SUPPLEMENTARY MATERIAL

Two new polyamine alkaloids from the *Bufo viridis* toad venom

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ABSTRACT

Two new polyamine alkaloids (bufonines A-B), together with four known alkaloids, bufotenidine (**3**), bufotenine (**4**), 1-(*β*-D-ribofuranosyl)-l*H*-1,2,4-triazone (**5**) and proline (**6**) were isolated from the *Bufo viridis* toad venom. Their structures were identified by UV, HR-ESI-MS, NMR spectral analyses, and comparison of theoretical and experimental ECD data. All compounds were tested in vitro cytotoxicity against three human cancer cell lines (HT-29, A549 and Hela). None of the compounds showed cytotoxicity towards all tested cell lines. To the best of our knowledge, this is the first report of alkaloid components from *Bufo viridis* toad venom.

Keywords: alkaloid; Bufo viridis; toad venom; cytotoxicity

Experimental

General

1D and 2D NMR spectra were acquired on VARIAN VNMRS 600 MHz NMR spectrometers (Varian, USA) in CD_3OD or D_2O as solvents with TMS as the internal standard. Semi-preparative HPLC separations were conducted on a DIONEX UltiMate 3000 instrument (Thermo Scientific, MA, USA) equipped with an X Charge RP-18 (5 μm, 10×250 mm) column or an X-Select CSH C18 (5 μm, 10×250 mm) column. Optical rotations were recorded on an Autopol VI automatic polarimeter (Rudolph Research Analytical, Flanders, NJ, USA) in MeOH. UV spectra were recorded on a DIONEX UltiMate 3000 UV/vis spectrophotometer equipped with Diode Array Detector (Thermo Scientific, MA, USA). The ECD spectra were obtained using A Chirascan spectropolarimeter (Applied Photophysics, UK) in methanol. The ECD calculations were conducted on the TmoleX 4.3 program and used b3-lyp functional theory method at the DFT/m4 level and def-TZVPP basis. HR-ESI-MS data were acquired on a Thermo Fisher QEXACTIVE mass spectrometer in methanol (Bremen, Germany). Silica gel (Qingdao Marine Chemical Ltd. Qingdao, P. R. China) column chromatography (CC) and Sephadex LH-20 (GE Healthcare, Sweden) CC were used for fractionation of the extracts.

Materials

The *Bufo viridis* was collected in Hetian, Xinjiang Province, China, in July 2021 and was identified by associate professor Chunfang Lu. By stimulating the skin glands of *Bufo viridis* to secrete toad venom and then collected it. A specimen sample (DS2021B) was deposited at our institution, Xinjiang Technical Institute of Physics and Chemistry, Chinese Academy of Sciences.

Extraction and isolation

The *Bufo viridis* toad venom (2 g) was extracted by 95% ethanol under ultrasonic condition (40 min, 40℃, 5 times). After evaporating the solvents under reduced pressure, 1.1 g of the dried extract was acquired. The crude extracts were eluted with a MeOH-H_2O (4:1) solvent system in a Sephadex LH-20 column to obtain five fractions (A1 to A5), and the alkaloid components were traced using TLC plates (silica, CH_2Cl_2–MeOH–H_2O, 40:20:1) and visualized after spraying

with Dragendorff reagent. The alkaloid-concentrated A1 fraction (203 mg) was divided into four fractions (A3A, A3B, A3C, A3D) by flash chromatography using an ODS column with a MeOH–H_2O gradient (10%–100% MeOH) as the mobile phase at a flow rate of 10 mL/min. The A3A fraction (78 mg) was separated by semi-prep HPLC (column: X Charge RP-18 5μm, 10×250 mm; solvent: MeOH–H_2O, 28:72; flow rate: 3.0 mL/min) to yield compounds **3** (2.4 mg, t_R =14.7 min), **4** (12.0 mg, t_R=18.0 min) and fraction Fr.A3A.1(24–25 min). The fraction Fr.A3A.1(23.7 mg) was further purified by semi-PHPLC (X-Select CSH C18 5μm, 10×250 mm; solvent: MeOH/H_2O=30:70; flow rate: 3.0 mL/min) to yield compounds **5** (2.9 mg, t_R=9.3 min) and **6**(2.2 mg, t_R=14.3 min). The A3D fraction (61 mg) was further purified by preparative TLC (silica gel), eluted with $CHCl_3$–MeOH–H_2O (82:16:2) to yield compounds **1** (4.3 mg) and **2** (8.7 mg).

bufonine A

Pale yellow amorphous powder, $[\alpha]_D^{25}$ +28.038 (c 0.01, MeOH); UV (MeOH)λ_{max} 205 nm; HR-ESI-MS *m/z* 317.1810 $[M+H]^+$ (calcd for $C_{13}H_{25}N_4O_5^+$, 317.1811); ^{1}H (600 MHz,D_2O) and ^{13}C NMR (150MHz, D_2O) data, see Table S1.

bufonine B

Brown powder, $[\alpha]_D^{25}$ +20.027 (c 0.01, MeOH); UV (MeOH)λ_{max} 207 nm; HR-ESI-MS *m/z* 331.1968 $[M+H]^+$ (calcd for $C_{14}H_{27}N_4O_5^+$, 331.1966); ^{1}H (600 MHz, CD_3OD) and ^{13}C NMR (150MHz, CD_3OD) data, see Table S1.

The cytotoxicity assay

The inhibitory effect of all the isolated compounds on the proliferation of selected cell lines (HT-29, A549 and Hela) was evaluated using the MTT assay. During the logarithmic growth phase, each cell line was inoculated in a 96-well culture plate at an appropriate density in the selected medium. Then, cells were incubated overnight and treated with compounds 1-6 at 37°C for 48 h. The medium was discarded and 100μL MTT (Sigma-Aldrich) was added and incubated in the dark at 37°C for 2 h. Doxorubicin was used as the positive control. The OD_{570} was measured with a SpectraMax M5 (Molecular Devices). The cell survival rate was calculated using the

following formula: Survival rate (%) = $OD_{compound}$-OD_{black}/$OD_{control}$- OD_{black} × 100. The IC_{50} values were calculated using GraphPad Prism 8.0.

List of Figures:

Table S1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data for compounds 1–2.

