Hematological Studies on Naturally Occurring Substances. VI.¹⁾ Effects of an Animal Crude Drug “Chan Su” (Bufonis Venenum) on Blood Coagulation, Platelet Aggregation, Fibrinolysis System and Cytotoxicity

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During the screening test of the animal crude drug “Chan su” (Chinese name, toad-cake), the venom of *Bufo bufo gargarizans* CANTOR (Bufonidae), on blood coagulation, platelet aggregation, fibrinolysis system and cytotoxicity, the ethyl acetate extract showed promotive action on platelet aggregation and remarkable cytotoxic activity on HeLa-S₃ cells. Nine kinds of bufadienolides were isolated from the ethyl acetate extract by bioactivity-guided fractionation and were identified by chemical and spectral analysis.

Keywords hematological study; Chan su; *Bufo bufo gargarizans*; blood coagulation; platelet aggregation; fibrinolysis system; cytotoxicity; HeLa-S₃ cell; bufadienolide; Bufonidae

“Chan su” (Bufonis Venenum) is a dry toad venom collected by squeezing the secretion directly from the parotid gland and sebaceous gland of Chinese toads, either *Bufo bufo gargarizans* CANTOR or *B. melanostictus* SCHNEIDER (Bufonidae). It is used in clinical therapy to stimulate heart contraction, for diuresis, to relieve pain and as an anti-tumor remedy in oriental medicine.²⁾

We previously reported the screening test of animal crude drugs on blood coagulation and the fibrinolysis system.³⁾ To carry out systematic studies on the physiological activities of the animal crude drug Chan su, we extracted and separated the material in the manner shown in Experimental to obtain the corresponding AcOEt-, MeOH- and H₂O extracts, respectively, and investigated the hematological effect and cytotoxicity on HeLa-S₃ cells. The MeOH- (56%) and H₂O extract (88%) showed relatively strong activity on the fibrinolytic system, and the AcOEt- (82%) and H₂O extract (64%) showed promotive action on platelet aggregation (see Fig. 1). As shown in Table I, the AcOEt extract was recognized to have a remarkable cytotoxic effect on HeLa-S₃ cells, and was subsequently submitted to column chromatography on silica gel to provide twelve fractions (fr. 1–12). Fractions 5–8 and 10, exhibiting more potent cytotoxicity against HeLa-S₃ cells, were purified by high performance liquid chromatography (HPLC) on a reversed phase column and recrystallized to obtain 9 kinds of bufadienolides: bufalin

(1), cinobufagin (2), resibufogenin (3), gamabufotalin (4), hellebrigenin (5), telocinobufagin (6), desacetylcinobufagin (7), bufotalin (8) and cinobufotalin (9), as shown in Chart 1. Each compound was identified by comparison of mp and $[\alpha]_D$, infrared (IR), fast atom bombardment mass spectra (FAB-MS), electron impact mass spectra (EI-MS), proton and carbon-13 nuclear magnetic resonance (¹H- and ¹³C-NMR) spectral data of authentic samples.^{4–8)}

Further, all of them were examined for blood coagulation by plasma recalcification time method, platelet aggregation by adenosine diphosphate (ADP)-induced aggregation of

TABLE I. Cytotoxic Activity of Extracts, Fractions and Bufadienolides Obtained from Chan Su on HeLa-S₃ Cells (IC₅₀ Value)

Sample name	IC ₅₀ (μg/ml)	Sample name	IC ₅₀ (μg/ml)
AcOEt extr.	0.0096	Fr. 11	0.0470
MeOH extr.	0.021	Fr. 12	0.235
Fr. 1	> 1	1	0.000615
Fr. 2	> 1	2	0.0208
Fr. 3	0.412	3	0.550
Fr. 4	0.0126	4	0.014
Fr. 5	0.00789	5	0.014
Fr. 6	0.00240	6	0.037
Fr. 7	0.0313	7	0.025
Fr. 8	0.0228	8	0.020
Fr. 9	0.0450	9	0.11
Fr. 10	0.00438

