SUPPLEMENTARY MATERIAL

Antileishmanial diterpenoid alkaloids from *Aconitum spicatum* (Bruhl) Stapf

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The crude extracts of tubers of *Aconitum spicatum* ( Bruhl) Stapf were investigated for *in vitro* antileishmanial activity against *Leishmania major*. The dichloromethane extract at pH 2.5 showed antileishmanial activity with IC\textsubscript{50} value of 27.10 ± 0.0 μg/mL. Chromatographic purification of the dichloromethane extract led to isolation of three C-19 norditerpenoid alkaloids indaconitine (1), chasmaconitine (2) and ludaconitine (3). Compounds 3 and 2 showed antileishmanial activity with IC\textsubscript{50} = 36.10 ± 3.4 and 56.30 ± 2.1 μg/mL, respectively. Compound 1 was less effective (IC\textsubscript{50} >100 μg/mL). The cytotoxicity of compounds 1, 2 and 3 studied against MCF7, HeLa and PC3 cancer cell lines and 3T3 normal fibroblast cell line did not show cytotoxicity at 30 μM.

Keywords: *Aconitum spicatum*, antileishmanial activity, *Leishmania major*, diterpenoid alkaloids

Experimental

Collection of plant material

The tubers of *A. spicatum* were collected from Panch Pokhari, Sindhupalchok district of central Nepal, at the altitude of 3800 m. The specimen of the plant was identified by comparing the
herbarium (voucher no.59185) deposited at National Herbarium Laboratory, Godawari, Lalitpur, Nepal.

**Chemicals**

Commercially available analytical grade solvents were used for solvent-solvent extraction and column chromatography. HPLC grade solvents were purchased from Merck and Fisher Scientific companies. Column chromatography was performed by using silica gel (Merck, 200-400 mesh). Pre-coated silica gel plates (Merck, silica gel 60 F<sub>254</sub>) were used for thin layer chromatography (TLC).

**Extraction and chromatographic separation of compounds**

The tubers were chopped into small pieces, and dried under shade. The dried tuber (6.0 Kg) was grinded into the fine powder, and was extracted thrice with 8 L of 1% HCl in methanol:water (85:15) for 72 h. The viscous dark red residue (1.2 kg) was obtained after concentration under reduced pressure. The crude extract (500 g) suspended in water, was extracted by solvent-solvent extraction to obtain n-hexane (4.2 g); acidic (prepared by adding CH<sub>3</sub>COOH) and basic dichloromethane (prepared by adding NH<sub>4</sub>OH) at pH 2.5 (14.0 g), pH 4 (0.16 g), pH 7 (3.2 g) and pH 11 (0.2 g); ethyl acetate (1.0 g) and n-butanol (21.6 g) extracts. The acidic dichloromethane extract at pH 2.5 (14.0 g) was column chromatographed on silica gel and eluted with n-hexane. Then polarity was gradually increased by adding ethyl acetate, followed by methanol. Two major extracts were compiled. The extract A was compiled from less polar eluents by increasing percentage of ethyl acetate in n-hexane. The extract B was compiled from polar eluents when percentage of methanol in ethyl acetate was gradually increased. The extract B was loaded onto silica gel column and eluted with dichloromethane/methanol to isolate
indaconitine (1), chasmaconitine (2) and ludaconitine (3). The extract A was repeatedly run into normal phase recycling HPLC (LC 908 column) eluting with ethyl acetate:n-hexane (20:80) to get emodin (4), trans-methyl-p-coumarate (5), p-hydroxybenzaldehyde (6) and ursolic acid (7). The dichloromethane extract at pH 4 (0.16 g) was chromatographed in silica gel column using n-hexane/ethyl acetate solvent system to obtain 5-(hydroxymethyl)-furan-2-carbaldehyde (8). β-Sitosterol glucoside (9) was obtained from dichloromethane extract at pH 11 (0.2 g) by silica gel column chromatography eluting with dichloromethane/methanol. The structures were identified by comparing their spectroscopic data with the literature. The modern spectroscopic techniques, such as UV, IR, $^1$H NMR, $^{13}$C NMR, COSY, HSQC, HMBC, EI, FAB and ESI MS were used for structure determination.