No.	1[a]		2[b]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1		181.2, C		178.1, C
2	4.22 (dd, *J*=8.4, 4.8 Hz)	57.1, CH	4.24 (dd, *J*=8.4, 4.8 Hz)	54.9, CH
3	1.71, m;1.86, m	31.6, CH_2	1.65, m; 1.82, m	30.9, CH_2
4	1.60, m	27.5, CH_2	1.55, m	26.4, CH_2
5	3.20 (t, *J*=6.9 Hz)	43.5, CH_2	3.14, m	42.0, CH_2
7		159.7, C		158.7, C
1′		179.5, C		175.7, C
2′	2.29 (q, *J*=7.1 Hz)	38.5, CH_2	2.20 (q, *J*=7.1 Hz)	37.1, CH_2
3′	1.60, m	28.0, CH_2	1.55, m	26.8, CH_2
4′	1.32, m	30.7, CH_2	1.30[c], m	29.9[c], CH_2
5′	1.60, m	27.2, CH_2	1.30[c], m	29.9[c], CH_2
6′	2.33(t, *J*=7.3 Hz)	37.4, CH_2	1.55, m	26.2, CH_2
7′		183.0, C	2.20, (q, *J*=7.1 Hz)	35.6, CH_2
8′				178.6, C

[a] Spectra were recorded in D_2O.
[b] Spectra were recorded in CD_3OD-d_4.
[c] Signals are overlapped.

Table S2. Cytotoxicity of compounds 1-6 against HT-29, A549 and Hela cell lines (n=3)

Sample No.	IC_{50} ±SD (μM)		
	HT-29	A549	Hela
1	>50	>50	>50
2	>50	>50	>50
3	>50	>50	>50
4	>50	>50	>50
5	>50	>50	>50
6	>50	>50	>50
Doxorubicin	1.15±0.03	0.86±0.07	0.55±0.05

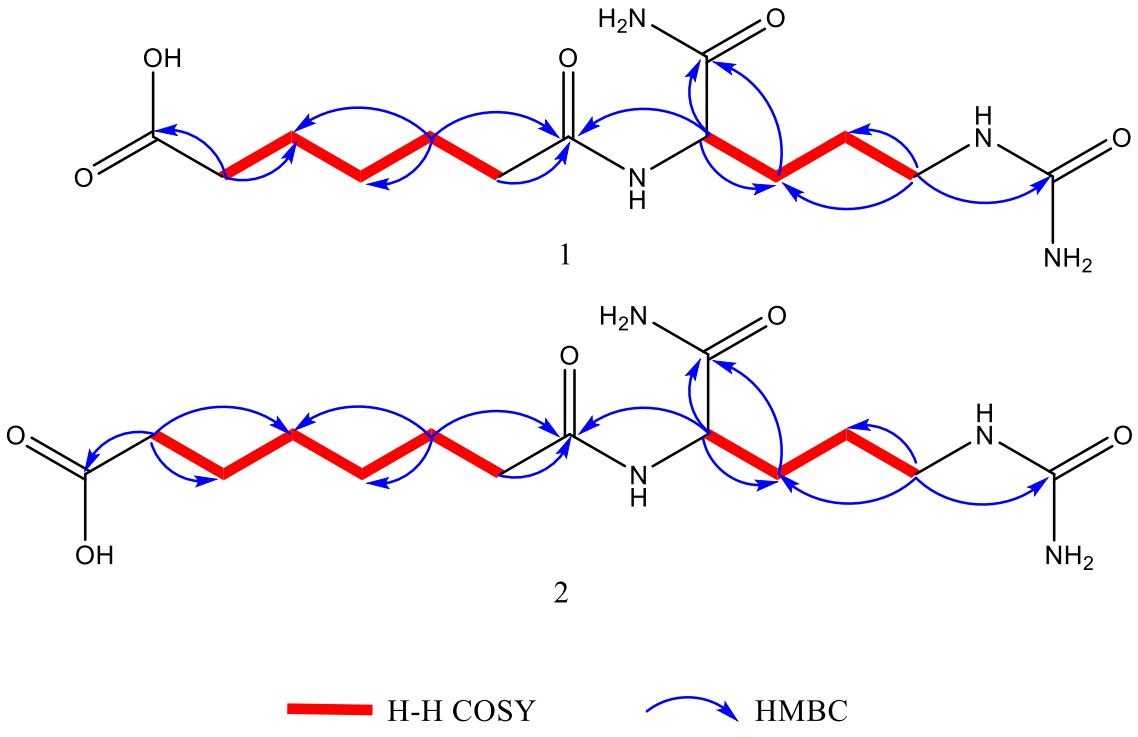

Figure S1. HMBC, and ^{1}H-^{1}H COSY correlations of the compounds **1-2**

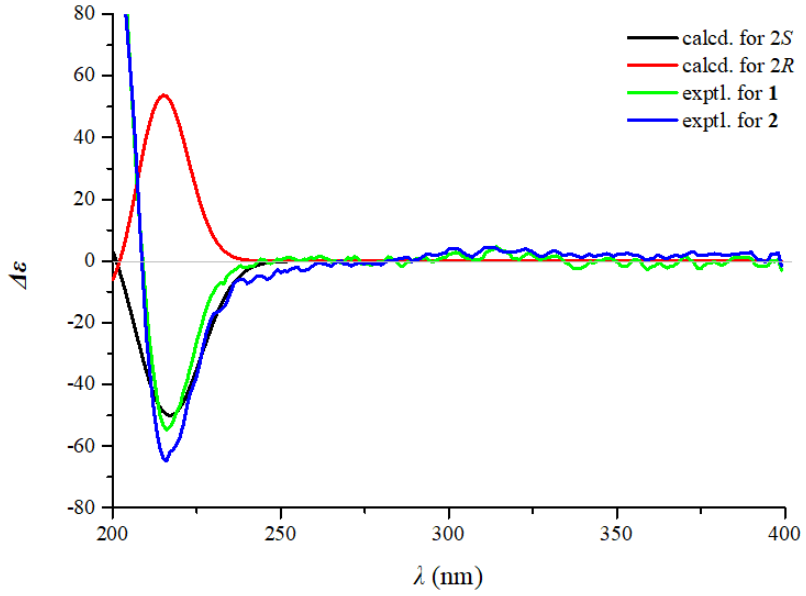

Figure S2. Experimental (exptl.) and calculated (calcd.) ECD spectra of the compounds **1-2**

Figure S3.1. ^{1}H NMR spectrum (600 MHz, D_2O) of Bufonine A (**1**)

