



|            |  |
|------------|--|
| Title      | Chemical and biochemical Studies of an Antidote Active Compound against Hemorrhagic Snake Venom from <i>Argusia argentea</i> |
| Author     | Dr. Hnin Thanda Aung   |
| Issue Date | 2013   |

# Chemical and Biochemical Studies of an Antidote Active Compound against Hemorrhagic Snake Venom from *Argusia argentea*

Hnin Thanda Aung<sup>1</sup>

## Abstract

*Argusia argentea* (Boraginaceae) is locally used in Okinawa as an antidote for jellyfish toxin and sea snakes venom poisoning. The crude methanol extract of the twigs and leaves of *Argusia argentea* was fractionated with ethyl acetate, 1-butanol and water successively. An acidic compound, rosmarinic acid RA (1), was isolated and purified from the ethyl acetate fraction by silica gel column chromatography, HPLC with ODS column and Cosmosil Cholesterol column. Snake venom neutralization activities of the active compound were examined by pharmacological assay methods. The results revealed that rosmarinic acid effectively inhibited venom-induced fibrinogenolytic, cytotoxic, edema, lethality, digestion of type IV collagen activity. Moreover, rosmarinic acid (1) inhibited both hemorrhage and neutrophil infiltrations caused by *protobothrops flavoviridis* venom in pathology sections. In this study, we investigated the structural requirements necessary for inhibition of snake venom activity through the use of compounds, which are structurally related rosmarinic acid (1). By examining anti-hemorrhagic activity of cinnamic acid analogs against *protobothrops flavoviridis* venom, it was revealed that the presence of the *E*-enoic acid moiety (-CH=CH-COOH) was critical. Furthermore, we examined the effect of benzenepolycarboxylic acids and substituted benzoic acids against *protobothrops flavoviridis* venom-induced hemorrhage. Pyromellitic acid (1,2,4,5-benzenetetracarboxylic acid) was found to be a potent inhibitor of hemorrhage, with an IC<sub>50</sub> value of 0.35 μM.

**Keywords:** *Argusia argentea*, Rosmarinic acid, snake venom, hemorrhage, *protobothrops flavoviridis*

## 1. Introduction

Snakebites are a serious medical problem in many regions of the world, especially in tropical and subtropical countries. [Warrell, Chippaux,

---

<sup>1</sup> Assistant Lecturer, Dr., Department of Chemistry, Mandalay University

Swaroop] Envenomations due to snakebites are commonly treated by parenteral administration of horse or sheep derived polyclonal antivenom aimed at the neutralization of toxins. [Panfoli] However, antivenom does not provide sufficient protection against venom-induced hemorrhage, necrosis, nephrotoxicity and furthermore, can often cause hypersensitivity reactions. [Calmette, Stahel, Corrigan, Sutherland, Cruz] Thus, there is an urgent need for new, effective, and inexpensive drugs. Natural products are important sources of novel therapeutic agents in anti-snake venom drug development. The goal of our research was isolation and identification of the active compound from *Argusia argentea*, which was responsible for snake venom neutralization and to investigate its mechanism of action and structural-activity relationship.

The methanolic extract of a sample of twigs and leaves of *A. argentea* showed activity against *Protobothrops flavoviridis* (Habu) venom-induced hemorrhage. The methanolic extract of *A. argentea* was fractionated with ethyl acetate, 1-butanol and water successively. An acidic compound, rosmarinic acid RA (1), was isolated and purified from the ethyl acetate fraction by silica gel column chromatography, HPLC with ODS column and Cosmosil Cholesterol column. Anti-hemorrhagic activity was assayed by using several kinds of snake venom. Snake venom neutralization activities of the active compound were examined by pharmacological assay methods of inhibition of fibrinogen hydrolytic activity, inhibition of venom cytotoxic action on HUVEC, inhibition of type IV collagen hydrolytic activity, inhibition of venom-induced edema, inhibition of venom-induced lethality and histological study of *P. flavoviridis* venom and the effect of rosmarinic acid (1).

Studies on structure-activity relationships are vital for understanding the molecular mechanisms of biochemical processes. In this study, we investigated the structural requirements necessary for inhibition of snake venom activity through the use of compounds, which are structurally related to rosmarinic acid (1). Firstly, the structural requirements of RA (1) for the inhibition of hemorrhage induced by the hemorrhagic snake venom of *P. flavoviridis* through the use of cinnamic acid analogues were determined. To better explore the structural features of CH=CH-COOH group compounds and to investigate the most potent snake venom anti-hemorrhage inhibitor, benzoic acid, benzenepolycarboxylic acids and substituted benzoic acids were discussed.

## 2. Materials and Methods

### 2.1 General

IR spectra were recorded on FT-IR-410 spectrophotometre.  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra were recorded on a JEOL ECA-500 ( $^1\text{H}$ : 500 MHz and  $^{13}\text{C}$ : 125 MHz). Chemical shifts for  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR are given in parts per million ( $\delta$ ) relative to solvent signal (methanol- $d_4$ :  $\delta_{\text{H}}$  3.30 and  $\delta_{\text{C}}$  49.0) as internal standard. EI- and FAB-MS were obtained with a JEOL JMS MS-700 and HX-110, respectively. *m*-Nitrobenzyl alcohol was used as a matrix for FAB-MS. Optical rotations were recorded on a JASCO P-1020 polarimeter (cell length 100 mm). Analytical TLC was performed on Silica gel 60 F<sub>254</sub> (Merck). Column chromatography was carried out on silica gel BW-820MH (Fuji Silysia Chemicals, Co. Ltd, Seto, Japan). Develosil ODS UG-5 ( $\phi 4.6 \times 250$  mm, Nomura Chemical, Seto, Japan), and Cosmosil Cholester ( $\phi 4.6 \times 250$  mm, Nacalai Tesque, Kyoto, Japan) columns were used for the analytical HPLC. Develosil ODS UG-5 ( $\phi 20 \times 250$  mm, Nomura Chemical, Seto, Japan), and Cosmosil Cholester ( $\phi 20 \times 250$  mm, Nacalai Tesque, Kyoto, Japan) columns were used for preparative HPLC.

### 2.1 Plant material

Fresh twigs and leaves of *A. argentea* were collected on Okinawa Islands, and they were moved to our laboratory below 4 °C.

