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Alkaloids and Triterpene from *Almeidea coerulea* (Nees and Mart.) a. St.-Hil. and Anti-leishmanial Activity

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ABSTRACT

The dichloromethane extract of Almeidea coerulea *stems yielded the (11-hydroxyrutaecarpine alkaloid reported for the first time from this species) and the triterpene (28-hydroxy-28, 29-dihydrolupeol). The dictamine, skimianine, sitosterol and stigmasterol were also isolated from methanol extract. Extracellular forms of* Leishmania amazonensis *(promastigotes) was tested with dichloromethane extract and 28-hydroxy-28, 29-dihydrolupeol with showed anti-leishmanial activity above 0.1 mg/mL and 75µg/mL (inhibited 50% promastigote growth), respectively.*

Key words: *Almeidea coerulea;* Rutaceae; indolopyridoquinazoline alkaloid; and anti-leishmanial activity

INTRODUCTION

Human leishmaniasis is a widespread parasitic disease caused by *Leishmania* sp (Grimaldi et al., 1991). The disease is endemic in Southern Brazil where it constitutes a serious heath problem, causing a spectrum of disease ranging from small cutaneous lesion to disseminated visceral leishmaniasis (Silveira et al., 1999). In Brazil, phlebotomine control is made by insecticide application in the houses and enclosures built in the peridomestic area (Teodoro et al., 1998, 2004). In leishmaniasis, the chemotherapy available so far is limited to the pentavalent antimonials such as sodium stibogluconate (Pentostan) and meglumine antimoniate (Glucantime), was introduced over 50 years ago. Both forms require lengthy parenteral administration and have severely toxic side effects, including renal and cardiac toxicity (Ministério da Saúde, 2000). Thus the drugs that are more effective, less toxic, and easier to use are urgently needed.

The family Rutaceae has been found to contain many secondary metabolites such as alkaloids, coumarins and lignans with a large spectrum of biological activites such as leishmanicidal activity (Lewis et al., 1983). Extracts from *Galipea longiflora*, Rutaceae species used by the Chimane Indians in Bolivia for the treatment of cutaneous leishmaniasis produced by *Leishmania braziliensis*, showed in vitro activity against *Leishmania* species (Fournet et al., 1996) and the butanol fraction of *Almeidea coerulea* was active against the trypomastigote form of *Trypanosoma*

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cruzi (Mafezoli et al., 2000). The aim of this work to study the isolation and the structural elucidation of one indolopyridoquinazoline alkaloid (**1**), two furoquinoline alkaloids (**2** and **3**), one lupane triterpene **4** and one mixture of steroids (**5** and **6**) and to evaluate the activity of the compound **4** against *Leishmania (L.) amazonensis.*

MATERIALS AND METHODS

General

The NMR spectra were obtained in a BRUKER DRX400 (9.4 T) and VARIAN GEMINI300 (7.05T), using deuterated solvent for field homogeneity, TMS as internal standard and temperature constant of 298K. For gHMBC the coupling constants were optimised for 4, 6, 8 and 12 Hz. IR: film NaCl plates; ESI-MSMS: low resolution on a triple quadrupole were recorded on a Micromass Quattro LC instrument equipped with a "Z-spray"ion source, and EI-MS on a CG/EM-SHIMADZU QP 2000 A. CC: silica gel 60 (70-230 and 230-400 mesh); TLC: silica gel plates F_{254} (0.25 mm in thickness).

Plant Materials

The aerial parts of *Almeidea coerulea* (Nees and Mart.) A.St.-Hil. were collected from Itacaré, Bahia, Brazil, in February 1993. The specimen was identified by Dr. Jose Pirani from the Department of Botany of the University of São Paulo (Brazil) and the voucher herbarium specimen (Pirani and Kallunki 2747) deposited in the University of Maringá (Brazil).