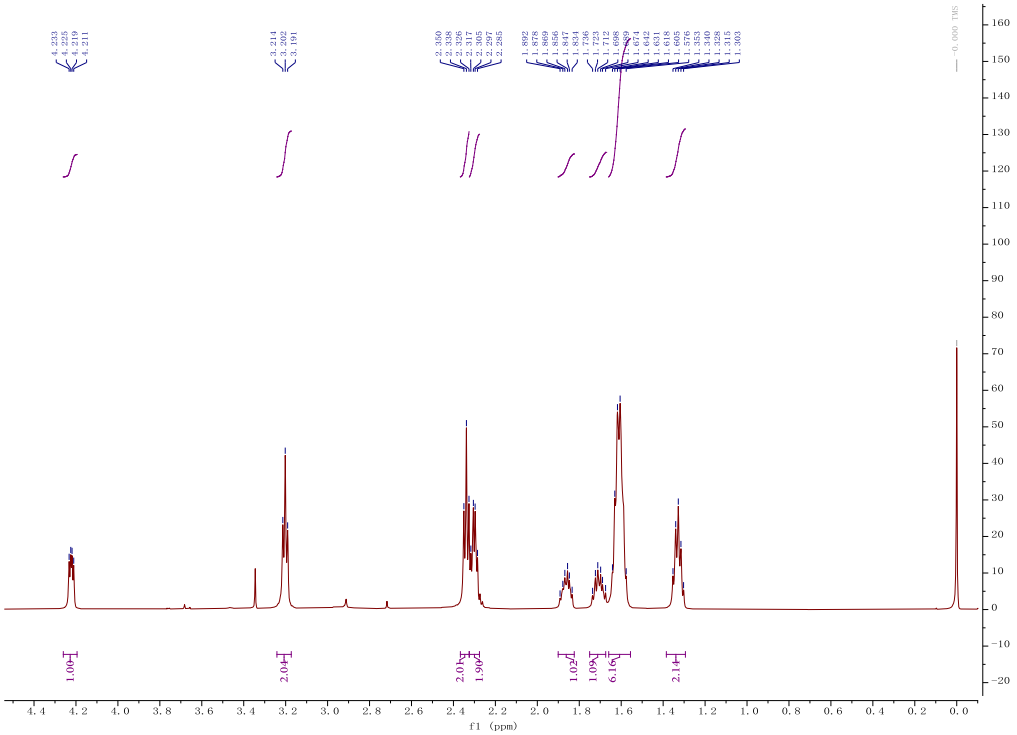

Figure S3.2. ^{13}C NMR spectrum (150 MHz, D_2O) of Bufonine A (**1**)

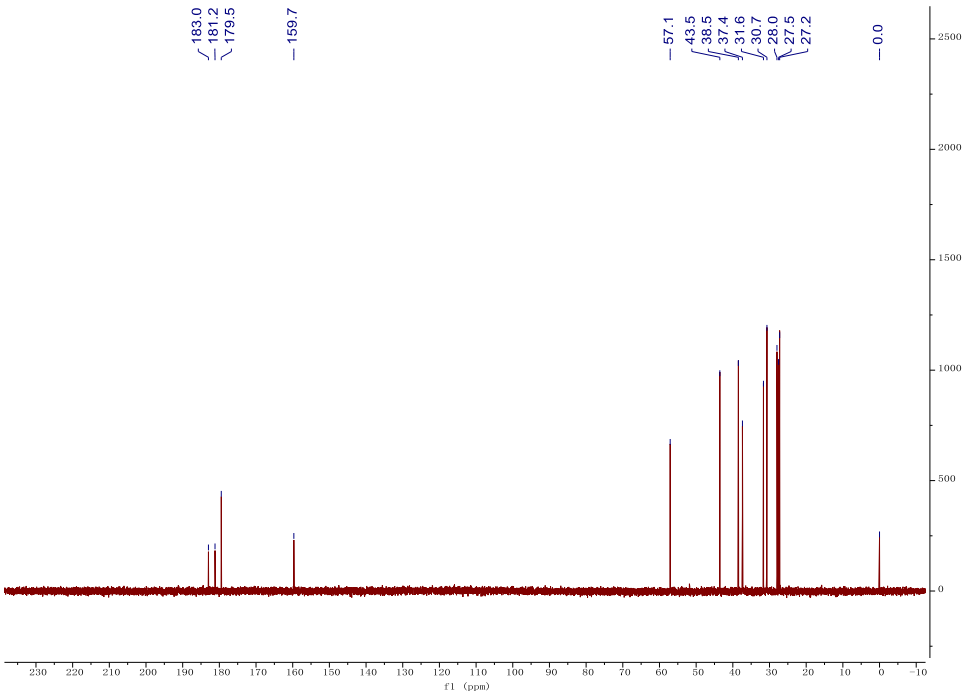

Figure S3.3. HMBC spectrum of Bufonine A (**1**)

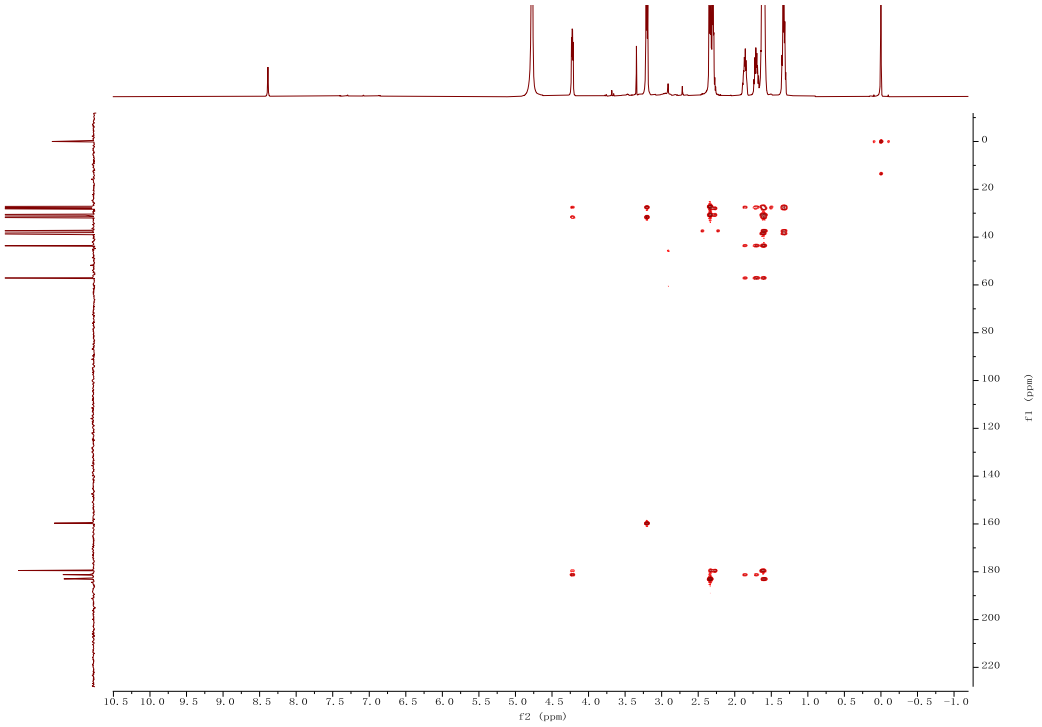

Figure S3.4. HSQC spectrum of Bufonine A (**1**)