### 2.2 Venoms and chemicals

*P. flavoviridis* (habu) venom (Okinawa), *P. elegans* venom, *Gloydius blomhoffii* venom and *Bitis arietans* venom were purchased from Japan Snake Institute, Gunma. *Crotalus atrox* venom was purchased from Sigma-Aldrich. Hemorrhagic toxin b (HTb) from *Crotalus atrox* venom was prepared by the method reported previously. Bilitoxin-2 and Ac<sub>1</sub>-proteinase were isolated using our methods reported previously from *Agkistrodon bilineatus* venom and *Deinagkistrodon acutus* venom, respectively. Hemorrhagic toxin-1 (HT-1) was obtained from *Bitis arietans* venom. Human and bovine fibrinogens were supplied by Sigma-Aldrich, Tokyo, Japan. Type IV collagen was purchased from Nitta Gelatin Inc. Cryo-preserved human umbilical vein endothelial cells (HUVEC), its respective cell culture media (HuMedia EB-2), other cell culture supplements, and reagents were obtained from Kurabo (Osaka, Japan). All other chemicals used herein were purchased from TCI, Tokyo, Japan.

### 2.3 Extraction of *A. argentea* and purification of antivenom compounds

Fresh twigs and leaves of *A. argentea* (wet 7 kg) were extracted with methanol. The methanolic extract was concentrated in vacuo, and dried extract (72 g) was obtained. The extract was then partitioned with ethyl acetate and 1-butanol against water successively to give ethyl acetate (16 g), 1-butanol (16 g) and water soluble fractions (40 g). The ethyl acetate extract (16 g) was fractionated by using a silica gel column with mixed solvents of chloroform and methanol (19:1 – 1:1) to give 21 fractions (H-1 – H-21). Among them, H-13 (1.39 g, elute with chloroform–methanol = 8: 2) was further separated with silica gel column using ethyl acetate–chloroform–formic acid (9: 1: 0.3 – 1: 1: 0.3) as eluents. H-13-10 (236 mg) possessing antihemorrhage activity was purified by HPLC with octadecylsilanized silica gel (ODS) column with methanol–water–formic acid (5: 4: 0.1) as a mobile phase. Then a compound (142 mg) was isolated and identified as RA (1) by NMR analysis.

**(+)-(E)-RA (1):** Amorphous solid.  $[\alpha]_D^{25} + 41.2^\circ$  (*c* 0.058, MeOH).  $^1\text{H-NMR}$  (500 MHz, methanol-*d*<sub>4</sub>)  $\delta$ : 7.55 (1H, d, *J* = 15.9 Hz; H-7), 7.04 (1H, br s; H-2), 6.95 (1H, d, *J* = 7.7 Hz; H-6), 6.78 (1H, d, *J* = 7.7 Hz; H-5), 6.76 (1H, s; H-2'), 6.70 (1H, d, *J* = 7.8 Hz; H-5'), 6.62 (1H, d, *J* = 7.8 Hz; H-6'), 6.26 (1H, d, *J* = 15.9 Hz; H-8), 5.19 (1H, br. d, *J* = 3.7 Hz; H-8'), 3.10 (1H, br. d, *J* = 13.4 Hz; H-7'a), 3.01 (1H, m; H-7'b).  $^{13}\text{C-NMR}$  (125 MHz, methanol-*d*<sub>4</sub>)  $\delta$ : 169.3 (C-9), 150.5 (C-4), 148.4 (C-7), 147.6 (C-3), 146.9 (C-3'), 146.0 (C-4'), 130.2 (C-1'), 128.5 (C-1), 123.9 (C-6), 122.6 (C-6'), 118.4 (C-2'), 117.3 (C-5), 117.1 (C-5'), 116.0 (C-2), 115.4 (C-8), 75.7 (C-8'), 38.8 (C-7'). HRFAB-MS (positive) *m/z*: 361.0881  $[\text{M}+\text{H}]^+$  (*m/z* 361.0923 calcd for C<sub>18</sub>H<sub>17</sub>O<sub>8</sub>).

### 2.4 Antihemorrhagic activity

Anti-hemorrhagic activity was assayed by the modified method of Bjarnason and Tu using ddY mice of 20 g average weight. Two groups of four mice were used for the experiment. All crude venom solutions of *P. flavoviridis* venom, *Crotalus atrox* venom, *G. blomhoffii* venom and *Bitis arietans* venom, were prepared at a concentration of 0.14 mg/ml in saline. Concentrations of purified hemorrhagic toxin solutions were as follows: HTb (0.41 mg/ml), bilitoxin-2 (0.0028 mg/ml), HT-1 (0.29 mg/ml), and Ac<sub>1</sub>-proteinase (1.04 mg/ml). A test solution was prepared by mixing the venom solution or the toxin solution (50  $\mu\text{L}$ ) and RA (1) (0.5 mg/ml in 10% DMSO-saline, 50  $\mu\text{L}$ ) followed by 10 min incubation at 37 °C. These test

solutions (100  $\mu$ L) were injected subcutaneously (s.c.) in the abdomen of mice. Similarly, a group of mice which were injected with a venom solution without RA (1) was used as a control group, and also a group which was only injected with 10% DMSO-saline (50  $\mu$ L) served as a blank group. Prior to this study, effects of DMSO at several concentrations were investigated, and DMSO at less than 10% was found to cause no significant inactivation of venom. After 24 h, mice were euthanized by inhalation of chloroform, the skin covering the abdomen was removed and hemorrhagic lesions were measured.

### **2.5 Collagen hydrolytic activity assay**

Collagen hydrolytic activity was assayed as follows. 0.1 M Sodium hydrogen carbonate (60  $\mu$ L, pH 12) was added to 0.3% type IV collagen (0.9 ml) and adjusted to pH 8. Aliquots of type IV collagen were incubated with *P. flavoviridis* venom (0.21  $\mu$ g/ml) in the presence or absence of RA (1) (0.5 mg/ml). At various time intervals, aliquots of 100  $\mu$ L of denaturing solution (10 mM phosphate buffer, pH 7.2, containing 10 M urea, 4% SDS, and 4%  $\beta$ -mercaptoethanol) were added. This solution was boiled for 3 min and run on SDS-PAGE using a 7.5% polyacrylamide slab gel electrophoresis.

