

INACTIVATION OF FORMOSAN SNAKE VENOMS IN VITRO BY THE CRUDE EXTRACT OF ARISTOLOCHIA RADIX

LI-HSUEH TSAI, HUNG-JUNG LIU, CHUNG-PING YANG and CHEN CHANG

The effectiveness of *Aristolochia radix* crude extract in inactivating venoms of several species of Formosan venomous snakes in vitro was investigated in this study. Constant amounts of *Aristolochia radix* crude extract solution were mixed with various amounts of venoms, and then injected intramuscularly into mice. Results, both in terms of mortality of the test animals and hemorrhage by injection of venoms and *Aristolochia radix* crude extract solution, showed a considerable degree of variation. In general, the crude extract of *Aristolochia radix* was very effective against venoms of several species of Formosan venomous snakes of family of Crotalidae (*Trimeresurus gramineus*, *Trimeresurus mucrosquamatus*, *Agkistrodon acutus*), but not effective against venoms of family of Elapidae (*Bungarus multicinctus*, *Naja naja atra*).

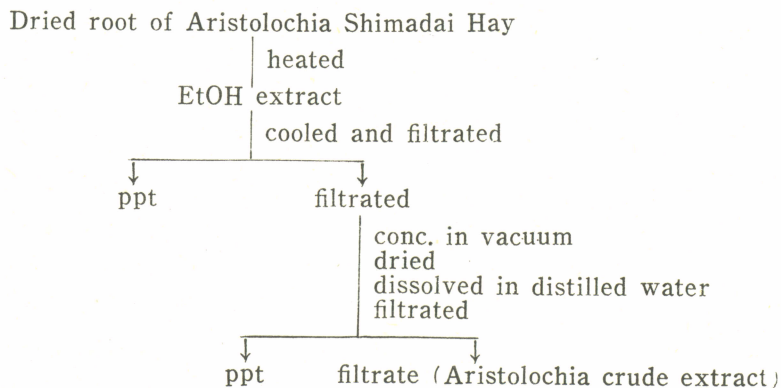
Key words: inactivation, Formosan snake venoms, *Aristolochia radix* crude extract.

Aristolochia radix "Ma-tou-ling" is one of the most important Chinese drugs. It was first recorded in "Hsin-Hsiu-Pen-Tsao⁽¹⁾". *Aristolochia Shimadai* Hay collected in Taiwan was recorded in "Manual of Medicinal Plants in Taiwan⁽²⁾". It had been used as folk medicine for treatment of snake bite in Taiwan and expectorant and emetic purposes^(2,3). Okonogi et al reported that tannic acid is very effective for the treatment of poisonous snake bite⁽⁴⁾. Sawai et al investigated the inactivation of snake venoms in vitro by dihydro-

thioctic acid (DHTA) and reported that DHTA was very effective against the poisonous venoms⁽⁵⁾. The purpose of the present study was to elucidate the effectiveness of *Aristolochia radix* crude extract to inactivate Formosan snake venoms in vitro.

MATERIALS AND METHODS

The dried root of *Aristolochia Shimadai* Hay was extracted with ethyl-alcohol (EtOH) and concentrated according to the process shown in the following chart.



Department of Physiology, Taipei Medical College, Taipei, Republic of China.

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The dried venoms were dissolved in the saline buffer solution. Potency tests were conducted by injecting various amounts of venom in a constant volume of 0.1 ml of the saline buffer solution, allowed to stand for one hour incubation at 37°C before, intramuscularly into the legs of the white mice (dd strain) weighing 18-20 gm. The saline buffer solution was prepared in 0.2 M phosphate buffer at pH 6.2. Inactivating tests were conducted by injecting 0.1 ml of 15% Aristolochia crude extract solution adding 0.2 M acid phthalate buffer mixed with various amounts of venom intramuscularly into the leg of the white mice. The pH of 15% Aristolochia crude extract solution as well as the pH of this solution adding 0.2 M acid phthalate buffer

mixed with venom were 4.1. The pH of solutions were measured with the Coleman pH meter (Parkin-Elmer Metrion IV). In these tests Aristolochia crude extract, venom, and buffered saline solution were mixed together and allowed to stand for one hour at 37°C before injection. Mice were observed for 24 hours after the injection. Not less than 5 mice were used for one test dose. The numbers of death and hemorrhage within 24 hours were used for the calculation of LD₅₀ and HD₅₀. The HD₅₀ and LD₅₀ of the venoms were calculated by the method of Litchfield and Wilcoxon (1949)⁽⁶⁾. The LD₅₀ tolerated by mouse, % loss of toxicity and % residual toxicity were calculated from the following formulas.

