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Snake Venom Inhibition Activity of Rosmarinic acid from *Argusia argentea*

Hnin Thanda Aung¹, Myint Myint Khine², Yoshiaki Takaya³, Masatakhe Niwa⁴

**Abstract**

A methanolic extract of *Argusia argentea* significantly inhibited hemorrhage induced by crude venom of *Protobothrops flavoviridis* (Habu). The extract was then separated according to antivenom activity by using silica gel column chromatography and HPLC equipped with an octadecylsilanized silica gel (ODS) column to afford rosmarinic acid (RA) (1) as an active principle. Anti-hemorrhagic activity was assayed by using several kinds of snake venom. RA (1) significantly inhibited the hemorrhagic effect of crude venoms of *P. flavoviridis*, *Crotalus atrox*, *Gloydius blomhoffii*, *Bitis arietans* as well as purified snake venom metalloproteinases, HT-b (*Crotalus atrox*), bilitoxin-2 (*Agkistrodon bilineatus*), HT-1 (*Bitis arietans*) and Ac₁-proteinase (*Deinagkistrodon acutus*). Inhibition against fibrinogen hydrolytic and collagen hydrolytic activities of *P. flavoviridis* venom were examined by SDS-PAGE. A histopathological study was done by microscopy after administration of venom in the presence or absence of RA (1). RA (1) was found to markedly neutralize venom-induced hemorrhage, fibrinogenolysis, cytotoxicity, lethality, edema 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 findings indicate that RA (1) can be expected to provide therapeutic benefits in neutralization of snake venom accompanied by heat stability.

**Keywords:** *Argusia argentea*, Rosmarinic acid, snake venom, hemorrhage, metalloproteinase

**Introduction**

Globally snakebite affects the lives of some 4.5 million people every year, and conservative estimates suggest that at least 100,000 people die from snakebite, and another 250,000 are permanently disabled[University

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of Melbourne]. Envenomation resulting from snakebite is an important public health hazard in many regions, particularly in tropical and subtropical areas [Gutierrez]. There are two main types of snake venoms namely neurotoxins, which attack the central nervous system and haemotoxins which target the circulatory system. They are usually complex mixtures of proteins including hemorrhagic metalloproteases, phospholipases A2 (PLA2), myotoxins, and other proteolytic enzymes, cytotoxins, cardiotoxins and others. Snake envenomation causes pathophysiological changes such as inflammation, increased body temperature, hemorrhage, necrosis, nephrotoxicity, cardiotoxicity, haemostatic changes and ultimately death [Theakston]. Envenomations due to snakebites are commonly treated by parenteral administration of horse- or sheep-derived polyclonal antivenoms aimed at the neutralization of toxins [Panfoli]. Although antiserum is the only available medical antidote against snakebite, it does not provide enough protection against venom-induced hemorrhage, edema, necrosis, or nephrotoxicity, and it often produces adverse hypersensitivity reactions [Calmette, Stahel, Corrigan, Sutherland, Cruz, L.S]. Hemorrhage is one of the most conspicuous consequences of snake envenoming, and it is sometimes lethal. Unfortunately, the only clinical treatment is antiserum against snake venoms. Snakebites often occur outdoors, far from medical institutions. Because of this, drugs to treat snakebites must be transported to remote locations ahead of time, requiring them to be stable against light, oxygen, and other forms of decomposition without the aid of refrigeration or a special container. It is therefore important to search for other compounds which can effectively neutralize the hemorrhagic and other harmful activities of snake venoms.