**Characterization of compounds**

**Indaconitine (1) [CAS No. 4491-19-4]**

![Image of Indaconitine (1)]

White amorphous (100 mg); melting point 197-198 °C; EI MS $m/z$: 629 [M]$^+$; molecular formula: C$_{34}$H$_{47}$NO$_{10}$; $^1$H NMR (500 MHz, CDCl$_3$) δ ppm: 3.11 (m, H-1), 2.03 (m, H-2), 2.33 (m, H-2), 3.73 (dd, $J$ = 9.5, 5 Hz, H-3), 2.06 (s, H-5), 4.00 (d, $J$ = 6.5 Hz, H-6), 3.02 (H-7), 2.89 (H-9), 2.07 (H-10), 2.06 (H-12), 2.60 (H-12), 4.88 (d, $J$ = 5 Hz, H-14), 2.40 (H-15), 2.97 (H-15), 3.37 (dd, $J$ = 8.5, 6.5 Hz, H-16), 2.99 (H-17), 3.50 (d, $J$ = 9 Hz, H-18), 3.62 (d, $J$ = 9 Hz, H-18),
2.33 (H-19), 2.89 (H-19), 2.44 (m, H-20), 2.53 (H-20), 1.07 (t, J = 7 Hz, H-21), 3.23 (s, OMe-1), 3.13 (s, OMe-6), 3.52 (s, OMe-16), 3.27 (s, OMe-18), 1.26 (s, COCH$_3$-8), 8.03 (d, J = 7 Hz, H-2’ and H-6’), 7.42 (H-3’ and H-5’), 7.54 (H-4’); $^{13}$C-NMR (125 MHz, CDCl$_3$) δ ppm: 82.2 (C-1), 33.5 (C-2), 71.6 (C-3), 43.1 (C-4), 40.8 (C-5), 83.1 (C-6), 48.7 (C-7), 85.5 (C-8), 44.7 (C-9), 47.4 (C-10), 50.2 (C-11), 35.2 (C-12), 74.7 (C-13), 78.8 (C-14), 39.5 (C-15), 83.5 (C-16), 61.6 (C-17), 77.0 (C-18), 47.4 (C-19), 48.8 (C-20), 13.3 (C-21), 55.8 (OMe-1), 57.7 (OMe-6), 58.7 (OMe-16), 59.1 (OMe-18), 169.8 (COCH$_3$-8), 21.5 (COCH$_3$-8), 166.2 (CO-14), 130.1 (C-1’), 129.6 (C-2’ and C-6’), 128.5 (C-3’ and C-5’), 133.1 (C-4’) (Khetwal et al. 1994).

EI Mass spectrum
IR spectrum
\(^1\)H NMR (500 MHz, CDCl\(_3\))
Chasmaconitine (2) [CAS No. 6846-46-4]