$$\text{LD}_{50} \text{ tolerated by mouse} = \frac{\text{LD}_{50} \text{ of venom in test solution}}{\text{LD}_{50} \text{ of original venom}}$$

$$\% \text{ loss of toxicity} = \frac{\text{LD}_{50} \text{ tolerated by mouse} - 1}{\text{LD}_{50} \text{ tolerated by mouse}} \times 100$$

$$\% \text{ residual toxicity} = \frac{\text{LD}_{50} \text{ of original venom}}{\text{LD}_{50} \text{ of heated venom}} \times 100$$

Venomous Snakes of Families of Crotalidae and Elapidae

Genus, Species Family	Latin name	Common name
Crotalidae	1. <i>Trimeresurus gramineus</i> (Shaw)	Bamboo Viper
	2. <i>Trimeresurus mucrosquamatus</i> (Cantor)	Taiwan Habu
	3. <i>Agkistrodon acutus</i> (Günther)	100 Pacer
Elapidae	4. <i>Bungarus multicinctus</i> (Blyth)	Taiwan Krait
	5. <i>Naja naja atra</i> (Cantor)	Taiwan Cobra

Heating of venoms

The dried venoms were dissolved in the saline buffer solution. Then the venom solution was heated in a water bath for 1 hour at 37°C. Two kinds of saline buffer solution were used. One contained 0.2 M phosphate buffer and the pH was adjusted to 6.2. The other contained 0.2 M acid phthalate buffer and the pH was adjusted to 4.1.

RESULTS

Tests with Crotalidae Venoms

Data on toxicity of these venoms were summarized in Table 1, which indicated that all Crotalid venoms were hemorrhagic in effect. The appearances of hemorrhages produced by injections of Crotalid snake venoms were shown in Fig. 1. As shown in Table 3, the

Table 1. Toxicity of Venoms of Three Species of Crotalid Snakes for White Mice

Venom Amounts ($\mu\text{g/gm}$)	<i>Trimeresurus gramineus</i> (Bamboo Viper)		<i>Trimeresurus mucrosquamatus</i> (Taiwan Habu)		<i>Agkistrodon acutus</i> (100 Pacer)	
	Mortality* (M)	Degree of Hemorrhage (DH)	Mortality	Degree of Hemorrhage	Mortality	Degree of Hemorrhage
32	10/10	3.0**	10/10	3.0	10/10	3.0
16	9/10	3.0	9/10	3.0	8/10	2.6
8	6/10	3.0	8/10	3.0	6/10	2.5
6	5/10	3.0	6/10	—	2/10	2.2
4	4/10	2.9	4/10	3.0	0/10	2.2
2	2/10	2.2	3/10	2.8	0/10	2.0
1	0/10	2.0	1/10	2.7	0/10	1.6
0.5	0/10	1.8	0/10	2.4	0/10	1.4
0.25	0/10	1.8	0/10	2.2	0/10	1.4
0.125	0/10	1.4	0/10	1.4	0/10	1.2
0.075	—	—	—	—	0/10	0.6
0.05	0/10	0.6	0/10	0.3	0/10	0.2
0.025	0/10	0.2	0/10	0	0/10	0
0.0125	0/10	0	—	—	—	—

* Mortality

Numerator indicates the number of mice which died.

Denominator indicates the number of mice used.

** Degree of hemorrhage (average value of 10 mice in each dose)

0: no hemorrhage at the site of injection.

1: hemorrhage in one third of the thigh.

2: hemorrhage in two thirds of the thigh.

3: hemorrhage extend to the trunk.

