Antileishmanial Steroidal Alkaloids from Roots of *Sarcococca coriacea*

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Summary: Bio-assay guided isolation of secondary metabolites from the plant *Sarcococca coriacea* (Hook. f.) yielded two steroidal alkaloids, alkaloid C (**1**) and *iso-N*-formylchonemorphine (**2**). The structures of compounds were identified by different spectroscopic techniques. Compounds **1** and **2** showed significant anti-leishmanial activities IC_{50} = 13.20 ± 0.05 μM and 24.76 ± 0.01 μM, respectively, in comparison with standard drugs pentamidine IC_{50} = 8.58 ± 0.04 μM and amphotericin B IC_{50} = 0.31 ± 0.05 μM.

Introduction

In the flora of Nepal, the genus *Sarcococca* comprises four species [1], which are rich sources of steroidal alkaloids and many of them have been reported to exhibit cholinesterase inhibition [2], and antibacterial [3], antitumor [4], antiulcer [5], and antileishmanial [6] activities. Our continuing study on the constituents of this genus has resulted in the isolation of a number pregnane- type steroidal alkaloids [2, 3, 7]. *Sarcococca coriacea* (Hook. f.) is an evergreen shrub widely distributed in Central Nepal at altitudes of 600-1600 m [8]. We have previously reported four steroidal alkaloids from the aerial parts of this plant [2, 9]. In our continuing studies on steroidal alkaloids from this plant, we recently initiated work on the roots of *S. coriacea* which has yielded two antileishmanial steroidal alkaloids.

Leishmaniasis is a disease caused by the protozoan parasite of the genus *Leishmania*. Different types of protozoa are responsible of leishmaniasis, such as *L. infantum*, *L. donovani*, *L. maxicana*, *L. chagasi*, *L. amazonesis*, *L. major*, *L. aethiopica*, *L. brasiliensis*, *L. tropica*, *etc.* Leishmaniasis is widespread all over the tropical and sub-tropical regions of Africa, Southern Europe, South and Central America, Asian, and Mediaterian regions. A number of synthetic drugs are used in the chemotherapy of leishmaniasis, many of which are not so effective. Some drugs such as tartar emetic urea, stibamine, megulamine antimoniate, sodium stibogluconate *etc* cause harsh undesirable effects to the patients. Failure of treatment is also common [10, 11]. Some of the drugs which are in current use, such as amphotericin B, and pentamidine, are toxic and lack adequate efficacy. In this scenario, there is an urgent need to develop effective and nontoxic oral drugs and topical applications in order to combat the painful disease.

Results and Discussion

During this study, different fractions obtained from 80% MeOH/H_2O extract of roots of plant *Sarcococca coriacea* were screened for antileishmanial activity. Out of them, dicholoromethane fraction of water soluble extract at pH-7 (Sc-B, experimental part) and dicholoromethane fraction of water soluble extract at pH-9 (Sc-C, experimental part), exhibited a significant antileishmanial activity IC_{50} = 13.74 ± 0.2 μg/mL and 4.75 ± 0.01 μg/mL, respectively, in comparison with standard drug amphotericin B 0.12 μg/mL. Fraction Sc-C which showed more activity (IC_{50} = 4.75 ± 0.01 μg/mL) was subjected to column chromatography and compounds (**1**) and (**2**) were obtained for the first time from this plant (Fig. 1).

Compound **1**, which was isolated as white needles showed a positive alkaloidal test with Dragendorff's reagent. Its molecular formula of $C_{24}H_{41}NO$ (4 degrees of unsaturation) was deduced from HREI MS M^+ at *m/z* 359.3128 ($C_{24}H_{41}NO$, calcd 359.3125) and ^{13}C-NMR. The EI MS exhibited

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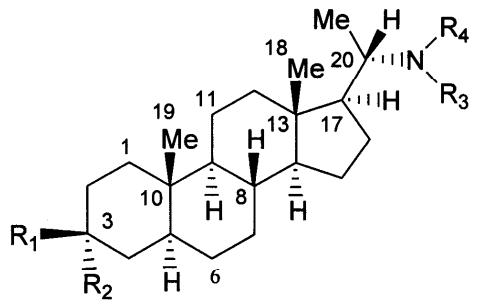