Fig. 1. Effects of Extracts of Chan Su on ADP-Induced Blood Platelet Aggregation (Rabbit)

Chart 1. Structures of Bufadienolides Isolated from Chan Su

TABLE II. Effects of Bufadienolides on Blood Coagulation, Platelet Aggregation and Fibrinolysis System

Bufadienolide	Coagulation (%)	Platelet aggregation (%)	Fibrinolysis (%)
1	1	-16	-31
2	-1	32	-29
3	-3	5	-8
4	3	-14	-23
5	-3	19	-22
6	-2	22	2
7	-6	0	-18
8	-10	13	-3
9	-5	14	4

TABLE III. Contents of Adrenaline and Noradrenaline in Chan Su

Sample	Contents of catecholamine (%)	
	Adrenaline	Noradrenaline
Powder of Chan su	2.94	0.13
AcOEt extr.	Trace	Trace
MeOH extr.	1.72	0.06
H ₂ O extr.	4.37	0.08

rabbit platelet, fibrinolysis system by fibrin plate method (see Table II) and cytotoxicity against HeLa-S₃ cells (see Table I).

It was found that compound 1 showed the most excellent cytotoxic activity, and 4 and 5 also exhibited relatively strong activity. Most compounds, however, had less activity in blood coagulation, platelet aggregation and the fibrinolysis system.

In addition, we determined adrenaline and noradrenaline content in Chan su by a HPLC method owing to the promotive effect of platelet aggregation.^{9a,b)} The data presented in Table III, in conjunction with Fig. 1, suggest that the promotive effect on platelet aggregation of Chan su is not only due to adrenaline but also to the synergism of interaction in bufadienolides or to other active substance existing in it.

Experimental

Spectroscopy All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO model DIP-4 automatic polarimeter. IR spectra were recorded by

Hitachi model 215, JASCO model IRA-1 and IR-S spectrometers. Mass spectral measurements were run on a JEOL model JMS-DX 302 and a JASCO model ZABHF spectrometer. NMR spectra were recorded with tetramethylsilane (TMS) as an internal standard on a JEOL model FT-NMR GSX-400 and a Varian FT-NMR GEMA-200 spectrometer at 50 and 200 MHz, respectively. HPLC was carried out on a Beckman model 110A pump equipped with a SSC Y-1000 UV detector and a Senshu pak. ODS-3251 column (i.d. 8 × 250 mm).

Materials The crude drug Chan su was imported from the Chinese market by Mikuni Co. (Osaka, Japan). All reagents were of fine grade.

Extraction of Chan Su The materials (315 g) were crushed and pulverized, then successively extracted with AcOEt, MeOH and water (3 l × 3, 2 h per extraction) under reflux. The AcOEt and MeOH solutions were evaporated *in vacuo* to dryness and the water solution was lyophilized to yield AcOEt (A: 6 g), MeOH (M: 82 g) and water (W: 112 g) extract, respectively.

Isolation of Bufadienolides 1—9 The AcOEt extract (6 g) was subjected to column chromatography on silica gel and eluted successively with acetone–chloroform–cyclohexane (3 : 3 : 4) to give 12 fractions (Frs. 1—12). Fractions 5 (0.51 g) and 6 (1.24 g) were separated by HPLC using MeOH–H₂O (7 : 3) as a solvent and were further purified by recrystallization to give compounds 1 (16 mg), 2 (28 mg) and 3 (46 mg). Fractions 10 (0.68 g) and 11 (0.92 g) were separated by HPLC using MeOH–H₂O (2 : 1) as a solvent and were further purified by recrystallization to give compounds 4 (18 mg), 5 (21 mg), 6 (24 mg) and 7 (12 mg). Fractions 7 (0.38 g) and 8 (0.46 g) were separated by HPLC using MeOH–H₂O (2 : 1) as a solvent and were further purified by recrystallization to give compounds 8 (11 mg) and 9 (14 mg).