### **2.6 Fibrinogen hydrolytic activity assay**

Fibrinogen hydrolytic activity was assayed by the method of Ouyang and Teng. A solution of 0.1% human fibrinogen in 50 mM Tris-HCl buffer (pH 7.5) (1 ml) and a venom solution (50  $\mu$ L of 0.21 mg/ml of *P. flavoviridis* venom or 5.5  $\mu$ g/ml of bilitoxin-2) were incubated in the presence or absence of RA (1) (0.5 mg/ml) at 37 °C. At various time intervals, aliquots of 100  $\mu$ L of denaturing solution (10 mM phosphate buffer, pH 7.2, containing 10 M urea, 4% sodium dodecyl sulfate (SDS), and 4%  $\beta$ -mercaptoethanol) were added. This solution was incubated at 37 °C for 6 h and then run on 10% polyacrylamide slab gel electrophoresis. Electrophoresis was carried out for 2 h with a current of 25 mA per slab gel. Bromophenol blue (BPB) solution was used as an indicator.

### **2.7 Histopathological study**

Histopathological study for RA (1) was performed by intramuscular (i.m.) injection of *P. flavoviridis* venom solution into the medial aspect of the thigh muscle of ddY strain white mice. Histopathological study of muscle was conducted in three groups. Group A was injected with the

venom (0.21 mg/ml, 100  $\mu$ l), while group B was injected with RA (1) (0.5 mg/ml, 100  $\mu$ L). Group C was injected with a mixture of the venom (0.41 mg/ml, 50  $\mu$ L) and RA (1) (0.25 mg/ml, 50  $\mu$ L). Test solutions were preincubated at 37 °C for 10 min before injection. The mice were killed by chloroform inhalation 24 h after injection. Tissue samples were immediately fixed in buffered formate fixative for 24 h at room temperature. The tissue was then washed for 4 h in running water, dehydrated in an autotechnicon, and stained with hematoxylin and eosin for observation under light microscope.

## 2.8 Cytotoxic action on HUVEC

The effects of RA (1) and *P. flavoviridis* venom on cultured human umbilical vein endothelial cells (HUVEC) were investigated using colorimetric cell viability assay [Ishiyama, Tominaga]. Frozen HUVEC were cultured and maintained in commercially available media, HuMedia-EB2, supplemented with fetal calf serum (2% v/v), hEGF (10 ng/ml), hFGF-B (5 ng/ml), hydrocortisone (1  $\mu$ g/ml), heparin (10  $\mu$ g/ml), gentamicin (50  $\mu$ g/ml), and amphotericin B (50 ng/ml). At confluency, cells were trypsinized, washed with the same medium and then resuspended in growth media. These cells were seeded in 96-multiwell plates (5  $\times$  10<sup>3</sup> cells per well in 100  $\mu$ l medium) and were allowed to attach and reach log phase of growth. Aliquots of venom and RA (1) to be assayed were diluted in saline and were sterilized by filtration with cellulose acetate 0.22  $\mu$ m membrane filters. Various concentrations of RA (1) (0.5, 0.25, 0.125, 0.06, and 0.03 mg/ml in 10% DMSO-saline) in the presence or absence of *P. flavoviridis* venom (0.14 mg/ml) were added to each well in 100  $\mu$ L medium. The plate was incubated at 37 °C under 5% CO<sub>2</sub> atmosphere for 17 h. Ten microliters of cell counting kit-8 was added to each well, and the microplate was incubated for 1 h, after which cell densities were measured at 450 nm using Bio-RAD Model 550 Microplate Reader.

## 2.9 Assay for edema activity

Hind-paw edema activity was assayed by the method of Ho *et al.* Four ddY strain white mice (20–23 g) were individually injected in the right foot pad with *P. elegans* venom (12.5  $\mu$ g in 50  $\mu$ l of 10% DMSO-saline). An equal volume of 10% DMSO saline was injected into the left paws as control. Inhibition assays were performed by preincubated RA (1) (0.5 mg/ml in 10% DMSO saline) with toxin for 10 min at 37 °C. The volume of

each paw was measured with a slide caliper. The degree of paw swelling was expressed as % increase of the initial paw volume.

### 2.10 Inhibition of venom lethal effect

The lethal toxicity of *P. flavoviridis* venom (LD<sub>50</sub>) was assayed by i.p. administration of different concentrations of venom dissolved in 10 % DMSO saline to groups ( $n = 4$ ) of ddY strain white mice (20 g). For venom inhibition study, various doses of venom were preincubated with 0.96 mg of RA (1) at 37 °C for 30 min followed by injection into the mice to test the inhibition of lethality.

## 3. Results

### 3.1 Extraction of *A. argentea* and purification of antivenom compounds

Twigs and leaves of *A. argentea* were extracted with methanol and the resulting extract was partitioned to afford ethyl acetate, 1-butanol, and water-soluble fractions, successively. Among them, the ethyl acetate fraction (2.5 mg/ml) showed significant antihemorrhagic activity against *P. flavoviridis* venom (final concentration 0.14 mg/ml in test solution). The fraction was fractionated by silica gel column chromatography to afford 21 fractions (H-1 to H-21), and their antihemorrhagic activity was examined. H-13, which inhibited hemorrhage induced by the venom, was further fractionated by repeated chromatography by using chromatography by using silica gel column and HPLC to afford H-13-10-1 as an antidote active compound against crude venom of *P. flavoviridis*. Its NMR and mass spectral data revealed the compound revealed the compound to be rosmarinic acid (RA) (1).

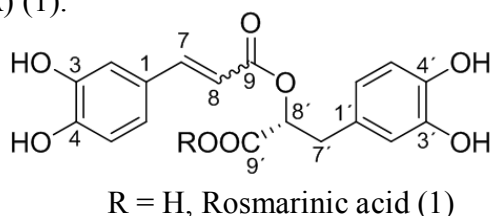


Fig. 1. Structure of rosmarinic acid (1).

### 3.2 Inhibitory activity of RA (1) on crude snake venoms and purified hemorrhagic toxins

Antihemorrhagic activity of RA (1) was studied by using crude venoms and purified hemorrhagic toxins. When crude venom (*P.*



*flavoviridis* venom, *Crotalus atrox* venom, *Gloydius blomhoffii* venom, and *Bitis arietans* venom) or purified toxin, (HTb, bilitoxin-2, HT-1 and Ac<sub>1</sub>-proteinase) was injected subcutaneously (*s.c.*) in the abdomen of mice, a distinct hemorrhagic lesion was observed (Fig 2b). No hemorrhagic spots were produced after *s.c.* injection of crude venom or purified toxin with RA (1) (Fig 2a). RA (1) effectively inhibited the hemorrhagic activities of crude venoms as well as purified hemorrhagic toxins.