White crystalline (300 mg); melting point 184 °C, EI MS m/z: 613 [M]+ molecular formula: C_{34}H_{47}NO_{9}; \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) δ ppm: 3.10 (m, H-1), 1.94 (m, H-2), 2.25 (m, H-2), 1.63 (m, H-3), 2.14 (s, H-5), 3.98 (d, J = 6.5 Hz, H-6), 2.99 (H-7), 2.90 (H-9), 2.16 (H-10), 2.05
(H-12), 2.71 (H-12), 4.89 ($d$, $J = 5$ Hz, H-14), 2.45 (H-15), 2.94 (H-15), 3.38 ($dd$, $J = 9$, 6 Hz, H-16), 2.96 (H-17), 3.20 ($d$, $J = 8.5$ Hz, H-18), 3.61 ($d$, $J = 8.5$ Hz, H-18), 2.48 (m, H-19), 2.52 (m, H-19), 2.43 (m, H-20), 2.57 (m, H-20), 1.10 ($i$, $J = 6$ Hz, H-21), 3.52 (s, OMe-1), 3.15 (s, OMe-6), 3.27 (s, OMe-16), 3.26 (s, OMe-18), 1.22 (s, COCH$_3$-8), 8.09 ($d$, $J = 7$ Hz, H-2’ and H-6’), 7.48 (m, H-3’ and H-5’), 7.60 (m, H-4’); $^{13}$C-NMR (125 MHz, CDCl$_3$) $\delta$ ppm: 85.8 (C-1), 27.0 (C-2), 35.5 (C-3), 40.0 (C-4), 50.1 (C-5), 86.8 (C-6), 50.1 (C-7), 86.8 (C-8), 46.3 (C-9), 42.0 (C-10), 51.3 (C-11), 37.5 (C-12), 76.2 (C-13), 80.2 (C-14), 40.7 (C-15), 85.0 (C-16), 62.8 (C-17), 81.3 (C-18), 54.9 (C-19), 50.0 (C-20), 13.6 (C-21), 59.4 (OMe-1), 58.4 (OMe-6), 56.2 (OMe-16), 59.4 (OMe-18), 171.3 (COCH$_3$-8), 21.7 (COCH$_3$-8), 167.7 (CO-14), 131.3 (C-1’), 130.7 (C-2’ and C-6’), 129.6 (C-5’ and C-3’), 134.3 (C-4’) (Yue et al. 1990).
EI Mass spectrum
IR spectrum
$^1$H NMR (500 MHz, CDCl$_3$)
Ludaconitine (3)

White crystalline (18 mg); EI MS m/z: 587 [M]+; molecular formula: C_{32}H_{45}NO_{9}; ^1H NMR (500 MHz, CDCl₃) δ ppm: 3.60 (m, H-1), 2.26 (m, H-2), 2.29 (m, H-2), 4.18 (m, H-3), 2.46 (s, H-5), 4.22 (d, J = 6.5 Hz, H-6), 2.18 (H-7), 2.61 (H-9), 2.42 (H-10), 5.01 (d, J = 5.5 Hz, H-14), 2.47 (H-15), 2.54 (H-15), 3.48 (H-16), 3.31 (H-17), 3.51 (H-18), 3.52 (H-18), 3.58 (H-19), 3.30 (H-
20), 1.39 (t, J = 7.5 Hz, H-21), 3.39 (s, OMe-1), 3.35 (s, OMe-6), 3.55 (s, OMe-16), 3.31 (s, OMe-18), 8.10 (d, J = 7 Hz, H-2’ and H-6’), 7.59 (H-3’ and H-5’), 7.46 (H-4’), $^{13}$C-NMR (125 MHz, CDCl$_3$) δ ppm: 81.3 (C-1), 30.1 (C-2), 70.6 (C-3), 43.8 (C-4), 44.3 (C-5), 83.0 (C-6), 56.1 (C-7), 74.6 (C-8), 45.7 (C-9), 41.5 (C-10), 52.1 (C-11), 36.7 (C-12), 76.4 (C-13), 80.5 (C-14), 43.8 (C-15), 84.1 (C-16), 66.9 (C-17), 78.1 (C-18), 51.0 (C-19), 50.7 (C-20), 11.0 (C-21), 55.3 (OMe-1), 58.5 (OMe-6), 59.4 (OMe-16), 59.4 (OMe-18), 167.8 (CO-14), 131.6 (C-1’), 131.0 (C-2’ and C-6’), 129.4 (C-3’ and C-5’), 134.2 (C-4’) (Khetwal et al. 1992).