HD₅₀ of *Trimeresurus gramineus* venom was 0.035 $\mu\text{g/gm}$, of *Trimeresurus mucrosquamatus* venom 0.075 $\mu\text{g/gm}$, and of *Agkistrodon acutus* venom 0.028 $\mu\text{g/gm}$. On the other hand the LD₅₀ of *Trimeresurus gramineus* venom was 4.8 $\mu\text{g/gm}$, of *Trimeresurus mucrosquamatus* venom 5.5 $\mu\text{g/gm}$, and of *Agkistrodon acutus* venom 8.6 $\mu\text{g/gm}$. The inactivating effect of 15% Aristolochia Crude Extract (ACE) against Crotalid Snake Venoms (CSV) were summarized in Table 2. Hemorrhages appeared remarkably in the injected legs of mice (C) which received injection of CSV without 15% ACE, however, hemorrhages reduced or disappeared in the injected legs of mice (E) which received injection of CSV with 15% ACE (Fig. 1). HD₅₀, LD₅₀, % loss of toxicity

and reduction of toxicity tolerated by mouse for CSV before and after adding 15% ACE were summarized in Table 3. The HD₅₀ of the venoms in the mice which received injections of CSV with 15% ACE, was 17.5 $\mu\text{g/gm}$ for *Trimeresurus gramineus*, 8 $\mu\text{g/gm}$ for *Trimeresurus mucrosquamatus* and 26.7 $\mu\text{g/gm}$ for *Agkistrodon acutus*. The LD₅₀ of *Trimeresurus gramineus* venom was 46 $\mu\text{g/gm}$, of *Trimeresurus mucrosquamatus* venom 38 $\mu\text{g/gm}$ and of *Agkistrodon acutus* venom 104 $\mu\text{g/gm}$ (Table 3). Among three kinds of CSV, reduction of toxicity by adding 15% ACE showed more than 84% loss of toxicity, and the toleration of mice to CSV was more than (or equal to) 7 LD₅₀. The results stated above showed that the inactivating effect of 15% ACE

Table 2. Inactivating Effects of 15% Aristolochia Crude Extract (ACE) against Venoms of Crotalid Snakes

Venom Amounts ($\mu\text{g/gm}$)	<i>Trimeresurus gramineus</i> (Bamboo Viper)				<i>Trimereurus mucrosquamatus</i> (Taiwan Habu)				<i>Agkistrodon acutus</i> (100 Pacer)			
	15% ACE*		O**		15% ACE		O		15% ACE		O	
	M	DH	M	DH	M	DH	M	DH	M	DH	M	DH
130									10/10	2.8		
114									8/10	2.6		
98									4/10	2.4		
92									2/10	2.2		
88	10/10	3.0							0/10	2.2		
—	—	—							0/10	—		
56	8/10	3.0							—	—		
48	7/10	3.0			10/10	3.0			—	—		
44	6/10	2.8			9/10	3.0			—	—		
40	2/10	2.8			6/10	3.0			—	1.6		
36	1/10	2.2	10/10	3.0	4/10	2.8			0/10	1.6	10/10	3.0
32	0/10	0.4	10/10	3.0	1/10	2.8	10/10	3.0	0/10	1.0	10/10	3.0
16	0/10	0	9/10	3.0	0/10	2.4	9/10	3.0	0/10	1.0	8/10	2.6
8	0/10	0	6/10	3.0	0/10	1.6	8/10	3.0	0/10	0.4	6/10	2.5
4	—	—	4/10	2.9	0/10	0.2	4/10	3.0	0/10	0	1/10	2.2
2	—	—	2/10	2.2	0/10	0	3/10	2.8	0/10	0	0/10	2.0
1	—	—	0/10	2.0	0/10	0	1/10	2.7	—	—	—	—
0.5	—	—	—	—	—	—	0/10	2.7	—	—	—	—

M is mortality of mice and DH is degree of hemorrhage

See foot notes of Table 1

* Injection of snake venoms with 15% ACE

** Injection of snake venoms without 15% ACE

Table 3. HD_{50} , LD_{50} , % Loss of Toxicity and Reduction of Toxicity Tolerated by Mouse for Venoms of Crotalid Snakes Before and After Adding 15% ACE