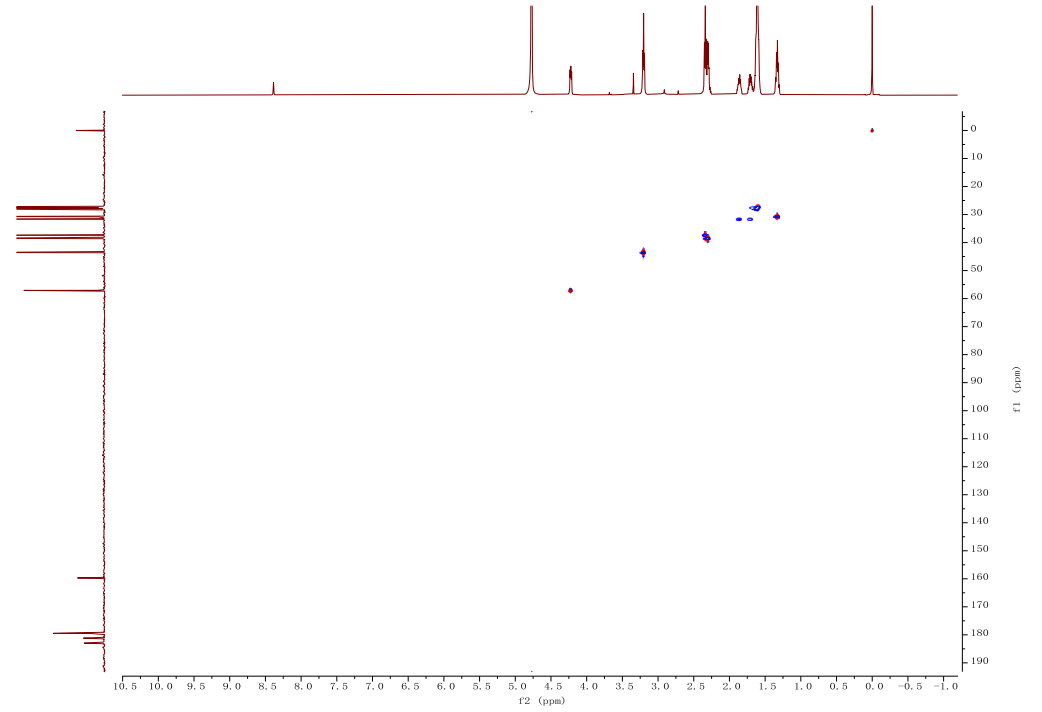

Figure S3.5. ^{1}H-^{1}H COSY spectrum of Bufonine A (**1**)

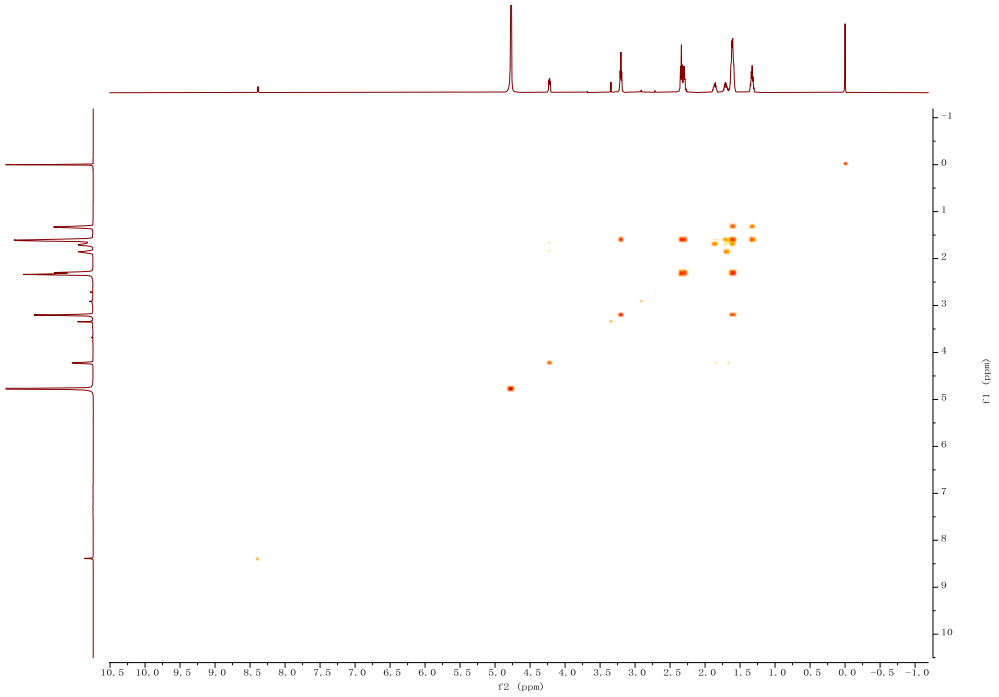

Figure S3.6. NOESY spectrum of Bufonine A (**1**)

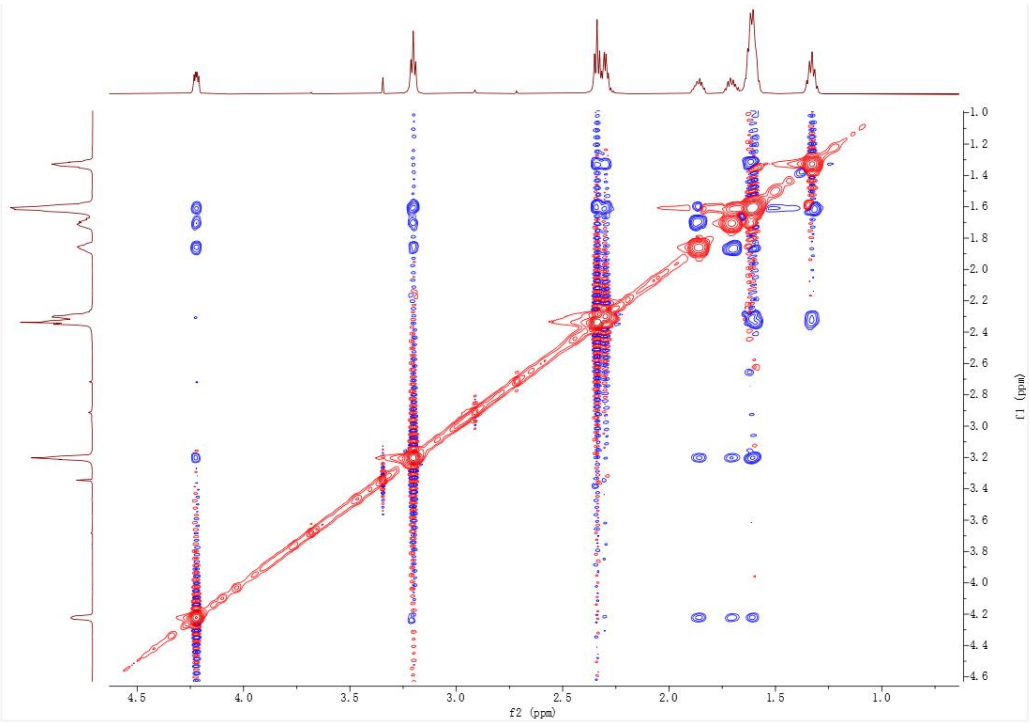

Figure S3.7. HRESIMS spectrum of Bufonine A (**1**)