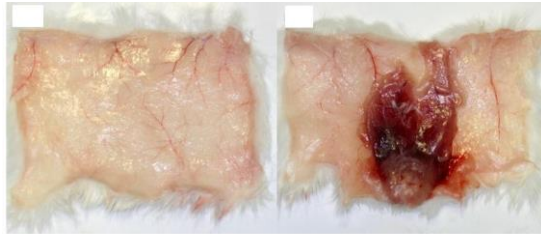


Fig.2. Inhibitory activity of RA (1) on *P. flavoviridis* venom (a)*P. flavoviridis* venom with RA (1), (b) *P. flavoviridis* venom without RA (1).

### 3.3 Inhibition of type IV collagen hydrolytic activity

Type IV collagen was incubated with *P. flavoviridis* venom for different periods of time. The venom completely degraded type IV collagen (104 kDa), especially over 1 h, and degradates with smaller molecular weights (43 and 35 kDa) appeared, as shown in Fig. 3a. In the presence of RA (1) (0.5 mg/ml), type IV collagen was not digested by incubation with the venom (Fig. 3b).

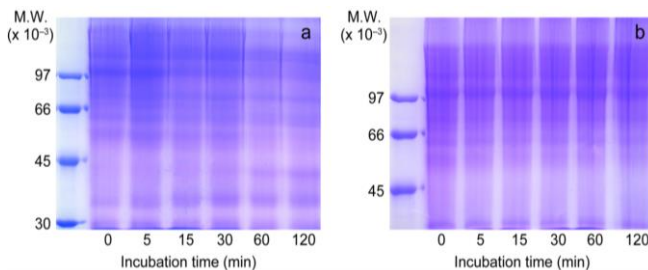


Fig. 3. Effect of RA (1) on type IV collagen hydrolytic activity of *P. flavoviridis* venom. (a) venom without RA (1), (b) venom with RA (1). Molecular weight makers of 97, 66, 43, and 30 kDa were used.

### 3.4 Inhibition of fibrinogen hydrolytic activity

To investigate the inhibition of fibrinogen hydrolytic activity, *P. flavoviridis* venom in the presence or absence of RA (1) was incubated with human fibrinogen at various time intervals. When human fibrinogen was incubated with *P. flavoviridis* venom, the A $\alpha$ -band of the fibrinogen disappeared on SDS-PAGE, whereas the B $\beta$ -chain and  $\gamma$ -chain were essentially unaffected (Fig. 4a). The venom with RA (1) did not reveal any apparent degradation of human fibrinogen (Fig. 4b).

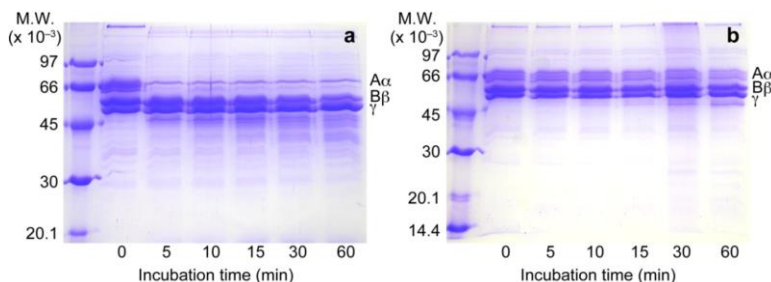


Fig. 4. Effect of RA (1) on human fibrinogen hydrolytic activities by *P. flavoviridis* venom. (a) *P. flavoviridis* venom without RA (1), (b) *P. flavoviridis* venom with RA (1). Molecular weight makers of 97, 66, 43, and 30 kDa were used.

### 3.5 Histopathological study of *P. flavoviridis* venom and the effect of rosmarinic acid

Histopathological study for RA (1) was conducted by intramuscular (*i.m.*) injection of *P. flavoviridis* venom solution into the medical aspect of the thigh muscle of ddy strain white mice. Both hemorrhage and neutrophil infiltrations were observed in a wide area (in the circle) after injection of *P. flavoviridis* venom (0.21 mg/ml) (Fig 5a). The result showed normal musculature devoid of hemorrhage and neutrophils in the muscle fibers after injection of RA (1) (0.5 mg/ml) (Fig 5b). There was no hemorrhage or neutrophil infiltration in the muscle fibers after injection of a mixture of the venom (0.41 mg/ml) and RA (1) (0.25 mg/ml; Fig. 5c).

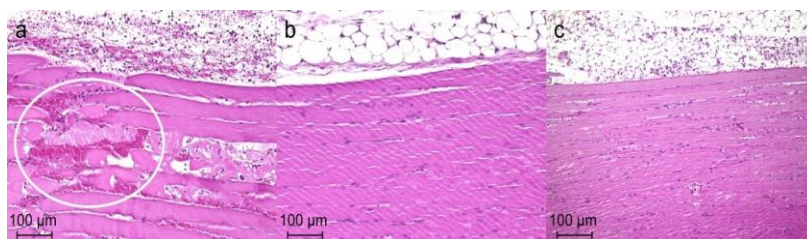


Fig. 5. Histopathological results of thigh muscle after (a) injection of *P. flavoviridis* venom (0.21 mg/ml) alone (b) injection of RA (1) (0.5 mg/ml) alone (normal muscle), (c) injection of a mixture of RA (1) (0.25 mg/ml) and the venom (0.41 mg/ml).

### 3.6 Inhibition of venom cytotoxic action on HUVEC

The inhibitory effect of RA (1) and *P. flavoviridis* venom on cultured human umbilical vein endothelial cells (HUVEC) were investigated using colorimetric cell viability assay. RA (1) alone had no effect on the viability of HUVEC, but it markedly protected HUVEC from the toxic effects of *P. flavoviridis* venom (0.14 mg/ml) at all concentrations of RA (1) tested (0.50, 0.25, 0.125, 0.06, and 0.03 mg/ml) (Fig. 6). The maximum (84.2%) protective effect of RA (1) was exhibited at 0.5 mg/ml.