Isolation of Constituents

The dichloromethane extract (8.1 g) and the methanol extract (22.1 g) of the stem the *A. coerulea* was submitted to vacuum chromatography over an microcrystalline cellulose (Avicel®) support and eluted with n-hexane, n-hexane-$CHCl_3$ (1:1), $CHCl_3$, $CHCl_3$-EtOAc (1:1), EtOAc, and MeOH with gave seven fractions of each extract. The fraction 3 (1,47 g) from dichloromethane extract was chromatographed in silica gel columns using hexane-CH_2Cl_2-EtOAc-Acetone gradient solvents to 156 fractions. The fractions 30 to 47 (239 mg) were rechromatographed in silica gel (230-400 mesh) successively to fractions 26 (17 mg) and 36 (13 mg). The fractions were purified by gel filtration columns on Sephadex LH-20 with MeOH-$ChCl_3$ (1:1) to obtain the compounds **1** (1.8 mg) and **4 (**22.3 mg). The fraction 2 (1.14 g) from methanol extract was chromatographed in silica gel columns using hexane-CH_2Cl_2-EtOAc-Acetone gradient solvents to obtain the compounds **3** (3mg) and mixture **5** and **6** (3 mg). The fractions 21 to 25 was chromatographed in gel filtration columns on Sephadex LH-20 eluting with MeOH-$CHCl_3$ (1:1) to the fraction 34 and was purified by silica gel column, hexane-acetone (17:3) to obtain the compound **2** (3.8 mg).

Culture and Maintenance of the Parasites

The promastigotes forms of *Leishmania (L.) amazonensis* strain MHOM/BR73/M2269 were cultured and tested in the 199 medium (Gibco, BRL) containing 10% fetal calf serum (Cultilab, Brazil), 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (0.1 mg/mL) (Sigma) at 25 ^oC. They were then used in the exponential phase of growth (day 4 of culture).

The compound **4** was dissolved in dimethylsufoxide (DMSO) and then diluted in the 199 medium. The final concentration of DMSO did not exceed 0.05%. For promastigote drug susceptibility determinations, the parasites were placed in each test well of a 24-well micro culture plate (Costar, Cambridge, MA) at $1x10^5$promastigotes/mL of culture medium and incubated in triplicates with various concentration of alkaloids 9 at 25^oC for 24, 48, 72 and 96h. The compound **4** was only added at the beginning of the cultivation. Controls cultures were incubated with the same dilution of DMSO or pentamidine isethionate (0.7µg/mL).

Promastigotes were counted using a hemocytometer, after it was passed five times through a 25-gauge needle in order to have separate clumps.

11-hydroxyrutaecarpine (**1**). Amorphous yellow solid; [UV MeOH ν_{max} nm (log ε)]: 240 (3,61), 273 (3,38), 356 (3,81). IV: νmáx.^{NaCl} cm^{-1}: 3443, 2923, 1653, 1580, 1470 and 1045. ^{13}C NMR ($(CD_3)_2SO$, 75,5 MHz): Table I. ESI-MSMS, *m/z* (rel. int. %): 304 $[M+H]^+$ (100).

28-hydroxy-28,29-dihydrolupeol (**4**) Amorphous yellow solid. ^1H NMR ($(CD_3)_2SO$, 300 MHz) δ: 4.26 (1H, d, *J*=5.4 Hz, 3-OH), 2.94-3.04 (1H, m, H-3, H-19), 1.20-2.00 (1H, m, H-5), 1.07 (3H, s, H-23), 0.95 (3H, s, H-29), 0.91 (3H, s, H-27), 0.98 (3H, s, H-26), 0.86 (3H, s, H-30), 0.76 (3H,

s, H-24), 0.64 (3H, s, H-25) ^{13}C NMR ($(CD_3)_2SO$, 75,5 MHz) δ: 77.1 (C-3), 71.1 (C-20), 55.0 (C-5), 50.9 (C-19), 49.8 (C-9), 48.7 (C-17), 48.4 (C-18), 43.0 (C-14), 41.1 (C-8), 38.6 (C-4), 38.5 (C-1, C-22), 36.7 (C-10), 36.2 (C-13), 34.2 (C-7), 33.2 (C-16), 31.8 (C-23), 28.3 (C-30), 28.1 (C-21), 27.6 (C-2), 27.4 (C-15), 27.2 (C12), 25.1 (C-29), 21.1 (C-11), 18.2 (C-6, C-28), 16.1 (C-24), 15.9 (C-25, C-26), 15.2 (C-27). ESI-MS, *m/z* (rel. int.): 483 $[M+k]^+$ (65).