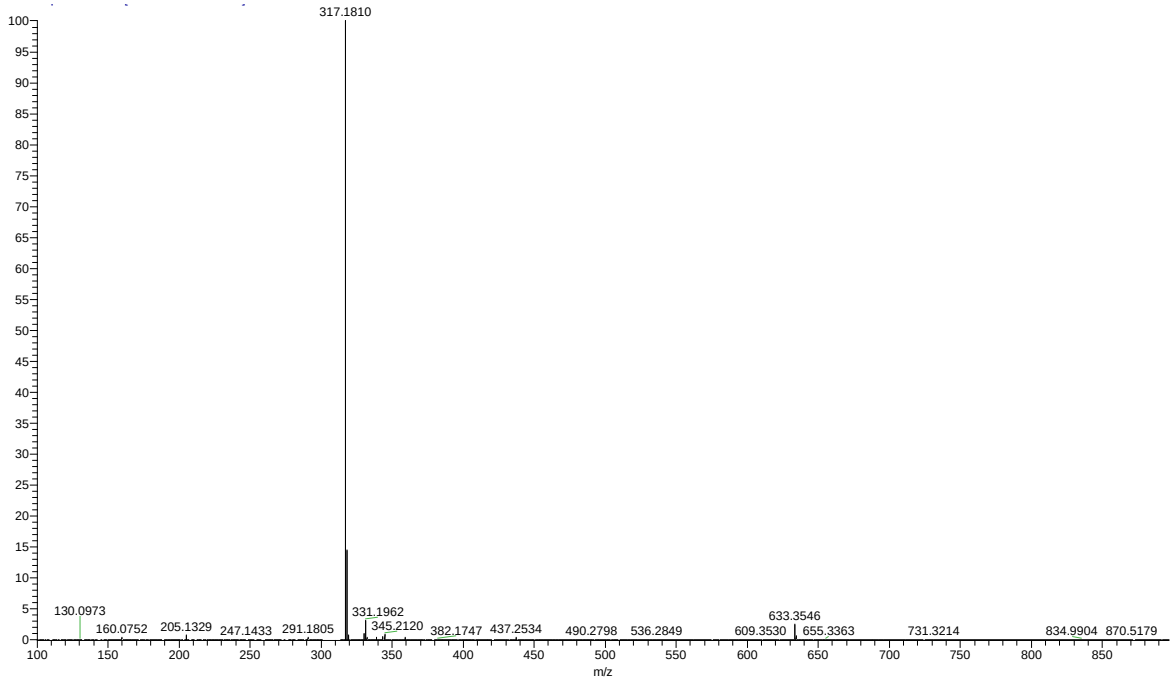

Figure S3.8. UV spectrum of Bufonine A (**1**)

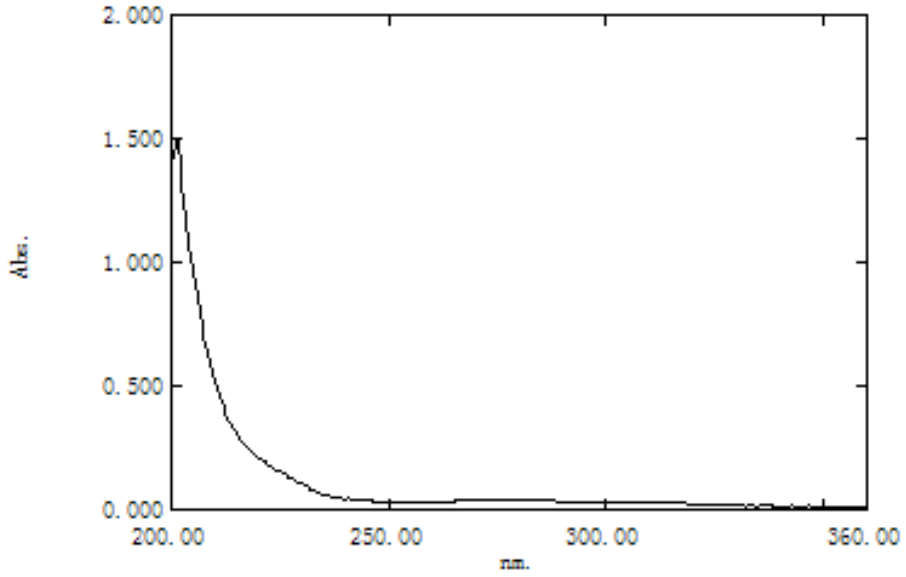

Figure S4.1. ^{1}H NMR spectrum (600 MHz, CD_3OD) of Bufonine B (**2**)

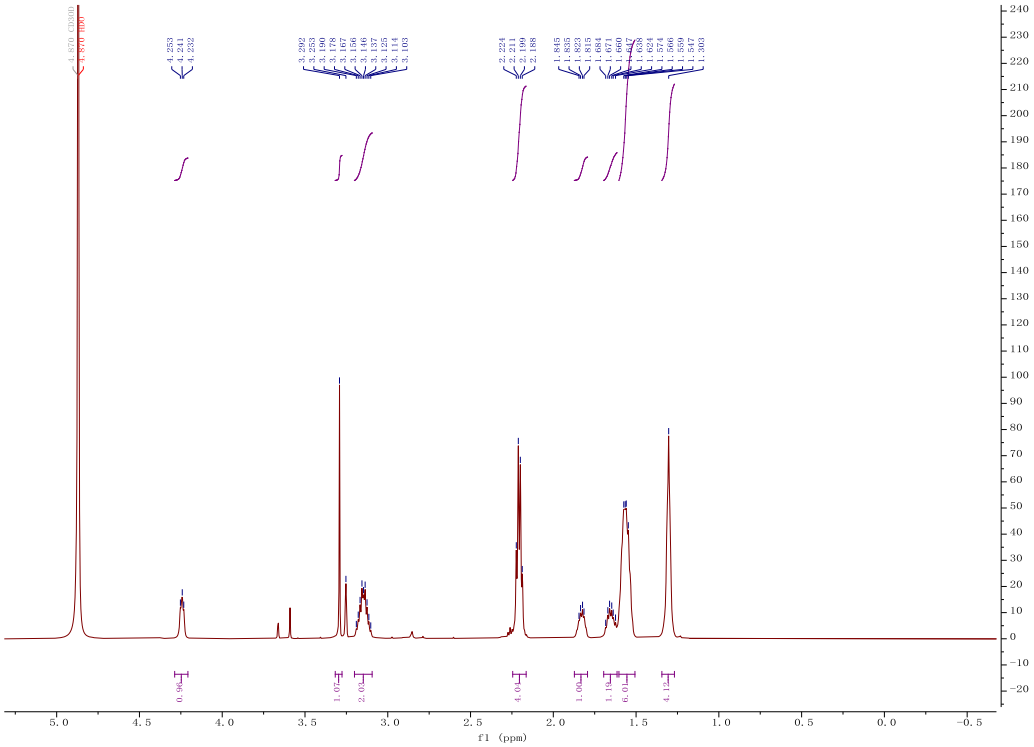

Figure S4.2. ^{13}C NMR spectrum (150 MHz, CD_3OD) of Bufonine B (**2**)