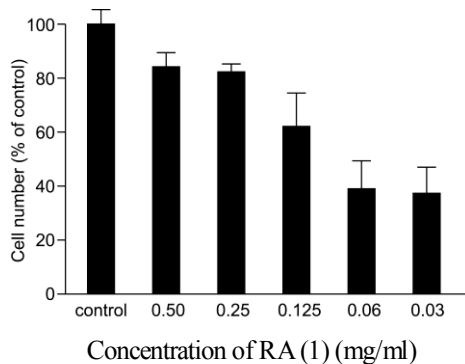


Fig. 6. Effects of RA (1) against the cytotoxic actions of *P. flavoviridis* venom on HUVEC.

### 3.7 Inhibition of venom-induced edema

The edema-forming activity was assayed using four mice. *P. elegans* venom induced an edema of 30 % in the mouse footpad, at a dose of 12.5  $\mu$ g. When *P. elegans* venom was preincubated with RA (1) (0.5 mg/ml), the edema-forming was reduced approximately by two-third.

### 3.8 Inhibition of venom lethal effect

The lethal toxicity (LD<sub>50</sub>) of *P. flavoviridis* venom was assessed by using 20 g of ddy mice. The venom of *P. flavoviridis* is highly lethal to mice with (*i.p.*) of 0.2 mg/20 g mouse. The control group died within 3 h after envenomation. All of the mice survived for 24 h after administration of a mixture of the venom and RA (1) (0.96 mg/20 g mouse). *P. flavoviridis* venom-induced lethality was significantly antagonized by RA (1) (0.96 mg/20 g mouse).

### 3.9 Antihemorrhagic activity of (*E*)- and (*Z*)-rosmarinic acid and contribution of the double bond to the activity

Only the *E*-isomer (**1a**) gave the exact antivenom activity that was significantly stronger (IC<sub>50</sub> 0.15 μM) than that of the natural rosmarinic acid (**1**) (IC<sub>50</sub> 0.60 μM). We further investigated the anti-hemorrhage activity of saturated rosmarinic acid (**5**) (Table 1.1), but it revealed that the compound showed no activity. From the results, the existence of the *E*-double bond seemed to be required to exhibit anti-hemorrhagic activity.

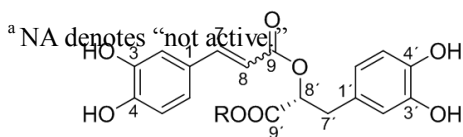
### 3.10 Antihemorrhagic activity of caffeic acid, and 3-(3,4-dihydroxyphenyl) lactic acid as components of rosmarinic acid and the contribution of the

| Compounds | IC <sub>50</sub> (μM) | phenyl) lactic acid as components of rosmarinic acid and the contribution of the |
|-----------|-----------------------|--|
|           |                       | carboxylic group   |

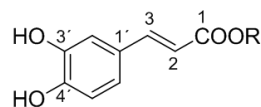
Rosmarinic acid (**1**) consists of caffeic acid (**3**) and 3-(3,4-dihydroxyphenyl)lactic acid (**4**) moieties. The activity of caffeic acid (**3**) (0.19 μM) was as potent as that of (*E*)-rosmarinic acid (**1a**) (Table 1.1). 3-(4-hydroxy-3-methoxyphenyl)lactic acid (**4'**) and rosmarinic acid methyl ester (**2**) did not show anti-hemorrhagic activity, so the presence of a free carboxylic group might be critical for anti-hemorrhagic activity. To test this hypothesis, methyl caffeate (**6**) was subjected to anti-hemorrhage tests. The compound exhibited no activity as expected. The caffeic acid moiety in rosmarinic acid (**1**) is esterified by the hydroxyl group of 3-(3,4-dihydroxyphenyl)lactic acid (**4**), but the activity might be performed by the double bond of caffeic acid moiety and the carboxylic group.

Table 1.1. Inhibitory activity of rosmarinic acid and its analogues.

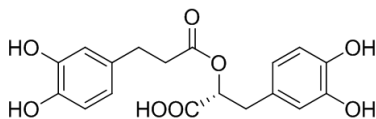
|   |      |
|---|------|
| Rosmarinic acid (RA) (1)                      | 0.60 |
| ( <i>E</i> )-Rosmarinic acid (1a)             | 0.15 |
| Caffeic acid (3)                              | 0.19 |
| 3-(4-Hydroxy-3-methoxyphenyl)lactic acid (4') | NA   |
| Saturated rosmarinic acid (5)                 | NA   |
| Rosmarinic acid methyl ester (2)              | NA   |
| Methyl caffeate (6)                           | NA   |



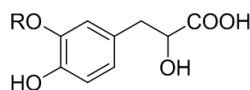
R=H, mixture of *E*- and *Z*-isomers  
Rosmarinic acid (1)  
R=H, *E*-isomer  
(*E*)-Rosmarinic acid (1a)  
R=CH<sub>3</sub>, mixture of *E*- and *Z*-isomers  
Rosmarinic acid methyl ester (2)



R=H Caffeic acid (3)  
R=CH<sub>3</sub> Methyl caffeate (6)



Saturated rosmarinic acid (5)



R=H 3-(3,4-Dihydroxyphenyl)lactic acid (4)  
R=CH<sub>3</sub> 3-(4-Hydroxy-3-methoxyphenyl)lactic acid (4')

### 3.11 Contribution of phenolic hydroxyl groups to antihemorrhagic activity

Caffeic acid (3) is suspected to play an important role to exhibit anti-hemorrhagic activity. Because of this, the participation of phenolic hydroxyl groups in relation to this activity should be discussed. Antihemorrhagic activities of various kinds of cinnamic acid analogues were investigated as demonstrated in Table 1.2. As a result, it was revealed that this activity does not depend on the numbers or positions of the phenolic hydroxyl groups when the configuration of a double bond is *E*. This is the result of cinnamic acid (7), which possesses no phenolic hydroxyl group, and thus does not show a significant change of activity from that of caffeic acid. Moreover, compounds with a methoxyl group on

phenyl group (**16** and **17**) exhibited just slightly lower activities than that of caffeic acid (**3**).

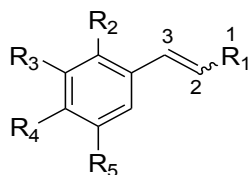
### 3.12 Contribution of the carboxyl group and side chain to anti-hemorrhagic activity

Compounds **13-15** with three phenolic hydroxyl groups, whose configurations of double bonds are all *Z*, did not show the activity (Table 1.2). When the carboxyl group is reduced to aldehyde and alcohol, the resultant compounds (**18** and **19**) were also inactive. Moreover, methyl cinnamate **20** showed no activity. On the other hand, compound **21**, which possessed a conjugated *E*-diene structure, showed similar activity (0.22  $\mu\text{M}$ ) to that of caffeic acid while 3-(3,4-dihydroxyphenyl)propanoic acid (saturated caffeic acid) (**22**) did not (Table 1.3). As the results, this evidence suggests that the configuration of the double bond and the existence of the carboxyl group were more effective to the activity than the positions of oxygen substituents on the phenyl group.