RESULTS AND DISCUSSION

Figures 2 and 3 show the activity of dichloromethane extract and compound **4** on promastigotes in concentrations from 0.001 to 6 mg/mL and from 10 to 100µg/mL, respectively. Dichloromethane extract was active against promastigote forms at concentrations above 0.1 mg/mL. Compound **4** inhibited 50% or more ($p<0.01$) promastigote growth in comparison to the non-treated controls at every period of time studied, except for 96h, showing the effect starting from 25µg/mL. The reference drug, pentamidine isethionate (0.7µg/mL) inhibited the growth of promastigotes more than 50%, starting from 24 h of cultivation. Compound **4** was active only above 75µg/mL, from 24 to 48 h. The dichloromethane extract from the stems of *A. coerulea* gave one alkaloid (**1**) and one triterpene (**4**) and the methanol extract gave two alkaloids (**2**) and (**3**) and one mixture of steroids (**5**) and (**6**). Four of them showed identical ^{1}H and ^{13}C NMR spectra as dictamine (**2**) (Chen et al., 1996) skimianine (**3**) (Chakravarty et al., 1999) and mixture of sitosterol and stigmasterol (**5/6**) (Pinto et al., 1994) and 28-hydroxy-28,29-dihydrolupeol (**4**), (Fig 1) which was established by ^{13}C by the spectral data and comparison with literature data (Wenkert et al., 1978; Razdan et al., 1998). The molecular formula of compound **1** was established as $C_{18}H_{13}N_3O_2$ by the combination of ESI-MSMS and ^{13}C NMR spectra. The IR spectrum of compound **1** showed absorption of a conjugated carbonyl group 1653 cm^{-1}. The ^{1}H NMR spectrum supported by a H-HCOSY experiment indicated the presence of one 1,2,4-substituted aromatic ring, two coupled methylenes, one 1,2-substituted aromatic ring and one phenolic hydroxyl and amino groups (Table 1). The ^{1}H NMR spectrum of compound **1** showed two symmetrical triplets at δ 4.40 and δ 3.09, corresponding to protons H-7 and H-8. The NH indolic group at δ 9.41 (*br s*) was supported by literature data of rutaecarpine (Ikuta et al., 1998).

Table 1 - ^{13}C NMR chemical shifts for compound **1** ($(CD_3)_2SO$, 75,5 MHz) and HMBC ($(CD_3)_2SO$, 100 MHz) and model **1A** ($CDCl_3$, 100 MHz). n.o. not observed

Position	**1A**	**1**		*HMBC*
1	126.7	126.5	7.62, *br d* (*J* = 7.2 Hz)	
1a	147.6	148.0		
2	134.3	134.7	7.78, *ddd* (*J* = 8.7, 7.2, 1.8 Hz)	
3	126.2	125.8	7.42, *ddd* (*J* = 8.1, 6.9, 1.2 Hz)	H-4
4	127.3	126.9	7.12, *dd* (*J* = 8.1,1.2 Hz)	
4[a]	121.2	121.0		
5	161.6	161.1		H-4
7	41.1	41.0	4.40, *t* (*J* = 6.9 Hz)	H-8
8	19.7	19.2	3.09, *t* (*J* = 6.9 Hz)	H-7
8[a]	118.3	119.1		H-8
9[a]	125.7	125.7		H-8, H-9
9	120.1	121.1	7.43, *d* (*J* = 8.7 Hz)	
10	120.7	111.6	6.61, *dd* (*J* = 8.7; 1.8 Hz)	
11	125.6	159.5		
12	112.1	97.1	6.82, *d* (*J* = 1.8 Hz)	H-10
12[a]	138.2	140.8		H-12
13[a]	127.2	n.o.		
14[a]	144.9	145.7		H-7
NH	9.15 *s*		9.41, *br s*	
OH-11			11.45 *s*	