The use of natural products, especially plants, for healing is as ancient and universal as medicine itself. In this study we have used one of the main traditional herbal plants called *Argusia* (or *Messerschmidia* or *Tournefortia*) *argentea* (Japanese name: Monpanoki) which is locally used in Okinawa as an antidote for poisoning from jellyfish and snakes venoms. A methanolic extract of the plant leaves was found to neutralize crude venom of *Protobothrops flavoviridis* which cause hemorrhage. We further separated the methanolic extract and isolated rosmarinic acid (RA) (1) as an active compound which inhibited the hemorrhagic effect of crude venoms of *P. flavoviridis*. Moreover, we submitted the compound to venoms of *P. flavoviridis*, *Crotalus atrox*, *Gloydius blomhoffii*, *Bitis arietans* as well as
purified snake venom metalloproteinases, HT-b (Crotalus atrox), bilitoxin-2 (Agkistrodon bilineatus), HT-1 (Bitis arietans) and Ac1-proteinase (Deinagkistrodon acutus). To investigate mechanistic evidence of RA's neutralization effects of snake venom, various pharmacological activities like lethality, hemorrhagic, fibrinogenolysis, cytotoxicity, edema and digestion of type IV collagen activity were studied. Moreover, histopathological study for myonecrosis was done by using microscope after administration of venom with or without RA (1).

Materials and Methods

General

IR spectra were recorded on FT-IR-410 spectrophotometre. \(^1\)H- and \(^{13}\)C-NMR spectra were recorded on a JEOL ECA-500 (\(^1\)H: 500 MHz and \(^{13}\)C: 125 MHz). Chemical shifts for \(^1\)H- and \(^{13}\)C-NMR are given in parts per million (\(\delta\)) relative to solvent signal (methanol-\(d_4\): \(\delta_H\) 3.30 and \(\delta_C\) 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\(_{254}\) (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.

Plant material

Fresh twigs and leaves of A. argentea were collected on Okinawa Islands, and they were moved to our laboratory below 4 \(\degree\)C.

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 Ac1-
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).

**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 octadecysilanized 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. [α]_D^{25} + 41.2° (c 0.058, MeOH). ¹H-NMR (500 MHz, methanol-d₄) δ: 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). ¹³C-NMR (125 MHz, methanol-d₄) δ: 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 [M+H]^+ (m/z 361.0923 calcd for C₁₈H₁₇O₈).
Antihemorrhagic activity

Anti-hemorrhagic activity was assayed by the modified method of Bjarnason and Tu\(^1\) 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\(_1\)-proteinase (1.04 mg/ml). A test solution was prepared by mixing the venom solution or the toxin solution (50 µL) and RA (1) (0.5 mg/ml in 10% DMSO-saline, 50 µL) followed by 10 min incubation at 37 °C. These test solutions (100 µL) 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 μL) 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 analyzed as follows. The area of the lesion was estimated by major and minor axes measurements, since the shape of the lesions are always amorphous, like an ellipse.

Collagen hydrolytic activity assay

Collagen hydrolytic activity was assayed as follows. 0.1 M Sodium hydrogen carbonate (60 µL, 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 μg/ml) in the presence or absence of RA (1) (0.5 mg/ml). At various time intervals, aliquots of 100 µL of denaturing solution (10 mM phosphate buffer, pH 7.2, containing 10 M urea, 4% SDS, and 4% β-mercaptoethanol) were added. This solution was boiled for 3 min and run on SDS-PAGE using a 7.5% polyacrylamide slab gel electrophoresis.

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 µL of 0.21 mg/ml of
P. flavoviridis venom or 5.5 µg/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 µL of denaturing solution (10 mM phosphate buffer, pH 7.2, containing 10 M urea, 4% sodium dodecyl sulfate (SDS), and 4% β-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.

**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 µl), while group B was injected with RA (1) (0.5 mg/ml, 100 µL). Group C was injected with a mixture of the venom (0.41 mg/ml, 50 µL) and RA (1) (0.25 mg/ml, 50 µL). 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.

**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 µg/ml), heparin (10 µg/ml), gentamicin (50 µg/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 × 10^3 cells per well in 100 µl 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 µm 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 µL medium. The plate was incubated at 37 °C under 5% CO₂ 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.

**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 µg in 50 µl 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.

**Inhibition of venom lethal effect**

The lethal toxicity of *P. flavoviridis* venom (LD₅₀) 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.

**Results**

**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). Antidote activities of RA (1) and fractions from *A. argentea* against *P. flavoviridis* venom were investigated as shown in Table 1.