EI Mass spectrum
IR spectrum
\textbf{\textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3})}
Emodin (4) [CAS No 518-82-1]

Orange crystalline (3 mg); melting point: 260 °C; EI MS m/z: 270 [M]+; molecular formula: C_{15}H_{10}O_{5}; ^1H NMR (300 MHz, C_{3}D_{6}O) δ ppm: 6.66 (d, J = 4 Hz, H-2), 7.25 (d, J = 4 Hz, H-4), 7.56 (s, H-5), 7.13 (s, H-7), 2.46 (s, CH_{3}-6); ^13C NMR (125 MHz, C_{3}D_{6}O) δ ppm: 166.3 (C-1), 108.8 (C-2), 166.2 (C-3), 109.6 (C-4), 121.4 (C-5), 149.5 (C-6), 124.9 (C-7), 163.2 (C-8), 191.7
(C-9), 182.1 (C-10), 136.4 (C-11), 110.0 (C-12), 114.0 (C-13), 134.2 (C-14), 21.9 (CH$_3$-6) (Liu et al. 2013).

EI Mass spectrum
IR spectrum

[Graph showing IR spectrum with peaks at various wavenumbers.]
$^1$H NMR (300 MHz, C$_3$D$_6$O)
trans-Methyl-p-coumarate (5) [CAS No 19367-38-5]

White crystalline (4 mg); melting point: 138 °C; EI MS m/z: 178 [M]+; molecular formula: C_{10}H_{10}O_{3}; $^1$H NMR (300 MHz, C_{3}D_{6}O) δ ppm: 6.34 (d, J = 15.9 Hz, H-2), 7.59 (d, J = 15.9 Hz, H-3), 7.54 (d, J = 8.7 Hz, H-5 and H-9), 6.88 (d, J = 8.7 Hz, H-6 and H-8), 3.70 (s, OCH$_3$-1); $^{13}$C NMR (125 MHz, C_{3}D_{6}O) δ ppm: 167.8 (C-1), 115.3 (C-2), 145.3 (C-3), 126.9 (C-4), 130.9 (C-5 and C-9), 116.6 (C-6 and C-8), 159.0 (C-7), 51.4 (OCH$_3$-1) (Chiang et al. 2003).
EI Mass spectrum
IR spectrum

![IR spectrum graph]

**Specifications:**
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- Scans: 10
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- Snip: J733
- Data Interval: 0.964/4
- Resolution: 2.0
$^1$H NMR (300 MHz, C$_3$D$_6$O)
$^{13}$C NMR (125 MHz, C$_3$D$_6$O)

$p$-Hydroxybenzaldehyde (6) [CAS No 123-08-0]

White crystalline (2 mg); melting point: 116 °C; EI MS $m/z$: 121[M-H]$^+$; molecular formula: C$_7$H$_6$O$_2$; $^1$H NMR (300 MHz, C$_3$D$_6$O) $\delta$ ppm: 7.79 ($d, J = 8.7$ Hz, H-2 and H-6), 7.00 ($d, J = 8.7$, H-3 and H-5), 9.84 ($s$, HCO); $^{13}$C NMR (75 MHz, C$_3$D$_6$O) $\delta$ ppm: 129.5 (C-1), 132.6 (C-2 and C-6), 116.5 (C-3 and C-5), 163.7 (C-4), 190.8.
EI Mass spectrum
$^1$H NMR (300 MHz, C$_3$D$_6$O)
$^{13}$C NMR (75 MHz, C$_3$D$_6$O)

Ursolic acid (7) [CAS No 77-52-1]

White crystalline (2 mg); melting point: 288 °C; EI MS $m/z$: 456 [M]$^+$; molecular formula: C$_{30}$H$_{48}$O$_3$; $^{13}$C NMR (125 MHz, C$_3$D$_6$O) $\delta$ ppm: 39.4 (C-1), 27.9 (C-2), 78.6 (C-3), 39.8 (C-4), 56.1 (C-5), 19.0 (C-6), 33.5 (C-7), 39.8 (C-8), 48.4 (C-9), 37.7 (C-10), 23.8 (C-11), 122.9 (C-12), 144.9 (C-13), 42.4 (C-14), 28.3 (C-15), 24.0 (C-16), 49.5 (C-17), 53.5 (C-18), 42.1 (C-19),
42.1 (C-20), 33.3 (C-21), 39.2 (C-22), 28.6 (C-23), 15.7 (C-24), 16.2 (C-25), 17.5 (C-26), 23.6 (C-27), 176.5 (C-28), 15.8 (C-29), 21.4 (C-30) (Babalola & Shode 2013).