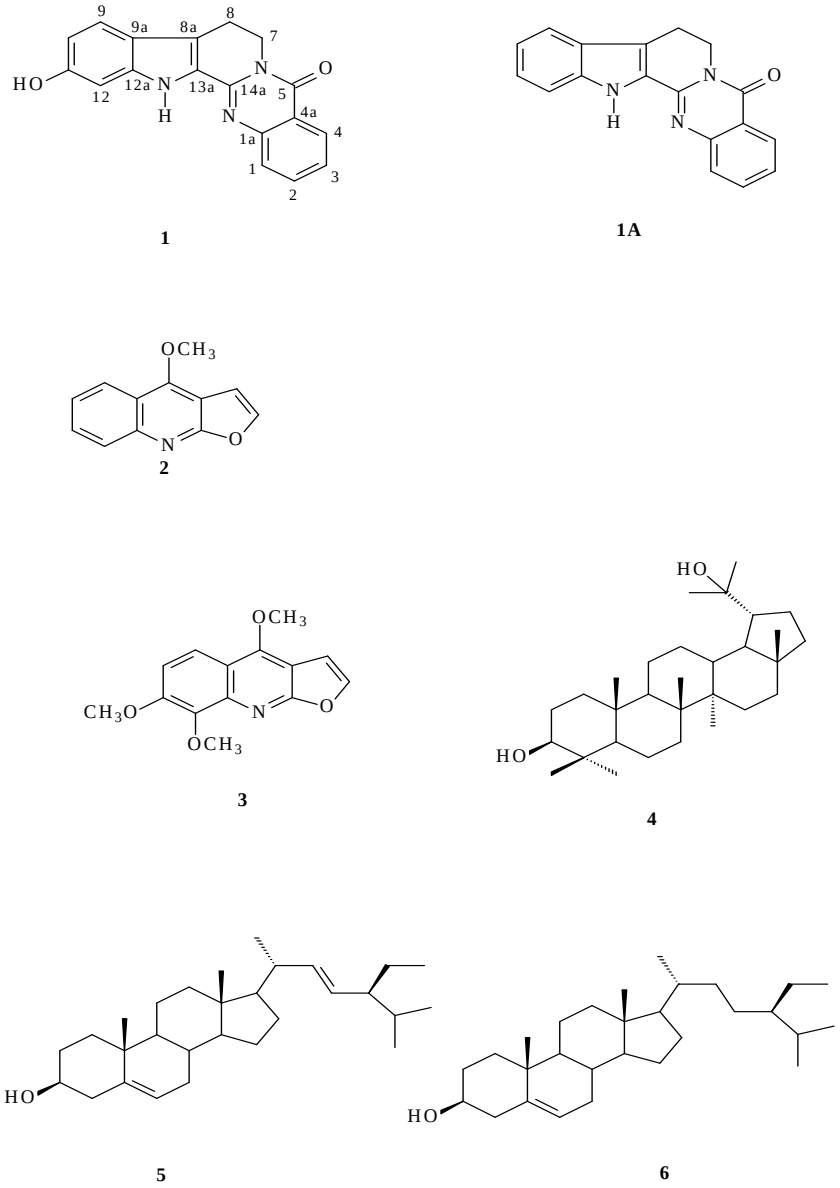

Figure 1 - Compounds from the aerial parts of *Almeidea coerulea.*

It appeared at 9.51 (*s*) and hydroxyl group at 11.54 (*s*). The nine carbon signals bearing protons were determined by gHSQC experiment, which establishes one bond correlations (Table 1). The remaining eight quaternary carbon signals were assigned based on long range H/C correlations from a ^{1}H-^{13}CgHMBC experiment and by comparison with rutaecarpine spectroscopic data[1]. Further analyses of HMBC correlations between the methylene protons at δ 4.40, *t* (*J* = 6.9 Hz, H-7) and and the two quaternary carbons at δ119.1 (C-8a) and δ 125.7 (C-9a) indicated that the junction of partial structures was C-8→ C-8a and C-14a→ C-13a.