Compound 1 was recrystallized from acetone–MeOH to give bufalin (1) as colorless needles. mp 225–234 °C. $[\alpha]_D^{22.5}$ -8.7° (*c* = 0.32, CHCl₃). EI-MS *m/z*: 386 (M⁺). ¹H-NMR (in CDCl₃, 400 MHz) δ: 0.70 (s), 0.95 (s), 2.46 (dd, *J* = 9.53, 6.76 Hz), 4.14 (brs), 6.26 (d, *J* = 9.84 Hz), 7.23 (d, *J* = 2.29 Hz), 7.84 (dd, *J* = 9.84, 2.29 Hz).

Compound 2 was recrystallized from ether–acetone to give cinobufagin (2) as colorless prisms. mp 210–215 °C. $[\alpha]_D^{22.5}$ -3.7° (*c* = 0.26, CHCl₃). EI-MS *m/z*: 442 (M⁺). FAB-MS *m/z*: 443 (M + H⁺). ¹H-NMR (in CDCl₃, 200 MHz) δ: 0.82 (s), 0.99 (s), 1.89 (s), 2.80 (d, *J* = 9.33 Hz), 3.66 (d, *J* = 1.32 Hz), 4.16 (brs), 5.46 (dd, *J* = 9.33, 1.32 Hz), 6.22 (d, *J* = 9.24 Hz), 7.17 (brs), 7.93 (d, *J* = 9.24 Hz).

Compound 3 was recrystallized from acetone–H₂O to give resibufogenin (3) as colorless amorphous substance. mp 153–156 °C. $[\alpha]_D^{22}$ -7.4° (*c* = 0.92, CHCl₃). EI-MS *m/z*: 384 (M⁺), FAB-MS *m/z*: 385 (M + H⁺). ¹H-NMR (in CDCl₃, 200 MHz) δ: 0.78 (s), 0.99 (s), 2.48 (d, *J* = 10.14 Hz), 3.53 (s), 4.14 (brs), 6.25 (dd, *J* = 9.74, 1.06 Hz), 7.25 (dd, *J* = 2.64, 1.06 Hz), 7.80 (dd, *J* = 9.74, 2.64 Hz).

Compound 4 was recrystallized from acetone–MeOH to give gamabufotalin (4) as colorless prisms. mp 260–264 °C. $[\alpha]_D^{22}$ 1.5° (*c* = 0.36, MeOH). EI-MS *m/z*: 402 (M⁺). FAB-MS *m/z*: 403 (M + H⁺). ¹H-NMR (in DMSO-*d*₆, 200 MHz) δ: 0.61 (s), 0.96 (s), 3.44 (brs), 3.89 (brs), 4.04 (d, *J* = 6.14), 4.14 (s), 4.14 (s), 6.29 (d, *J* = 9.67 Hz), 7.55 (d, *J* = 2.53 Hz), 7.90 (dd, *J* = 9.67, 2.53 Hz).

Compound 5 was recrystallized from acetone to give hellebrigenin (5) as colorless plates. mp 154–159/232–236 °C. $[\alpha]_D^{22}$ 21.9° (*c* = 0.42, CHCl₃). EI-MS *m/z*: 416 (M⁺). FAB-MS *m/z*: 417 (M + H⁺). ¹H-NMR (in DMSO-*d*₆, 200 MHz) δ: 0.55 (s), 2.44 (d, *J* = 8.43 Hz), 4.02 (brs), 4.33 (brs), 5.20 (s), 5.37 (brs), 6.30 (d, *J* = 9.84 Hz), 7.52 (d, *J* = 2.01 Hz), 7.92 (dd, *J* = 9.84, 2.01 Hz), 10.05 (s).