Venoms	HD_{50} ($\mu\text{g/gm}$)		LD_{50} ($\mu\text{g/gm}$)		Reduction of toxicity	
	O	15% ACE	O	15% ACE	LD_{50} tolerated by mouse	% loss of toxicity
<i>Trimereurus gramineus</i> (Bamboo Viper)	0.035 (0.025-0.049)	17.5 (15.4-19.8)	4.8 (3.4-6.7)	46 (40.7-52)	9.5 (8-12)	89
<i>Trimeresurus mucrosquamatus</i> (Taiwan Habu)	0.075 (0.054-0.105)	8 (7.3-8.7)	5.5 (3.9-7.7)	38 (35-41.4)	7 (5.4-9)	84
<i>Agkistrodon acutus</i> (100 Pacer)	0.028 (0.02-0.039)	26.7 (24.3-29.4)	8.6 (6.1-12)	104 (95-114)	12 (9.5-15.5)	92

HD_{50} : 50% hemorrhagic dose; LD_{50} : 50% lethal dose.

O: venoms without 15% ACE 15% ACE: venoms with 15% ACE

Parentheses following HD_{50} and LD_{50} denoted their 95% confidence limits.

against CSV in vitro were remarkable.

Tests with Elapid Venoms

Elapid Snake Venoms (ESV) were primarily neurotoxic in effect. The inactivating effect of 15% ACE against ESV were summarized in Table 4. LD₅₀, % loss of toxicity and reduction of toxicity were summarized in Table 5. The venom of *Bungarus multicinctus* produced no local hemorrhage and the venom of *Naja naja atra* produced a very slight degree of hemorrhage at the site of injection. The LD₅₀ of *Bungarus*

multicinctus venom was 0.88 µg/gm and of *Naja naja atra* venom was 0.95 µg/gm. On the other hand for the mice which received injection of ESV with 15% ACE, the LD₅₀ of *Bungarus multicinctus* venom was 0.75 µg/gm and of *Naja naja atra* venom was 2 µg/gm. In the case of *Naja naja atra* venom reduction of toxicity by adding 15% ACE was statistically significant, but in the case of *Bungarus multicinctus* venom it was not statistically significant.

Table 4. The Inactivating Effect of 15% Aristolochia Crude Extract (ACE) against Venoms of Elapid snakes

Venom Amounts (µg/gm)	<i>Bungarus multicinctus</i> (Taiwan Krait)				<i>Naja naja atra</i> (Taiwan Cobra)			
	15% ACE		O		15% ACE		O	
	M	DH	M	DH	M	DH	M	DH
4	5/5	0	5/5	0	5/5	0.8	5/5	0.8
3	5/5	0	5/5	0	3/5	0	5/5	0
2	5/5	0	5/5	0	3/5	0	4/5	0
1	5/5	0	4/5	0	1/5	0	2/5	0
0.95	4/5	0	—	—	0/5	0	2/5	0
0.9	4/5	0	2/5	0	0/5	0	—	—
0.8	3/5	0	2/5	0	0/5	0	2/5	0
0.7	3/5	0	0/5	0	—	—	—	—
0.6	0/5	0	—	—	—	—	1/5	0
0.5	—	—	—	—	—	—	1/5	0
0.4	—	—	—	—	—	—	0/5	0

See foot notes of Table 1 and Table 2.

Table 5. LD₅₀, % Loss of Toxicity and Reduction of Toxicity Tolerated by Mouse for Venoms of Elapid Snakes Before and After Adding 15% ACE

Venoms	LD ₅₀ (µg/gm)		Reduction of toxicity	
	O	15% ACE	LD ₅₀ tolerated by mouse	% loss of toxicity
<i>Bungarus multicinctus</i>	0.88 (0.51-1.6)	0.75 (0.63-0.89)	0	0
<i>Naja naja atra</i>	0.95 (0.95-1.38)	2 (1.1-3.8)	2.2	54.5

O: venoms without 15% ACE 15% ACE: venoms with 15% ACE.
Parentheses following LD₅₀ denoted their 95% confidence limits.

Effects of Incubation and pH on Toxicity

Table 6. Effect of Incubation on Toxicity

Per cent residual toxicity of venoms after incubation at 37°C for 1 hour.