Fig. 1: Structure of compounds **1** and **2.**

Comp.	R_1	R_2	R_3	R_4	Unsaturation
1	H	OMe	Me	Me	Δ 5,6
2	$N(Me)_2$	H	H	CHO	-

an M^+ at *m/z* 359, which was further confirmed by FAB MS (*m/z* 360). The presence ofbase peak at *m/z* 72 in EI MS indicated the presence of 20-dimethyl amino pregnane type skeleton [7]. The ^{1}H-NMR spectrum of compound **1** displayed two up-field singlets at δ 0.65 and 0.98, characteristic of C-18 and C-19 angular methyls, respectively. A doublet at δ 0.86 ($J_{21,\ 20}$ = 6.4 Hz) was assigned to the C-21 secondary methyl, while a downfield 6H singlet at δ 2.14 was ascribed to NMe_2 protons. A 1H multiplet at δ 2.42 was assigned to the C-20 methine proton, while a broad signal at δ 3.04 ($W_{1/2}$ = 6.0 Hz) was ascribed to the C-3 *equatorial* methine proton [12]. A 3H singlet at δ 3.33 was assigned to the protons of methoxy group. A downfield signal at δ 5.34 was due to the C-6 methine proton. The ^{13}C–NMR (broad-band and DEPT) spectra of compound **1** showed a total of 24 carbons, including six methyl, eight methylene, seven methine and three quaternary carbons. The one - bond ^{1}H-^{13}C correlations were determined by HMQC spectrum, while the long - range ^{1}H-^{13}C connectivities were established through HMBC techniques. This compound was previously isolated from *Sarcococca saligna* [13].

Compound **2** was similar to the compound **1**, with only the difference of presence of a dimethylamino substituent at C-3 and formamide at C-20 instead of methoxy and dimethyl amino group in **1** and absence of double bond between C5-C6. Molecular formula of compound **2** was deduced from the HREI MS, M^+ at *m/z* 374.6155 ($C_{24}H_{42}NO$, calcd 374.6152). Two intense signals at *m/z* 84 and 110 in EI MS indicated the presence of a 3-(dimethylamino) pregnane skleton [14]. ^{1}H-NMR displayed resonance at δ 2.30 (6H, s) which was ascribed to *N*-Me_2 protons. A broad multiplet at δ 3.97 ($W_{1/2}$ = 16.6 Hz) was assigned to the C-3 *axial* methine proton [12], geminal to an amino group, while a downfield splitted signal at δ 7.90/7.98 was characteristic of a formamide proton. The ^{13}C-NMR (broad-band and DEPT) spectra showed 24 carbon signals, including five methyl, nine methylene, eight methine, and two quaternary carbons. Two-dimensional NMR spectroscopy, such as COSY, HMQC and HMBC were used to confirm the structure of the compound. This compound was previously isolated from *Sarcococca brevifolia* [15].

Compounds **1** and **2** were subjected to anti leishmanial activity test and found to be active with IC_{50} = 13.20 ± 0.05 μM and 24.76 ± 0.01 μM, respectively, in comparison with standard drugs pentamidine IC_{50} = 8.58 ± 0.04 μM and amphotericin B 0.31 ± 0.05 μM (Table-1).

Table-1: *In vitro* antileishmanial activities of steroidal alkaloids.

Compounds	IC_{50} (μM)
1	**13.20 ± 0.05**
2	**24.76 ± 0.29**
Amphotericin B [a]	**0.31 ± 0.05**
Pentamidine [a]	**8.58 ± 0.04**

[a] Standard drugs

Experimental

General

All reagents used were of analytical grade. TLC: precoated SiO_2 plates (*Merck GF-254*), Visualization with Dragendorff's spraying reagent. UV Spectra: Hitachi UV-3200 spectrophotometer, in MeOH, λ_{max} in nm. IR Spectra: *Jasco A-302* IR spectrophotometer, in $CHCl_3$, cm^{-1}. Mass spectra: *Jeol HX-110* mass spectrometer, in *m/z* (rel. %). NMR Spectra: Bruker Avance *AM-400* and *AC-300* spectrometers, chemical shifts δ in ppm rel. to $SiMe_4$ (= 0 ppm) as internal standard, coupling constants *J* in Hz.

Plant Material

S. coriacea (Hook. f.) were collected at an altitude of 1600 m from Champadevi, Kathamndu, Nepal, during July 2005. The plants were identified by Prof. Dr. Krishna Kumar Shrestha, Central Department of Botany, Tribhuvan University, Kritipur, Kathmandu, Nepal. A voucher specimen (No. Sc-1/ 05) was deposited in same section.

Extraction and Isolation

The air - dried roots (16 kg) of *S. coriacea* were extracted with 80% aq. MeOH (60 L). The concentrated extract (1.5 kg) was dissolved in cold distilled H_2O (9 L) and defatted with petroleum ether (30 L) to afford, after concentration, fraction SC- A (90 gm). The aqueous layer was then extracted with CH_2Cl_2 (30 L) to obtain a 'neutral' fraction (45 g, SC-B). The aqueous fraction was then made alkaline (pH 9-10) with aqueous NH_3 solution, and extracted again with CH_2Cl_2 (30 L) to obtain an 'alkaline' fraction (40 g, SC-C).