EI Mass spectrum
IR spectrum
$^1$H NMR (300 MHz, C$_3$D$_6$O)
$^{13}$C NMR (125 MHz, C$_3$D$_6$O)

5-(Hydroxymethyl)-furan-2-carbaldehyde (8) [CAS No 67-47-0]

![Chemical Structure](attachment:image.png)

Yellowish brown (9 mg); melting point: 35 °C; EI MS $m/z$: 126 [M]$^+$; molecular formula: C$_6$H$_6$O$_3$; $^1$H NMR (500 MHz, C$_3$D$_6$O) $\delta$ ppm: 7.35 ($d, J = 3.5$ Hz, H-3), 6.56 ($d, J = 3.5$ Hz, H-4), 9.58 ($s$, HCO-2), 4.63 (H-5'); $^{13}$C NMR (75 MHz, C$_3$D$_6$O) $\delta$ ppm: 153.3 (C-2), 123.7 (C-3), 110.1 (C-4), 162.8 (C-5), 178.0 (HCO-2), 57.5 (C-5') (Etse et al. 1988).
EI Mass spectrum
IR spectrum
$^1$H NMR (500 MHz, C$_3$D$_6$O)

**TELEANGIETAL (L.TAMANG)/DR. IQBAL/DR. PRATTS/C3D6O**

**ICBS/U.O.K**

**AVANCE AV-500**

**LAB NO: 109-B**

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- **DD:** 10000.0 Hz
- **PF1:** 0.000544 Hz
- **W1:** 1.664496 sec
- **DS:** 360.0 Hz
- **TOO:** 1.500000 sec

**CHANNEL F1**

- **W1:** 0.00 sec
- **F1:** -1.500 sec
- **F2:** 506.133569 Hz
- **DD:** 37164
- **Mag:** 900.130000Hz

**^{13}C NMR**

- **DF:**
  - **0.30 Hz**
- **PC:** 1.00
**13^C NMR (75 MHz, C₃D₆O)**

\[ \beta - \text{Sitosterol glucoside (9)} \] [CAS No 474-58-8]

White amorphous (12 mg); melting point: 286 °C; EI MS \( m/z \): 414 [M-Glu]+; molecular formula: C_{35}H_{59}O_{6}; \( ^{13} \)C NMR (150 MHz, CD_{3}OD) \( \delta \) ppm: 141.8 (C-5), 122.8 (C-6), 33.0 (C-7), 33.2 (C-8), 51.7 (C-9), 38.5 (C-10), 22.2 (C-11), 41.1 (C-12), 43.5 (C-13), 58.1 (C-14), 25.3 (C-15), 27.1 (C-16), 57.4 (C-17), 12.4 (C-18), 19.4 (C-19), 37.4 (C-20), 19.5 (C-21), 35.1 (C-22), 30.7 (C-23), 47.2 (C-24), 30.3 (C-25), 20.3 (C-26), 19.9
(C-27), 24.1 (C-28), 12.4 (C-29), 102.4 (C-1'), 71.6 (C-2'), 77.8 (C-3'), 75.1 (C-4'), 78.0 (C-5'), 62.8 (C-6') (Yayli et al. 2003).