Compound 6 was recrystallized from acetone to give telocinobufagin (6) as colorless needles. mp 154–157/204–206 °C. $[\alpha]_D^{22}$ 5.4° (*c* = 0.48, CHCl₃). EI-MS *m/z*: 402 (M⁺). FAB-MS *m/z*: 403 (M + H⁺). ¹H-NMR (in DMSO-*d*₆, 200 MHz) δ: 0.60 (s), 0.82 (s), 2.45 (d, *J* = 9.44 Hz), 4.01 (brs), 4.18 (s), 4.82 (s), 5.22 (d, *J* = 4.03 Hz), 6.29 (d, *J* = 9.81 Hz), 7.52 (d, *J* = 2.52 Hz), 7.93 (dd, *J* = 9.81, 2.52 Hz).

Compound 7 was recrystallized from acetone–ether to give desacetylcinobufagin (7) as colorless particles. mp 180–220 °C. $[\alpha]_D^{22}$ 18.4° (*c* = 0.24, CHCl₃). EI-MS *m/z*: 400 (M⁺). FAB-MS *m/z*: 401 (M + H⁺). ¹H-NMR (in CDCl₃, 200 MHz) δ: 0.80 (s), 0.99 (s), 2.61 (d, *J* = 9.23 Hz), 3.58 (d, *J* = 1.19 Hz), 4.15 (brs), 4.75 (dd, *J* = 9.23, 1.19 Hz), 6.22 (d, *J* = 9.79 Hz), 7.24 (dd, *J* = 2.59, 0.96 Hz), 7.99 (dd, *J* = 9.79, 2.59 Hz).

Compound 8 was recrystallized from acetone to give bufotalin (8) as colorless prisms. mp 226–229 °C. $[\alpha]_D^{22.5}$ 4.0° (*c* = 0.16, CHCl₃). EI-MS *m/z*: 444 (M⁺). FAB-MS *m/z*: 445 (M + H⁺). ¹H-NMR (in CDCl₃, 200 MHz) δ: 0.78 (s), 0.95 (s), 1.87 (s), 2.65 (dd, *J* = 15.49, 8.95 Hz), 2.88 (d, *J* = 8.95 Hz), 4.15 (brs), 5.54 (m), 6.20 (dd, *J* = 9.39, 0.93 Hz).

Compound 9 was recrystallized from acetone–ether to give cinobufotalin

(9) as colorless plates. mp 249–255 °C. $[\alpha]_D^{22.5}$ 13.3° ($c=0.28$, CHCl_3). EI-MS m/z : 458 (M^+). FAB-MS m/z : 459 ($\text{M}+\text{H}^+$). $^1\text{H-NMR}$ (in CDCl_3 , 200 MHz) δ : 0.83 (s), 0.98 (s), 1.90 (s), 2.81 (d, $J=9.43$ Hz), 3.26 (d, $J=1.34$ Hz), 4.21 (brs), 5.45 (dd, $J=9.43$, 1.34 Hz), 6.22 (d, $J=9$, 12 Hz), 7.1 (d, $J=1.10$ Hz), 7.92 (d, $J=9$, 12 Hz).

Blood Coagulation A water soluble test sample (5 mg) was dissolved in 250 μl of saline or a water insoluble sample (5 mg) was dissolved in 250 μl of DMSO. A mixture containing 30 μl of test solution and 150 μl of platelet poor plasma (PPP) obtained from a healthy Japanese white male rabbit in the manner reported previously³⁾ was incubated for 60 s with stirring, then dropped into 150 μl of 0.025 M CaCl_2 solution at 37 °C. Saline or DMSO was used as a control. Coagulation time was evaluated by an automatic recording apparatus (KC-4A, Amelung GmbH, Lemgo) based on the viscosity change of the plasma. Each sample was tested at least 3 times on the plasma of each of 3–5 test animals. The average time was calculated by the following equation: ratio of anti-coagulation (%) = [(coagulation time of each test solution/coagulation time of control) – 1] $\times$ 100.