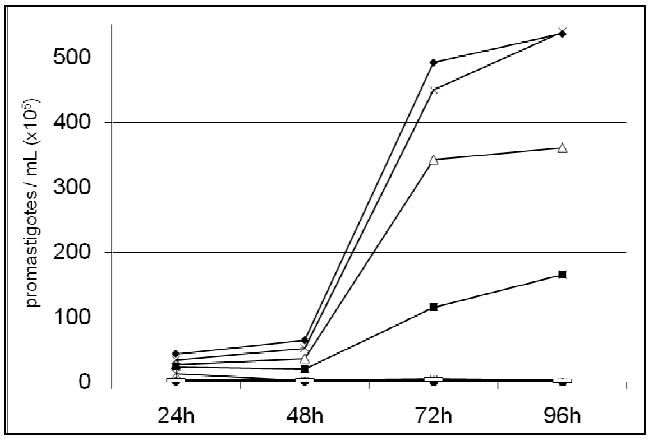

Figure 2 - Effects of dichloromethane extract from *Almeidea coerulea* and control on the growth of *Leishmania amazonensis* promastigote forms. The results represent the mean ± standard error of at least three independent experiments, which were performed in triplicate. The bars indicate standard deviations.♦ positive control; ×0.001mg/mL; △ 0.01mg/mL; ■ 0.1mg/mL; ✳ 1mg/mL; ● 6mg/mL; pentamidine 0.7µg.

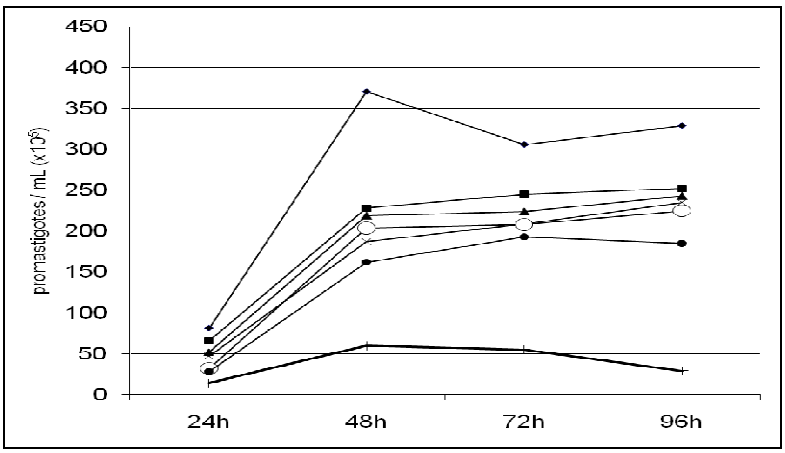

Figure 3 - Effects of *28-hydroxy-28,29-dihydrolupeol* **(4)** from *Almeidea coerulea* and control on the growth of *Leishmania amazonensis* promastigote forms. The results represent the mean ± standard error of at least three independent experiments, which were performed in triplicate. The bars indicate standard deviations. ◆ positive control; ■ 10µg/mL; ▲ 25µg/mL; × 50µg/mL; ○ 75µg/mL; ●100µg/mL; ┼ pentamidine 0.7µg.

The HMBC correlation between the aromatic proton at δ 7.12, *dd* (*J* = 8.1,1.2 Hz, H-4) and carbonyl carbon at δ 161.1 (C-5) confirmed that the junction of partial structures shoud be C-5→ C-4a and H-14→ C14a. The correlation of aromatics protons at methylene δ 7.43, *d* (*J* = 8.7 Hz, H-9), δ 6.61, *d* (*J* = 8.7 Hz, H-10) and 6.82, *d* (*J* = 1.8 Hz, H-12) and aromatic carbon δ 125.7 (C-9a), δ 97.1 (C-12) and δ 140.8 (C-12a) respectively confirmed the position of hydroxyl group at C-11. Based on the above evidence, the structure of 1 was established as 11-hydroxyrutaecarpine.