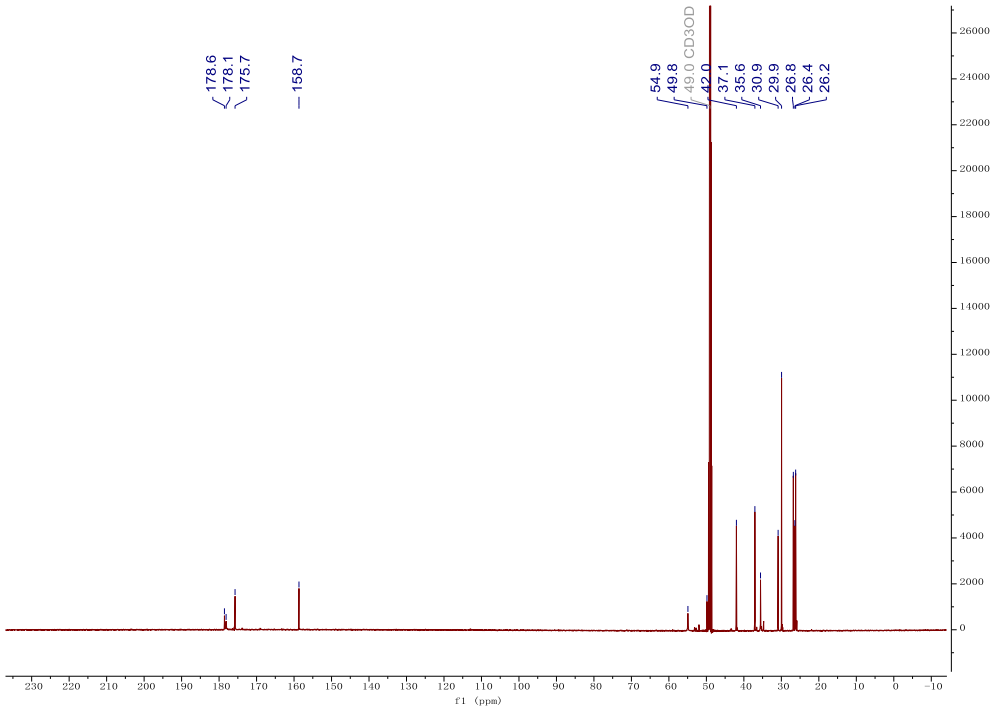

Figure S4.3. HMBC spectrum of Bufonine B (**2**)

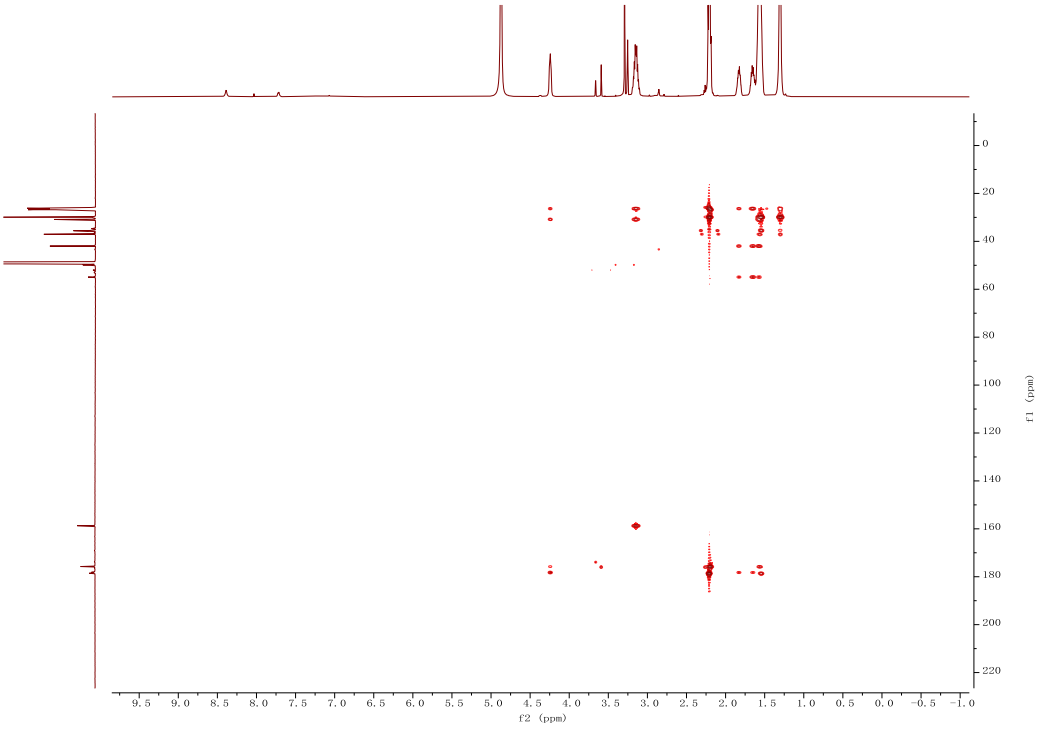

Figure S4.4. HSQC spectrum of Bufonine B (**2**)