Platelet Aggregation By the same method as the blood coagulation test, fresh blood (9 ml) containing 3.8% sodium citrate (1 ml) was centrifuged at 1000 rpm for 20 min at room temperature to obtain a clear supernatant as platelet-rich plasma (PRP). PPP was prepared by centrifugation of the remaining blood at 3000 rpm for 10 min. A water soluble sample (5 mg) was dissolved in 1 ml of saline, 25 μl of which was added to 175 μl of PRP; a sample (5 mg) of low saline solubility was dissolved in 200 μl of MeOH, 5 μl of which was added to 195 μl of PRP, then dropped into 25 μl 50 mM CaCl_2 and incubated at 37 °C for 1 min with stirring. The platelet aggregation was induced in a final concentration of 2 μM ADP and determined by a light transmission method slightly modified from that of Okuyama *et al.*^{10a)} as reported previously,^{10b)} using a platelet aggregometer of PAM-8T (Mebanix Corporation). Saline or MeOH was used as a control. As shown in Fig. 1 the ratio of platelet aggregation potency is assigned as 0% PRP transmission and 100% PPP transmission.¹¹⁾ The activity of anti-platelet aggregation (%) was calculated by the following equation: ratio of anti-platelet aggregation (%) = [(platelet aggregation potency of sample/platelet aggregation potency of control) – 1] $\times$ 100.

Fibrinolytic Activity Preparation of the fibrin plate was carried out according to the procedure previously reported.³⁾ A mixture of 100 μl of each test solution, 30 μl of urokinase solution (600 U/ml) and 10 μl of aliquot was placed in each of the two wells in two plates. The control used phosphate buffer in place of the sample. After incubation at 37 °C for 18 h, the diameters of the sample- and control-lysed area were measured. This procedure was repeated at least 3 times. The value of activity (%) was calculated by the following equation: fibrinolytic activity (%) = [(lysed area of test solution/lysed area of control) – 1] $\times$ 100.

Cytotoxic Activity HeLa-S₃ cells were adjusted to the concentration of 7.5×10^3 cells/ml and 0.2 ml of cells suspension were seeded into each well of a 96 well tissue culture plate (Coster). The cells were precultured

for 24 h at 37 °C in a 5% CO_2 incubator. Then, 10 μl of various concentrations of samples was put into each well and the plates were incubated for 72 h at 37 °C in a 5% CO_2 incubator. Following incubation, the medium in the well was discarded. The cells were fixed with MeOH for 2 min and then stained for 30 min with a solution of 0.05% methylene blue in 10 mM Tris-HCl buffer (pH 8.5). The plates were washed 3 times with distilled water and left to dry at room temperature. Each well as filled with 200 μl of 3% HCl in order to dissolve the dye on the adherent cells. The optical density of HCl solution was measured on an automatic photometer (Dynatech Laboratories Inc., Tokyo, Japan) at 660 nm. Percents of growth inhibition rates (%) were calculated by the following formula: percent of growth inhibition (%) = $[1 - A_{660}(\text{treated cells}) / A_{660}(\text{control cells})] \times 100$. IC₅₀ values were calculated from the growth inhibition curve drawn on a piece of logarithmic-probability graph paper.

Determination of Adrenaline Content Chan su powder and its extracts (50 mg of each) were dissolved in 5 ml of Ringer solution (NaCl 6.0 g, KCl 0.075 g, CaCl_2 0.1 g, NaHCO_3 0.1 g) and were centrifuged at 3000 rpm for 20 min, then filtered to obtain a supernatant as the test solution. The adrenaline content determined by two point calibration method of HPLC (flow rate: 1 ml/min) was carried out on a Capcell pak C-18 column (i.d. 4.6×250 mm) (Shiseido) using phosphate buffer solution (pH 3.0) as solvent.

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