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REFERENCES

Chakravarty, A.K.; Sarkar, T.; Masuda, K.; Shiojima, K. (1999) Carbazole alkaloids from roots of *Glycosmis arborea*. *Phytochemistry*, **50,** 1263-1266.

Chen, S.I.; Wu, S.J.; Leu, L.Y.; Tsai, I.W.; Wu, T.S. (1996) Alkaloids from root bark of *Zanthoxylum simulans*. *Phytochemsitry*, **42**, 217-219.

Grimaldi, G.; Momem, H.; Naiff, R.D.; McMahon-Pratt, D.; Barrett, T.V. (1991), Leishmaniasis in Bahia, Brazil: evidence that *Leishmania amazonensis* produces a wide spectrum of clinical disease. *Am. J. Trop. Hyg.*, **44**, 645-661.

Ikuta, A. Nakamura, T.; Urabe, H. (1998) Indolopyridoquinazoline, furoquinoline and canthinone type alkaloids from *Phellodendron amurense* callus tissues *Phytochemistry*, **48**, 285-291.

Lewis, J. Biological activity of some Rutaceous compounds. In: Waterman, P. G., Grundon, M.F. (Eds.), Chemistry and Chemical Taxonomy of Rutales, Academic Press, London, 1983; pp. 301-318.

Mafezoli, J.; Vieira, P.C.; Fernandes, J.B.; Silva, M. F. G.; Alburquerque, S. (2000) In vitro activity of Rutaceae species against the trypomastigote form of *Trypanosoma cruzi*. *J. Ethnopharmacol.*, **73**, 335-340.

Ministério da Saúde – Manual de Controle da Leishmaniose Tegumentar Americana, Fundação Nacional de Saúde, 5ª. ed., 62p. 2000, Brasília, Brasil.

Pinto, A.C.; Simoni, M. L. P. S. C.; Socorro, M.P.; Cunha, C.; Coelho, R.B.; Patitucci, M.L.; Lago, R.C.A. (1994) Misturas Naturais de Esteróides, uma Alternativa para Aplicação de Padrões em Análises por Cromatografia Gasosa de Alta Resolução *Quím. Nova* **1994**, *17*, 333.

Razdan, K.T.; Harkar, S.; Qadri, B.; Qurishi, A.M.; Khuroo, A. M. (1988) Lupene derivatives from *Skimmia laureola Phytochemistry*, **27**, 1890-1892.

Silveira, T.G.V.; Arraes, S.M.A.A.; Bertolini, D.A.; Teodoro, U.; Lonardoni, M.V.C.; Roberto, A.C.B.S.; Ramos, M.; Sobrinho, N.A.; Ishikawa, E.; Shaw, J. (1999). Observações sobre o diagnóstico laboratorial e a epidemiologia da leishmaniose tegumentar no Estado do Paraná, sul do Brasil. *Rev. Soc. Bras. Med. Trop.*, **32**, 413-423.

Teodoro, U.; Galati, E. A. B.; Kühl, J. B.; Lozovei, A. L. and Barbosa, O. C. (1998), Controle de flebotomíneos com DDT, em área endêmica de leishmaniose tegumentar no Estado do Paraná, Sul do Brasil. *Braz. Arch. Biol. Technol.*, **41**, 359-364.

Teodoro, U.; Soccol, V.T.; Kühl, J.B.; Rodrigues dos Santos, D.; Silvestre dos Santos, E.; Rodrigues dos Santos, A.; Abbas, M.; Cassia Dias, A.C. (2004), Reorganization and cleanness of peridomiciliar area to control sand flies (Diptera, Psychodidae, Phlebotominae) in South Brazil. *Braz. Arch. Biol. Technol.*, **47**, 205-212.

Wenkert, E.; Baddeley, G.V.; Burfitt, I.R.; Moreno, L.M. (1978) C-13 nuclear magnetic-resonance spectroscopy of naturally-occurring substances: LVII. Triterpenes related to lupane and hopane. *Org. Magn. Reson.*, **11**, 337-343.

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