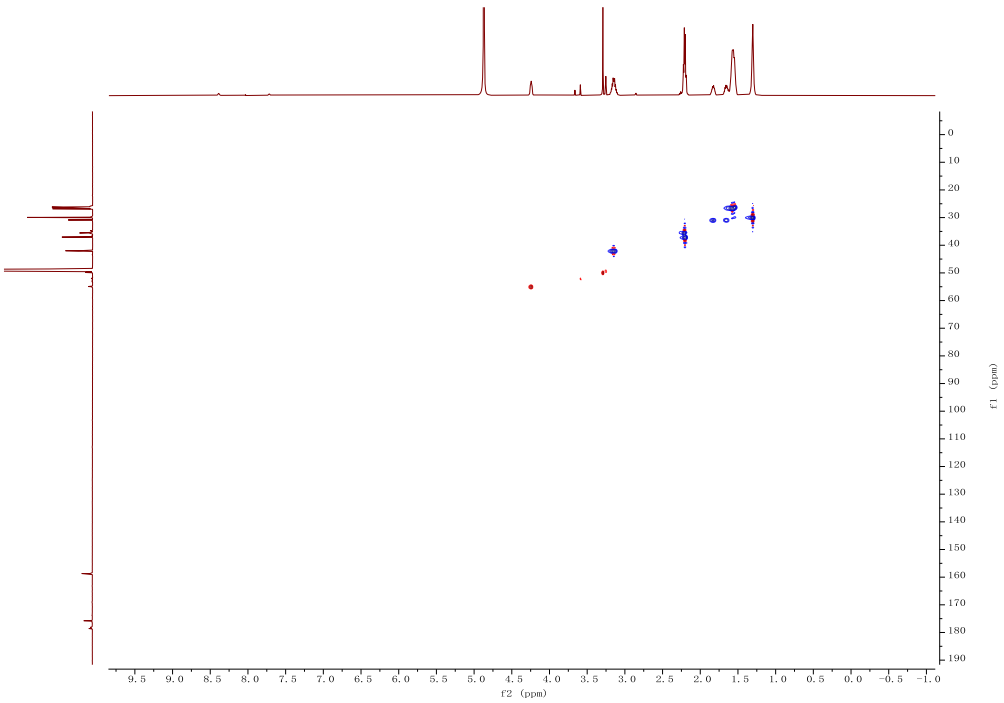

Figure S4.5. ^{1}H-^{1}H COSY spectrum of Bufonine B (**2**)

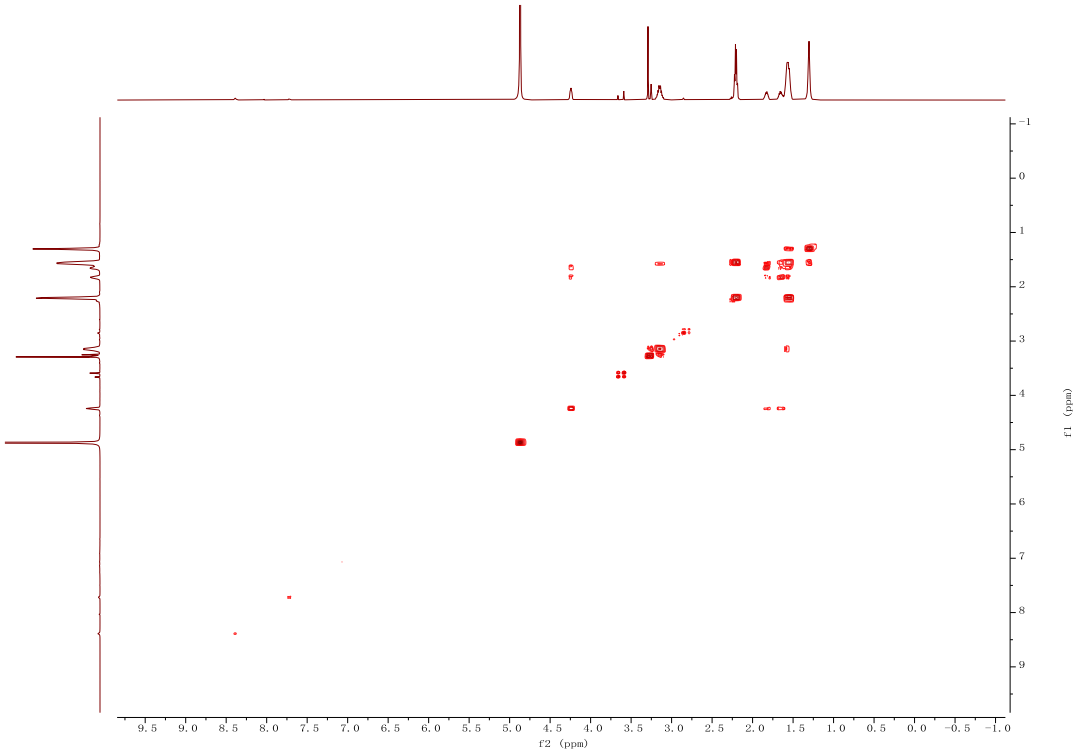

Figure S4.6. NOESY spectrum of Bufonine B (**2**)

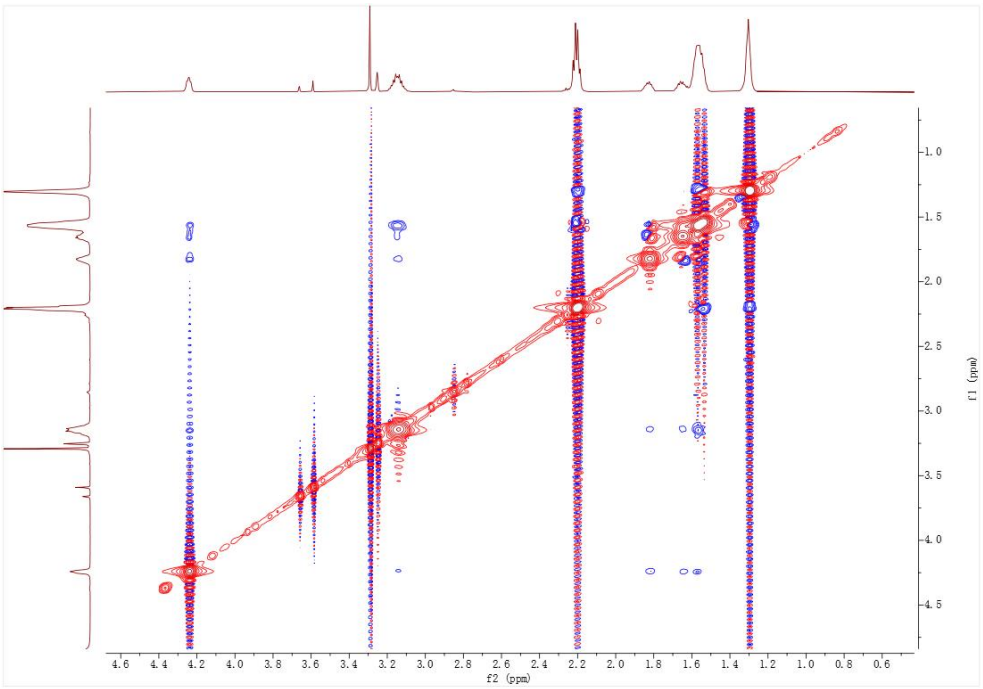

Figure S4.7. HRESIMS spectrum of Bufonine B (**2**)