### 3.13 Anti-hemorrhagic activity of chlorogenic acid

As shown in Table 1.3, chlorogenic acid (**23**), an ester of caffeic acid and quinic acid, did not change the  $\text{IC}_{50}$  value (0.20  $\mu\text{M}$ ) significantly in comparison to that of caffeic acid (**3**).

Table 1.2. Inhibitory activity of cinnamic acid and its analogues.



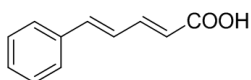
| Compounds                           | <i>2EZ</i> | R <sub>1</sub> | R <sub>2</sub> | R <sub>3</sub> | R <sub>4</sub> | R <sub>5</sub> | $\text{IC}_{50}$<br>( $\mu\text{M}$ ) |
|-------------------------------------|------------|----------------|----------------|----------------|----------------|----------------|---------------------------------------|
| Cinnamic acid ( <b>7</b> )          | <i>E</i>   | COOH           | H              | H              | H              | H              | 0.18                                  |
| 2-Hydroxycinnamic acid ( <b>8</b> ) | <i>E</i>   | COOH           | OH             | H              | H              | H              | 0.19                                  |

Table 1.3. Inhibitory activity of cinnamic acid analogues.

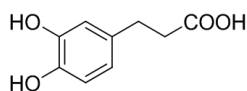
|   |          |                    |    |                  |                  |    |                 |
|---|----------|--------------------|----|------------------|------------------|----|-----------------|
| 3-Hydroxycinnamic acid ( <b>9</b> )         | <i>E</i> | COOH               | H  | OH               | H                | H  | 0.18            |
| 4-Hydroxycinnamic acid ( <b>10</b> )        | <i>E</i> | COOH               | H  | H                | OH               | H  | 0.19            |
| 2,4-Dihydroxycinnamic acid ( <b>11</b> )    | <i>E</i> | COOH               | OH | H                | OH               | H  | 0.21            |
| 3,4,5-Trihydroxycinnamic acid ( <b>12</b> ) | <i>E</i> | COOH               | H  | OH               | OH               | OH | 0.18            |
| 2,3,4-Trihydroxycinnamic acid ( <b>13</b> ) | <i>Z</i> | COOH               | OH | OH               | OH               | H  | NA <sup>a</sup> |
| 2,4,5-Trihydroxycinnamic acid ( <b>14</b> ) | <i>Z</i> | COOH               | OH | H                | OH               | OH | NA              |
| 3,4,5-Trihydroxycinnamic acid ( <b>15</b> ) | <i>Z</i> | COOH               | H  | OH               | OH               | OH | NA              |
| Ferulic acid ( <b>16</b> )                  | <i>E</i> | COOH               | H  | OCH <sub>3</sub> | OH               | H  | 0.20            |
| 3,4-Dimethoxycinnamic acid ( <b>17</b> )    | <i>E</i> | COOH               | H  | OCH <sub>3</sub> | OCH <sub>3</sub> | H  | 0.24            |
| Cinnamaldehyde ( <b>18</b> )                | <i>E</i> | CHO                | H  | H                | H                | H  | NA              |
| Cinnam alcohol ( <b>19</b> )                | <i>E</i> | CH <sub>2</sub> OH | H  | H                | H                | H  | NA              |
| Methyl cinnamate ( <b>20</b> )              | <i>E</i> | COOCH <sub>3</sub> | H  | H                | H                | H  | NA              |

| Compounds   | IC <sub>50</sub> (μM) |
|---|-----------------------|
| ( <i>2E,4E</i> )-5-Phenylpenta-2,4-dienoic acid ( <b>21</b> ) | 0.22                  |
| 3-(3,4-Dihydroxyphenyl)propanoic acid ( <b>22</b> )           | NA                    |
| Chlorogenic acid ( <b>23</b> )                                | 0.20                  |

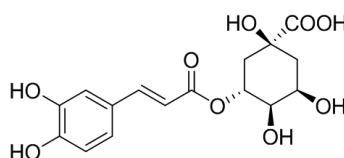
<sup>a</sup> NA denotes "not active."



(*2E,4E*)-5-Phenylpenta-2,4-dienoic acid (**21**)



3-(3,4-Dihydroxyphenyl)propanoic acid (**22**)



Chlorogenic acid (**23**)

### 3.13 Contribution of the phenyl group to anti-hemorrhagic activity

As mentioned above, the hydroxyl group(s) on the phenyl group is not critical to exhibit anti-hemorrhagic activity, but the *E*-enoic acid moiety is. Then the anti-hemorrhagic activity of the simplest compounds, crotonic acid (**24**) and sorbic acid (2,4-hexadienoic acid) (**25**) was tested. As a result, as shown in Table 1.4, these two compounds also exhibited comparable

activity as that of cinnamic acid (7). From this result, it revealed that phenolic group is not also so effective to the activity.

Table 1.4. Anti-hemorrhagic activity of aliphatic enoic acids

$$\text{R}-\text{CH}=\text{CH}-\text{COOH}$$

| Entry  | R                      | IC <sub>50</sub> (μM) |
|--|------------------------|-----------------------|
| Crotonic acid ( <b>26</b> )                          | CH <sub>3</sub>        | 0.22                  |
| Sorbic acid ( <b>27</b> )                            | CH <sub>3</sub> -CH=CH | 0.21                  |
| (2 <i>E</i> ,4 <i>E</i> )-Muconic acid ( <b>28</b> ) | HOOC-CH=CH             | 0.11                  |

### 3.14 Anti-hemorrhagic activity of benzoic acid

The anti-hemorrhagic activity of benzoic acid (26), the smallest compound possessing a benzene ring and the enoic acid moiety in a molecule, was investigated. As a result, benzoic acid (26) (IC<sub>50</sub> 0.20 μM) showed almost the same activity as caffeic acid (3) (IC<sub>50</sub> 0.19 μM). Based on this result, it is estimated that benzoic acid analogues can also exhibit the same anti-hemorrhagic activity as compounds with an *E*-enoic acid moiety.

