depressed in sylvaticin (**2**) and rollidecin (**4**), both acetogenins with four hydroxy groups. Further studies should confirm this interesting finding in experimen-



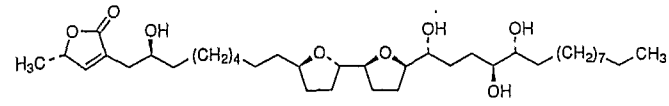
1 Rolliniastatin-1



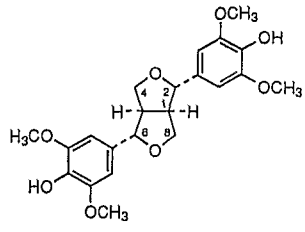
2 Sylvaticin



3 Squamocin



4 Rollidecin B



5 Lirioresinol B

Extracts and compounds	*L. braziliensis* (2903)	*L. amazonensis* (PH-8)	*L. donovani* (HS-70)	Percent reducton of the parasite number in infected murine blood (%) at 250 µg/ml
Hexanic extract	>100	>100	>100	31
Dichloromethanic extract	100	100	100	74
Methanolic extract	>100	>100	>100	9
Rolliniastatin-1 (1)	5	5	5	89
Sylvatacin (2)	10	10	10	–
Squamocin (3)	5	5	5	67
Rollidecin B (4)	50	50	50	–
Lirioresinol B (5)	>100	>100	>100	–
Liriodenine (6)	5	5	5	53
Pentamidine	5	5	5	–
Gentian violet				100

Table 1 *In vitro* activity of *R. emarginata* crude extracts, acetogenins and liriodenine towards three strains of promastigote forms of *Leishmania* spp. (IC_{100} µg/ml) and bloodstream forms of *Trypanosoma cruzi*.

tal murine models of *in vitro* tests on *T. cruzi* amastigotes forms in order to the knowledge of the antiprotozoal activity of these fourth acetogenins isolated from *R. emarginata*.

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