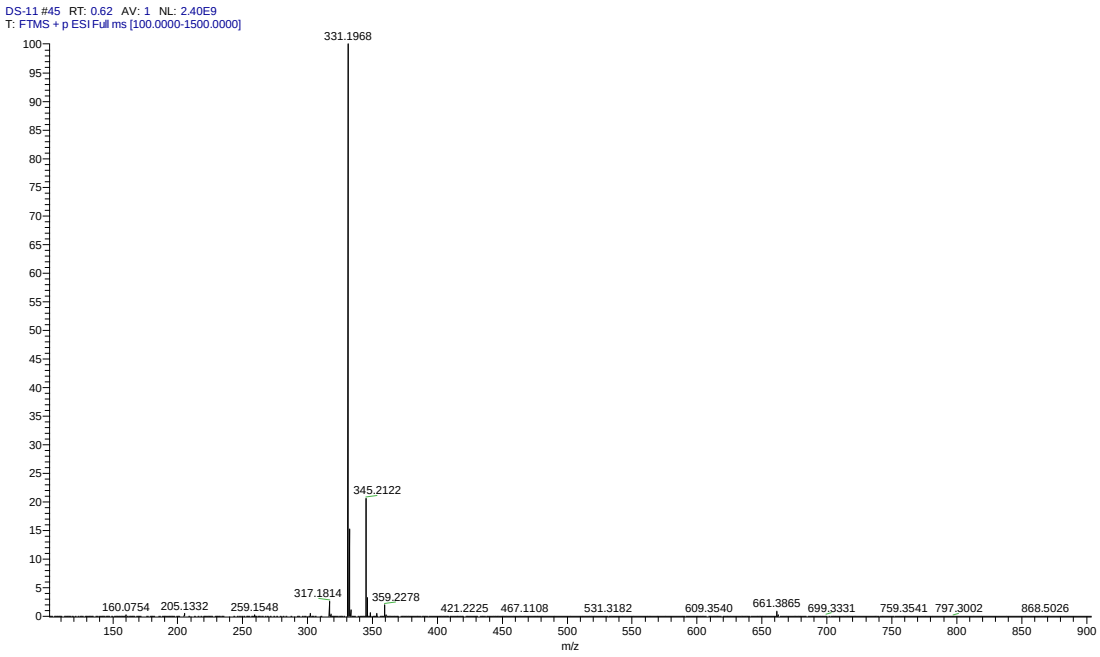

Figure S4.8. UV spectrum of Bufonine B (**2**)

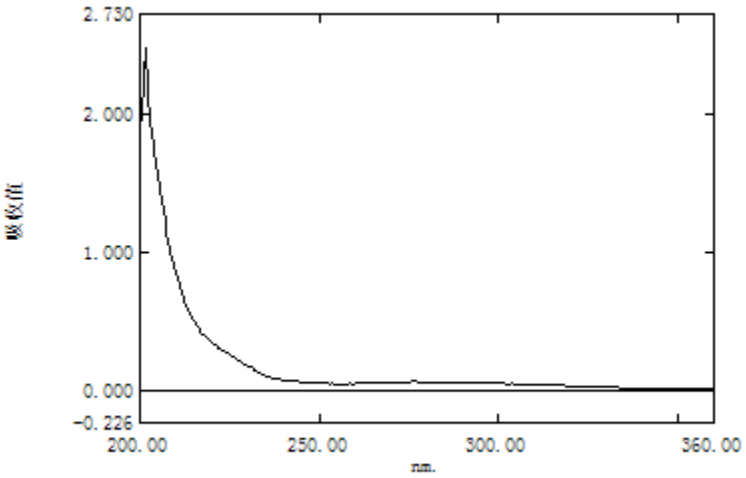

Figure S5.1. Total Ion Chromatography (TIC) of crude extract and (+) HPLC-ESI-MS/MS spectrum of bufonine A (**1**)

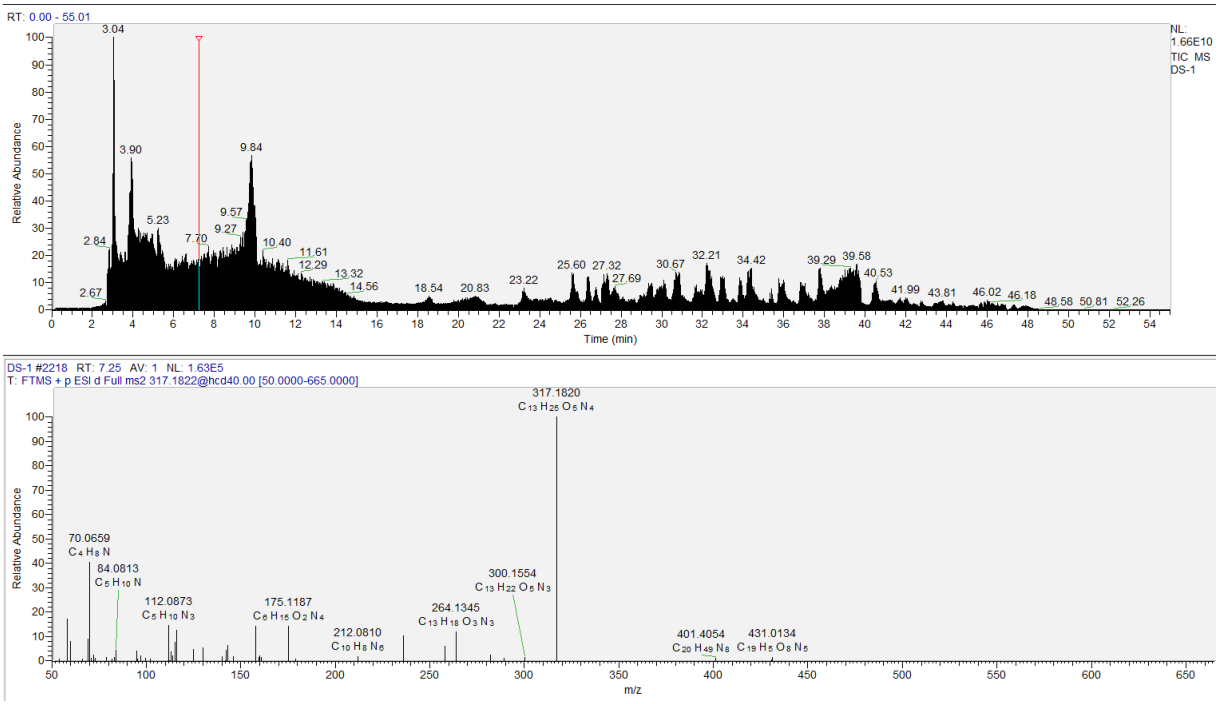

Figure S5.2. Total Ion Chromatography (TIC) of crude extract and (+) HPLC-ESI-MS/MS spectrum of bufonine B (**2**)