### 3.15 Anti-hemorrhagic activity of benzenepolycarboxylic acids

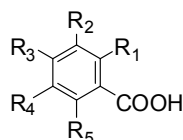
First, three benzenedicarboxylic acids, phthalic acid (27), isophthalic acid (28), and terephthalic acid (29) were tested. As shown in Table 1.5, anti-hemorrhage potencies of these compounds were very similar and two-fold higher than those of benzoic acid (26) and caffeic acid (3). The inhibitory activity did not depend on the positions of carboxyl groups. Then the activities of two benzenetricarboxylic acids, trimesic acid (1,3,5-benzenetricarboxylic acid) (30) and trimellitic acid (1,2,4-benzenetri carboxylic acid) (31), were tested. Their IC<sub>50</sub> values were almost the same and much smaller, respectively, than those of benzenedicarboxylic acids (27–29). This evidence demonstrated that the number of carboxyl groups on the benzene ring greatly influenced anti-hemorrhagic activity. Other benzenepolycarboxylic acids, pyromellitic acid (1,2,4,5-benzenetetracarboxylic acid) (32), benzenepentacarboxylic acid (33), and mellitic acid (benzenehexacarboxylic acid) (34), were revealed to have IC<sub>50</sub> values of 0.035, 0.043 and 0.043 μM, respectively. The potency of the tetracarboxylic acid (32) was higher than those of tricarboxylic acids, as expected. But



lower potency was observed for the penta-and hexacarboxylic acids (33 and 34) than for tetracarboxylic acid (32). The exact cause has not been confirmed yet, but it is estimated that the carboxyl groups caused steric hindrance to bind the active site of the snake venom (metalloproteinase) or the intramolecular hydrogen bonding among the carboxyl groups, though the mechanism of action of these compounds has not been fully understood.

| Entry                                     | R <sub>1</sub> | R <sub>2</sub> | R <sub>3</sub> | R <sub>4</sub> | R <sub>5</sub> | IC <sub>50</sub><br>( $\mu$ M) |
|---|----------------|----------------|----------------|----------------|----------------|--------------------------------|
| Benzoic acid (26)                         | H              | H              | H              | H              | H              | 0.20                           |
| Phthalic acid (27)                        | COOH           | H              | H              | H              | H              | 0.10                           |
| Isophthalic acid (28)                     | H              | COOH           | H              | H              | H              | 0.10                           |
| Tetraphthalic acid (29)                   | H              | H              | COOH           | H              | H              | 0.090                          |
| Benzene-1,3,5-tricarboxylic acid (30)     | H              | COOH           | H              | COOH           | H              | 0.067                          |
| Benzene-1,2,4-tricarboxylic acid (31)     | COOH           | H              | COOH           | H              | H              | 0.062                          |
| Benzene-1,2,4,5-tetracarboxylic acid (32) | COOH           | H              | COOH           | COOH           | H              | 0.035                          |

Table 1.5. Inhibitory activity of aromatic benzoic acids



|                                  |      |      |      |      |      |       |
|----------------------------------|------|------|------|------|------|-------|
| Benzenepentacarboxylic acid (33) | COOH | COOH | COOH | COOH | H    | 0.043 |
| Mellitic acid (34)               | COOH | COOH | COOH | COOH | COOH | 0.043 |

4.

### Discussion

Several compounds are reported to be constituents of the plant, such as phenylpropanoids, flavonoids, alkaloids, steroids, and triterpenes; however, none of these are reported to be an antivenom active constituent in the plant. [Ogihara] This is therefore the first scientific proof of antivenom activity of *A. argentea* which has been traditionally used as a folk medicine in the Okinawa Islands. In this study, the inhibitory activities of RA (1) from *A. argentea* against the action of snake venom were investigated. RA (1) effectively inhibited snake venom induced hemorrhage by crude venoms of *P. flavoviridis*, *Crotalus atrox*, *Gloydius blomhoffii*, and *Bitis arietans* or purified toxins (HTb, bilitoxin-2, HT-1 and Ac<sub>1</sub>-proteinase). Envenomation by snakebites often produces persistent hemorrhage due to considerable degradation of fibrinogen and other coagulation factors, thus preventing clot formation. [Laing] The pathogenesis of venom-induced hemorrhage involves direct damage to endothelial cells in microvessels by hemorrhagic toxins. [Markland] Snake venom metalloproteinases (especially snake venom metallo- proteinase from *P. flavoviridis* venom) degrade the most important components of the basement membrane, such as laminin, type IV collagen and nidogen/ entactin. [Baramova, Mashiko] In this study, an attempt was made to determine the protective effects of RA (1) on digestion of human fibrinogen, digestion of type IV collagen and cytotoxic action on HUVEC induced by *P. flavoviridis* venom. The pure compound showed anti-fibrinogenolytic activity by inhibiting the digestion of the A $\alpha$ -chain of human fibrinogen. RA (1) also effectively inhibited HUVEC against the toxic action of *P. flavoviridis* venom at various concentrations and digestion of type IV collagen. Moreover, the pathological study of thigh muscles showed that RA (1) inhibited hemorrhage and neutrophil infiltrations. *P. flavoviridis* venom-induced lethality was significantly antagonized by RA (1) (0.96 mg), whereas the venom is highly lethal to mice with 0.2 mg. The compound inhibited the edema-forming effect of *P. elegans* venom and lethal action induced by *P. flavoviridis* venom.

The structural features necessary for high potency snake venom inhibition included the presence of an *E*-enoic acid moiety in cinnamic acid relatives. Moreover, aliphatic acids, crotonic acid (IC<sub>50</sub> 0.22  $\mu$ M), sorbic acid (IC<sub>50</sub> 0.21  $\mu$ M) and trans,-trans-muconic acid (IC<sub>50</sub> 0.11  $\mu$ M), were

also comparable to caffeic acid. This evidence suggests the phenyl group is not necessary for activity, but the enoic acid is. To better explore the structural features of CH=CH-COOH group compounds and to investigate the most potent snake venom anti-hemorrhage inhibitor, benzoic acid, benzenepoly carboxylic acids and substituted benzoic acids are examined. Pyromellitic acid (1,2,4,5-benzenetetracarboxylic acid) (32) was found to be a potent inhibitor of hemorrhage, with an IC<sub>50</sub> value of 0.035 μM.