Venom	pH=4.1			pH=6.2		
	LD ₅₀ (μg/gm)		Per cent residual toxicity (%)	LD ₅₀ (μg/gm)		Per cent residual toxicity (%)
	Incubation 37°C 1 hour	No incubation		Incubation 37°C 1 hour	No incubation	
<i>Trimeresurus gramineus</i>	6.2 (4.4-8.7)	5.2 (3.7-7.3)	83	4.8 (3.4-6.7)	4.75 (3.4-6.7)	99
<i>Trimeresurus mucrosquamatus</i>	8.5 (6.0-11.9)	7.7 (4.5-10.8)	90	5.5 (3.9-7.7)	5.3 (4 -7.6)	96
<i>Agkistrodon acutus</i>	18 (13-25.2)	17.5 (13-24.5)	97	8.6 (6.1-12)	8.6 (6.1-12)	100
<i>Bungarus multicinctus</i>	0.9 (0.5-1.6)	0.9 (0.5-1.6)	100	0.88 (0.51-1.6)	0.88 (0.51-1.6)	100
<i>Naja naja atra</i>	0.95 (0.65-1.4)	0.95 (0.65-1.4)	100	0.95 (0.65-1.38)	0.95 (0.65-1.38)	100

Parentheses following LD₅₀ denoted their 95% confidence limits.

Various amounts of venom in a constant volume of 0.1 ml of the saline buffer solution at pH 4.1 and pH 6.2 were allowed to stand for one hour incubation at 37°C and without incubation before injection intramuscularly into the legs of the white mice. By comparing the toxicity of the same venom with 1 hour incubation with that without incubation at the same pH, the Elapid Snake Venoms (ESV) were found heat stable either at pH 4.1 or pH 6.2 (Table 6). The Crotalid Snake Venoms (CSV) were stable at pH 6.2 after the incubation at 37°C for

1 hour and their toxicity reduced slightly after the incubation at pH 4.1 but not to the level of statistical significance.

When we compared the toxicity between the same venom after 1 hour incubation at pH 4.1 and that at pH 6.2, CSV in the acid medium at pH 4.1 were unstable, their reduction of toxicity were to the level of statistical significance. From the results stated above it was found that the thermal effect under 37°C incubation for one hour on toxicity of snake venoms were very slight or none. However, the effect of pH on

Table 7. Effect of pH on Toxicity

Venoms	LD ₅₀ (μg/gm)		Reduction of toxicity	
	pH=4.1 ^a	pH=6.2 ^b	LD ₅₀ tolerated by mouse	% loss of toxicity
<i>Trimeresurus gramineus</i>	6.2 (4.4-8.7)	4.8 (3.4-6.7)	1.29	22.5
<i>Trimeresurus mucrosquamatus</i>	8.5 (6.0-11.9)	5.5 (3.9-7.7)	1.54	25
<i>Agkistrodon acutus</i>	18 (13-25.2)	8.6 (6.1-12)	2.1	52
<i>Bungarus multicinctus</i>	0.9 (0.5-1.6)	0.88 (0.51-1.6)	1	0
<i>Naja naja atra</i>	0.95 (0.65-1.4)	0.95 (0.65-1.38)	1	0

a: LD₅₀ of venom in test solution. b: LD₅₀ of original venom.
Parentheses following LD₅₀ denoted their 95% confidence limits.

CSV were rather remarkable. In comparison of Table 3 and Table 7, the results were as following. The LD₅₀, in the mice which received injections of CSV with 15% ACE, for the venom of *Trimeresurus gramineus* was 46 (40.7–52) µg/gm, for *Trimeresurus mucroqu-amatus* venom 38 (35–41.4) µg/gm and for *Agkistrodon acutus* venom 104 (95–114) µg/gm (Table 3). With 1 hour incubation at pH 4.1 (the same pH as the venom added with 15% ACE) the LD₅₀ of *Trimeresurus gramineus* venom was 6.2 (4.4–8.7) µg/gm, of *Trimeresurus mucroqu-amatus* venom 8.5 (6.0–11.9) µg/gm, and of *Agkistrodon acutus* venom 18 (13–25.2) µg/gm. Reduction of toxicity after adding 15% ACE was statistically highly significant as compared with the toxicity of the venom at the same condition (one hour incubation at pH 4.1). Therefore, it seems that the inactivating effect of ACE on snake venoms is probably due to its constituents but not to its pH change.