The 'alkaline' fraction (SC-C) was adsorbed on SiO_2 (*Merck* silica gel 60, 70-230 mesh) and eluted with different gradients of petroleum ether, acetone and a few drops of diethyl amine, to afford fraction A-I. Fraction I (0.80 g) was further subjected to neutral Al_2O_3 column chromatography and eluted with petroleum ether / ethyl acetate which resulted in the isolation of compounds **1** (20 mg) and **2** (10 mg).

Alkaloid C (**1**)

$[\alpha]_D^{25}$: - 28 (*c* 0.03, $CHCl_3$) {Reported: -32 ($CHCl_3$)}, M. Pt. 154 °C {Reported: 152-153 °C}, UV (MeOH) nm (log ε): λ_{max} 242 (2.53), EI MS *m/z* (rel. int. %): 359 (M^+, 11), 344 (36), 72 (100), FAB^{+ve} MS: *m/z* 360, HREI MS *m/z* : 359.3128 (Calcd for $C_{24}H_{41}N$ O, 359.3125). ^{1}H-NMR, δ (400 MHz, $CDCl_3$): 0.65 (3 H, s, Me- 18), 0.98 (3 H, s, Me-19), 0.86 (3H, d, $J_{21,\ 20}$ = 6.4 Hz, Me – 21), 2.14 (6 H, s, H-*N*Me_2), 2.42 (1 H, m, H-20), 3.04 (1 H, m, H-3), 3.33 (3H, s, OCH_3), 5.34 (1 H, d, H-6). ^{13}C-NMR, δ (100 MHz, $CDCl_3$): 38.7 (C-1), 28.0 (C-2), 80.4 (C-3), 39.6 (C-4), 140.8 (C-5), 121.6 (C-6), 31.8 (C-7), 31.9 (C-8), 50.2 (C-9), 36.9 (C-10), 21.0 (C-11), 39.8 (C-12), 41.4 (C-13), 54.8 (C-14), 24.1 (C-15), 37.2 (C-16), 56.9 (C-17), 12.1 (C-18), 19.3 (C-19), 61.1 (C-20), 9.8 (C-21), 39.0 (*N*$(Me)_2$) , 55.6 (OCH_3).

Iso-N-formylchonemorphine (**2**)

$[\alpha]_D^{28}$ - 20 (*c* = 0.05, MeOH) {Reported: -14 (*c* = 0.2, $CHCl_3$), IR ($CHCl_3$) ν_{max} cm^{-1}: 3260 (N-H), 2934 (C-H), 1665 (C=O), EI MS *m/z* (rel. int. %): 374 (M^+, 69.6), 359 (5), 110 (81.7), 84 (100), FAB^{+ve} MS: *m/z* 375, HREI MS *m/z*: 374.6155 (Calcd for $C_{24}H_{42}NO$, 374.6152), ^{1}H-NMR, δ (300 MHz, $CDCl_3$): 0.73 (3H, s, H-18), 0.81 (3H, s, H-19), 1.13 (3H, d, $J_{21,\ 20}$ = 6.5 Hz, H-21), 2.30 (6H, s, *N*-Me_2), 3.97 (1H, br. m, $W_{1/2}$ = 16.6 Hz, H-3αx), 7.90/7.98 (1H, s, H-NCO). ^{13}C-NMR, δ (100 MHz, $CDCl_3$): 38.8 (C-1), 24.9 (C-2), 65.6 (C-3), 31.7 (C-4), 47.7 (C-5), 27.7 (C-6), 30.0 (C-7), 36.7 (C-8), 55.7 (C-9), 36.8 (C-10), 22.0 (C-11), 40.6 (C-12), 43.2 (C-13), 57.2 (C-14), 25.1 (C-15), 33.2 (C-16), 57.9 (C-17), 12.6 (C-18), 12.7 (C-19), 46.6 (C-20), 21.7 (C-21), 41.7 (*N*$(Me)_2$), 162.4 (NCHO).

Antileishmanial assay (in vitro)

Leishmania promastigotes were grown in bulk early in modified NNN biphasic medium using normal physiological saline. *Leishmania* parasite promastigotes were cultured with RPMI 1640 medium (Sigma) supplemented with 10% heat inactivated foetal bovine serum (PAA Laboratory). Parasites at log phase are centrifuged at 2000 rpm for 10 minutes, and washed three times with saline at same speed and time. Parasites are diluted with fresh culture medium to a final density of 10^6 cells /mL.

In a 96-well micro titer plate, 180 μL of medium was added in different wells. 20 µL of the experimental compound was added in medium and serially diluted. 100 μL of parasite culture was added in all wells. Two rows should be left for negative and positive control. Negative controls received medium, while the positive control contains varying concentrations of standard antileishmanial compound *e.g.* amphotericin B (Fluka), and pantamidine (ICN). The plate was incubated between 22-25 °C for 72 h. The culture was examined microscopically on an improved neubauer counting chamber and IC_{50} values of compounds possessing antileishmanial activity were calculated by Software Ezfit 5.03 Perella Scientific. All assays were run in duplicate [16, 17].

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