EI Mass spectrum
IR spectrum
$^1$H NMR (500 MHz, CD$_3$OD)
In vitro antileishmanial activity

*Leishmania major* promastigotes were grown in bulk in modified Novy-MacNeal-Nicolle biphasic medium using normal physiological saline. *Leishmania* promastigotes were cultured with Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, St. Louis, USA), supplemented with 10% heat inactivated Fetal Calf Serum (FCS) (PAA Laboratories GmbH, Austria). Parasites at log phase were centrifuged at 2000 rpm for 10 min and washed three times with saline. Parasites were diluted with fresh culture medium to a final density of 1x10^6 cells/mL.

In a 96-well micro titer plate, 180 µL medium was added in different wells. The extract and compounds were dissolved in phosphate buffer saline (pH 7.4 containing 0.5% MeOH and...
0.5% DMSO) to make a stock concentration of 1000 mg/mL. The stock solution (20 μL) was added in medium and serially diluted to obtain working concentrations (1-100 μg/mL). Parasite culture (100 μL) was added in all wells. Two rows were left for negative and positive controls. Negative controls received only medium while the positive controls contained different concentrations of standard antileishmanial compounds namely amphotericin B (MP Biomedical Inc.) and pantamidine (ICN Biomedical Inc). The plate was incubated at 25 °C for 72 h. The culture was examined microscopically on an improved Neubaure counting chamber and IC₅₀ values were calculated using Software Ezfit 5.03 Perella Scientific. All assays were run in duplicate (Choudhary et al. 2005). Table S1 shows the data resulted from the antileishmanial activity.

Table S1. *In vitro* antileishmanial activity of the different extracts and pure compounds isolated from *A. spicatum*.

| Treatments                   | IC₅₀ (μg/mL) ± S.D. | SI    |
|------------------------------|---------------------|-------|
| *n*-Hexane extract           | > 100               | -     |
| Dichloromethane extract (pH 2.5) | 27.10 ± 0.0         | 36.9ᵃ |
| Dichloromethane extract (pH 7) | > 100               | -     |
| Ethyl acetate extract        | > 100               | -     |
| *n*-Butanol extract          | > 100               | -     |
| Indaconitine (1)             | > 100               | -     |
| Chasmaconitine (2)           | 56.3 ± 2.1          | 0.53ᵇ |
| Ludaconitine (3)             | 36.1 ± 3.4          | 0.83ᵇ |
| Amphotericin B (Standard)    | 0.29 ± 0.05         | -     |
| Pentamidine (Standard)       | 5.09 ± 0.09         | -     |
\[ \text{SI}^a = \text{IC}_{50} \text{ value for extract on brine shrimp divide by the IC}_{50} \text{ value for the extract on} \]

*Leishmania major*

\[ \text{SI}^b = \text{IC}_{50} \text{ value for compound on 3T3 cells divide by the IC}_{50} \text{ value for the extract on} \]

*Leishmania major*

**Brine shrimp cytotoxicity**

Test plant extracts were dissolved in pure dimethyl sulfoxide (DMSO) and re-dissolved in seawater to make concentrations were 100, 500, 1000 μg/mL. After hatching and maturation of *Artemia salina*, 10 larvae were placed in each plate using pipette and incubated at 25-27°C for 24 h under illumination. After 24 h, the numbers of dead naupli in each plate were counted. The experiment was done in triplicate. DMSO was used as negative control and thymol as positive control. The percentage lethality was determined by comparison of mean surviving larvae of the test and control Plates (Meyer et al. 1982).

**MCF7, 3T3, HeLa and PC3 cell lines cytotoxicity**

Cytotoxicity of compounds was evaluated by using the MTT assay (Mosmann 1983). The cell-lines were cultured in Dulbecco’s Modified Eagle’s Medium, supplemented with 5% of fetal bovine serum (FBS), 100 IU/mL of penicillin, and 100 μg/mL of streptomycin. Then, 1x10^4 cells/well loaded into each well of 96-well plates. After overnight incubation, it was added with different concentrations of compounds. After 72 hrs, MTT was added to each well and incubated further for 4 hrs, and then the absorbance was taken at 570 nm.

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