DISCUSSION

In general, Crotalid venoms are primarily hemotoxic and hemorrhagic and Elapid venoms are neurotoxic. The results showed that inactivating effects of Aristolochia extract against the Crotalid venoms in vitro were remarkable, but those against the Elapid venoms were very slight or none. It needs further study to see whether or not Aristolochia radix extract can inactivate Crotalid snake venoms in vivo. Li et al reported⁽⁸⁾ that two most important chemical components of Aristolochia radix extract were obtained in crystal forms, they were allantoin and aristolochic acid. Allantoin has been used topically in suppurating wounds, resistant ulcers, to stimulate growth of

healthy tissue⁽⁹⁾. The antimicrobial activities of Aristolochic acid have been estimated and also the inactivating effects on bacterial toxins and enzymes related to pathogenicity have been investigated⁽¹⁰⁾. It is highly probable that aristolochic acid or allantoin may inactivate Crotalid snake venoms. Studies on the inactivating effect of chemical components of Aristolochia radix extract against the Crotalid snake venoms in vivo are in progress.

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臺灣馬兜鈴根抽取物溶液試管內對於 臺灣蛇毒解毒之研究

蔡麗雪 劉鴻榮 楊中平 張 鎮

本實驗研究臺灣馬兜鈴根之抽取物溶液於試管內對於臺灣毒蛇之蛇毒解毒效果。對照組係分別注射 0.1 ml 各種不同濃度及種類之蛇毒。實驗組則分別注射 0.1 ml 之經過15%馬兜鈴根抽取物溶液處理之各種不同濃度及種類之蛇毒。兩組注射部位均為小白鼠之後腿部肌肉，注射後經24小時觀察其死亡率及注射部位之出血程度，並求個別之 HD_{50} 及 LD_{50} 加以比較之。

赤尾鯢 HD_{50} 對照組為 0.035 (0.025–0.049) $\mu\text{g/gm}$ ，實驗組為 17.5 (15.4–19.8) $\mu\text{g/gm}$ ， LD_{50} 對照組為 4.8 (3.4–6.7) $\mu\text{g/gm}$ ，實驗組為 46 (40.7–52) $\mu\text{g/gm}$ ，龜殼花 HD_{50} 對照組為 0.075 (0.054–0.105) $\mu\text{g/gm}$ ，實驗組為 8 (7.3–8.7) $\mu\text{g/gm}$ ， LD_{50} 對照組為 5.5 (3.9–

7.7) $\mu\text{g/gm}$ ，實驗組為 38 (35–41.4) $\mu\text{g/gm}$ 。百步蛇 HD_{50} 對照組為 0.028 (0.02–0.039) $\mu\text{g/gm}$ ，實驗組為 26.7 (24.3–29.4) $\mu\text{g/gm}$ ， LD_{50} 對照組為 8.6 (6.1–12) $\mu\text{g/gm}$ ，實驗組為 104 (95–114) $\mu\text{g/gm}$ 。雨傘節 LD_{50} 對照組為 0.88 (0.51–1.6) $\mu\text{g/gm}$ ，實驗組為 0.75 (0.63–0.89) $\mu\text{g/gm}$ 。飯匙倩 LD_{50} 對照組為 0.95 (0.065–1.38) $\mu\text{g/gm}$ ，實驗組為 2 (1.1–3.8) $\mu\text{g/gm}$ 。

由結果顯示臺灣馬兜鈴根之抽取液試管內對於臺灣毒蛇臺灣蛇科如赤尾鯢、龜殼花、百步蛇，蛇毒解毒的效果顯著，而對蝙蝠蛇科如雨傘節、飯匙倩之效果極微或缺如。

(註： HD_{50} ：50%致出血量； LD_{50} ：50%致死量)