## 5. Conclusion

The antivenom active compound, RA (1), from the methanolic extract of *A. argentea* significantly inhibited hemorrhage induced by *P. flavoviridis* venom. RA (1) was found to markedly neutralize venom-induced lethality, paw edema, hemorrhage, fibrinogenolysis, cytotoxicity and digestion of type IV collagen activity. Moreover, RA (1) inhibited both hemorrhage and neutrophil infiltrations caused by *P. flavoviridis* venom in pathology sections. These results demonstrate that RA (1) possesses potent snake venom neutralizing properties. In this study, the structural requirement for cinnamic acid analogues to exhibit anti-hemorrhagic activity was investigated. As a result, compounds possessing a carboxylic group and an *E*-double bond showed high potency with respect to their activities. Moreover, even the compounds without the phenyl group exhibit the activity. It does not seem that the carboxylic group and the double bond need to be conjugated in order for this to occur. Among the compounds tested in the study, pyromellitic acid (32) (0.035 μM) was the most effective, demonstrating that the four carboxyl groups on the benzene nucleus are important for inhibitory activity. This is the first report of benzenepolycarboxylic acids with potential antihemorrhagic properties and their structure-activity relationship. The strong antihemorrhagic compounds described in this paper will lead to drugs that prevent hemorrhage induced by *P. flavoviridis* venom.

## Acknowledgements

I would like to acknowledge Rector Dr. Khin Swe Myint for his great help in academic progress and research pursuits.

I would like to give special thanks Prof. Dr. Myint Myint Sein for their emotional support and insightful suggestions.

I would like to express my deepest gratitude to Prof. Dr. Masatake Niwa and associate professor Dr Yoshiaki Takaya for their guidance, patience, insightful discussions and encouragement.

I am greatly indebted to Dr Mya Aye for their endless support, insightful suggestions and guidance.

I would like to thank to my colleagues for sharing their academic knowledge, kindness and help.

### References

- Baramova, E. N.; Shannon, J. D.; Bjarnason, J. B.; Fox, J. W. Identification of the cleavage sites by a hemorrhagic metalloproteinase in Type IV collagen. *Matrix* **1990**, *10*, 91-97.
- Baramova, E. N.; Shannon, J. D.; Bjarnason, J. B.; Fox, J. W. Degradation of extracellular matrix proteins by hemorrhagic metalloproteinases. *Arch. Biochem. Biophys.* **1989**, *275*, 63-71.
- Bjarnason, J.B.; Tu, A. T. Hemorrhagic toxins from western diamondback rattlesnake (*Crotalus atrox*) venom: Isolation and characterization of five toxins and the role of zinc in hemorrhagic toxin e. *Biochemistry* 1978, *17*, 3395-3404.
- Calmette, A. Contribution a l'étude du venin des serpents. Immunization des animaux et traitement de l'envenimation. *Ann. Inst. Pasteur* 1894, *8*, 275-291.
- Chippaux, J.P. Snake-bites: Appraisal of the global situation. *Bull. World Health Organ.* **1998**, *76*, 515-524.
- Corrigan, P.; Russell, F.E.; Wainchal, J. Clinical Reactions to Antivenin. In: *Toxins: Animal, Plant and Microbial*; Rosenberg, P., Ed.; Pergamon Press, Oxford, New York, 1978, 457-467.
- Cruz, L. S.; Vargas, R.; Lopes, A. A. Snakebite envenomation and death in the developing world. *Ethn Dis.* 2009, *19*, 42-46.
- Ho, C. L.; Hwang, L. L.; Chen, C. T. Edema-inducing activity of a lethal protein with phospholipase A<sub>1</sub> activity isolated from the black-bellied hornet (*Vespa basalis*) venom. *Toxicon* 1993, *31*, 605-613.
- Ishiyama, M.; Miyazono, Y.; Sasamoto, K.; Ohkura, Y.; Ueno, K. A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. *Talanta* 1997, *44*, 1299-1305.
- Laing, G. D.; Moura-da-Silva, A. M. Jarahagin and its multiple effects on hemostasis. *Toxicon* **2005**, *45*, 987-996.
- Markland, F. S. Snake venoms and the hemostatic system. *Toxicon* **1998**, *36*, 1749-1800.
- Mashiko, H.; Takahashi, H. Hemorrhagic factors from snake venoms. II. Structures of haemorrhagic factors and types and mechanisms of haemorrhage. *J. Toxicol. Toxin Rev.* **1998**, *17*, 493-512.
- Ogihara, K.; Nakazato, R.; Nishi, Y.; Higa, M.; Yogi, S. DPPH-radical scavenging constituents from the twigs of *Messerschmidia argentea* (III). *Bull Fac Sci, Univ Ryukyus* **2002**, *74*, 73-80.
- Ouyang, C.; Teng, C. M. Fibrinogenolytic enzymes of *Trimeresurus mucrosquamatus* venom. *Biochim. Biophys. Acta* 1976, *420*, 298-308.
- Panfoli, I.; Calzia, D.; Ravera, S.; Morelli, A. Inhibition of hemorrhagic snake venom components: old and new approaches. *Toxins* 2010, *2*, 417-427.

- Stahel, E.; Wellauer, R.; Freyvogel, T. A. Vergiftungem durch einheimische (*Vipera vipera berrus* and *Vipera aspis*) Eine retrospective studies on 113 patients. *Schweiz. Med. Wochenschr* 1985, *115*, 890-896.
- Sutherland, S. K. Premedication, adverse reactions and the use of venom detection kits. *Med. J. Aust.* 1992, *157*, 734-739.
- Swaroop, S.; Grab, B. Snakebite mortality in the world. *Bull. World Health Organ.* **1954**, *10*, 35-76.
- Theakston, R. D. G.; Reid, H. A. Development of simple standard assay procedures for the characterization of snake venom. *Bull. World Health Organ.* 1983, *61*, 949-956.
- Tominaga, H.; Ishiyama, M.; Ohseto, F.; Sasamoto, K.; Hamamoto, T.; Suzuki, K.; Watanabe, M. A water soluble tetrazolium salt useful for colorimetric cell viability assay. *Anal. Commun.* 1999, *36*, 47-50.
- Warrell, D.A. The global problem of snakebite: Its prevention and treatment. In *Recent Advances in Toxinology Research*; Gopalakrishnakone, P., Tu, C.K., Eds.; National University of Singapore: Singapore, 1992; pp. 121-153.