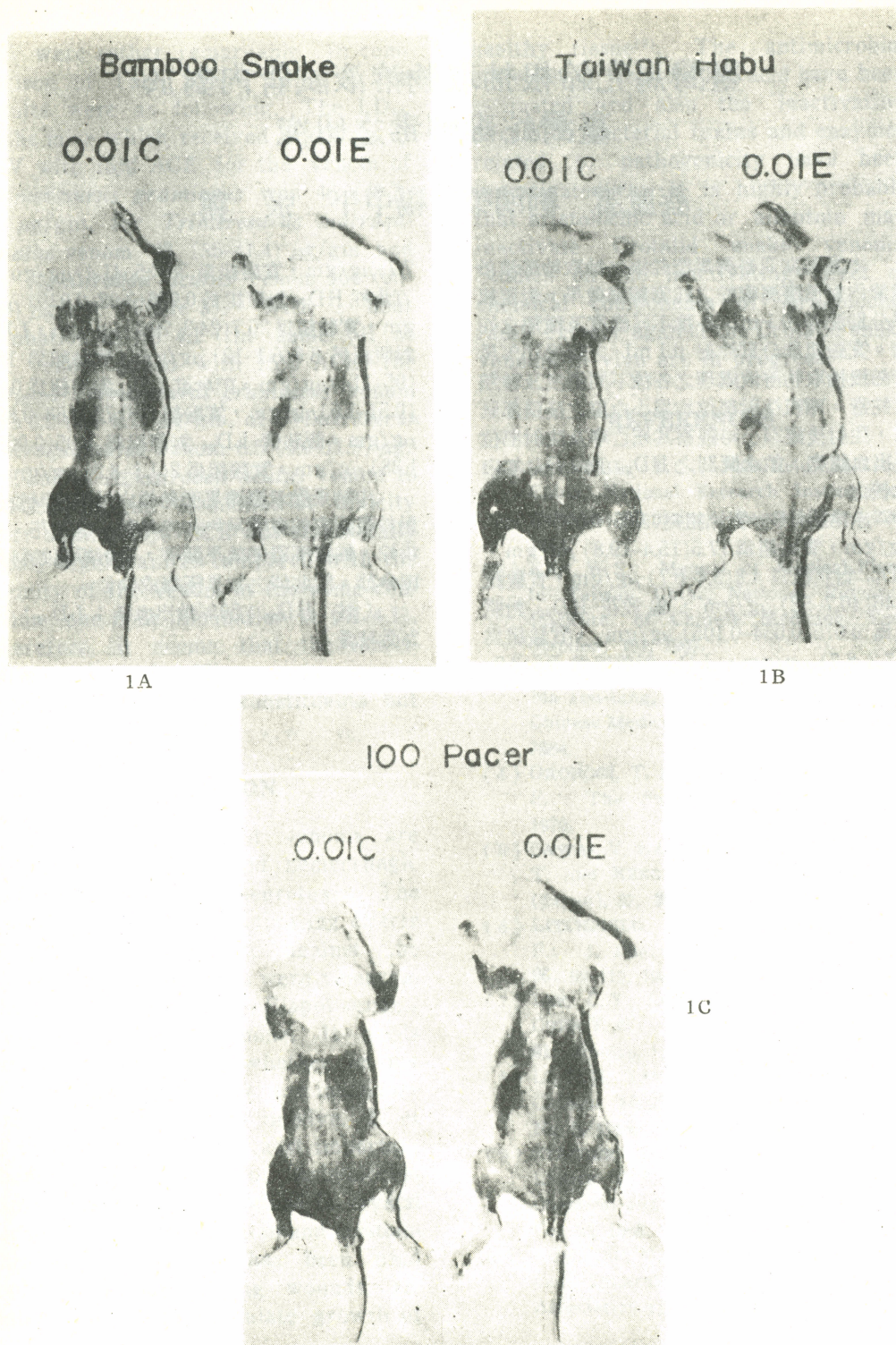


Figure 1 The hemorrhages of mice 24 hours after the injection of Crotalid venom with and without 15% ACE.
 0.01C: hemorrhages of mice 24 hours after the injection of 0.01 mg/20 gm ($0.5 \mu\text{g/gm}$) of Crotalid venom.
 0.01E: hemorrhages of mice 24 hours after the injection of 0.01 mg/20 gm ($0.5 \mu\text{g/gm}$) of Crotalid venom with 15% Aristolochia crude extract.