Table 1. Antivenom activities of fractions from methanol extract of *A. argentea* against crude venom of *P. flavoviridis*.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Concentration (mg/ml)</th>
<th>Activity(^a)/IC(_{50}) (μm)</th>
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</thead>
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<tr>
<td>Methanol extract</td>
<td>2.5</td>
<td>+++</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>2.5</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>++</td>
</tr>
<tr>
<td>1-Butanol fraction</td>
<td>2.5</td>
<td>–</td>
</tr>
<tr>
<td>Water fraction</td>
<td>2.5</td>
<td>+</td>
</tr>
<tr>
<td>H-13</td>
<td>0.5</td>
<td>+++</td>
</tr>
<tr>
<td>H-13-10</td>
<td>0.5</td>
<td>+++</td>
</tr>
<tr>
<td>RA (1)</td>
<td>0.60</td>
<td></td>
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Concentration of venom was 0.14 mg/ml. +++; 100–75% inhibition, ++; 75–50% inhibition, +; 50 to >0% inhibition. All experiments were carried out with four mice\(^a\).
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 Ac1-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 (Table 2).
Table 2. Inhibition of hemorrhage by RA (1) against crude snake venoms and hemorrhagic metalloproteinases.

<table>
<thead>
<tr>
<th>Origins</th>
<th>Venoms</th>
<th>Concentrations of venoms (mg/ml)</th>
<th>Inhibitory activity of hemorrhage by RA (1)</th>
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</thead>
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<tr>
<td>Crude venoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. flavoviridis</em></td>
<td>0.14</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td><em>Crotalus atrox</em></td>
<td>0.14</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td><em>Gloydius blomhoffii</em></td>
<td>0.14</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td><em>Bitis arietans</em></td>
<td>0.14</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Hemorrhagic metalloproteinases (purified venoms)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Crotalus atrox</em></td>
<td>Ht-b</td>
<td>0.41</td>
<td>+++</td>
</tr>
<tr>
<td><em>Agkistrodon bilineatus</em></td>
<td>bilitoxin 2</td>
<td>0.0028</td>
<td>+++</td>
</tr>
<tr>
<td><em>Bitis arietans</em></td>
<td>HT-1</td>
<td>0.29</td>
<td>+++</td>
</tr>
<tr>
<td><em>Deinagkistrodon acutus</em></td>
<td>Ac1-p</td>
<td>1.04</td>
<td>+++</td>
</tr>
</tbody>
</table>

*a* Concentration of RA (1) for all experiments was 0.5 mg/ml.

*b* +++: 100 – 75% inhibition, ++: 75 –50% inhibition, +: 50 – >0% inhibition. All experiments were carried out with four mice.

**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.

7.5% SDS-PAGE of time-dependent digestion of type IV collagen by *P. flavoviridis* venom in the presence or absence of RA (1). (a) venom without RA (1), (b) venom with RA (1). Molecular weight makers of 97, 66, 43, and 30 kDa were used.

**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 Aα-band of the fibrinogen disappeared on SDS-PAGE, whereas the Bβ-chain and γ-chain were essentially unaffected (Fig. 4a). The venom with RA (1) did not reveal any apparent degradation of human fibrinogen (Fig. 4b). RA (1) also inhibited Aα-chain hydrolysis by bilitoxin-2 (Fig. 4c and 4d).
Fig. 4. Effect of RA (1) on human fibrinogen hydrolytic activities by *P. flavoviridis* venom and bilitoxin-2.

10% SDS-PAGE of time-dependent digestion of human fibrinogen by *P. flavoviridis* venom and bilitoxin-2 in the presence or absence of RA (1). (a) *P. flavoviridis* venom without RA (1), (b) *P. flavoviridis* venom with RA (1), (c) bilitoxin-2 without RA (1); (d) bilitoxin-2 with RA (1). Molecular weight makers of 97, 66, 43, 30, 20.1, and 14.4 kDa were used.

**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).

**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) (Table 3, Fig. 6). The maximum (84.2%) protective effect of RA (1) was exhibited at 0.5 mg/ml.

![Graph](image)

Fig. 6. Effects of RA (1) against the cytotoxic actions of *P. flavoviridis* venom on HUVEC.
Table 3. Effects of rosmarinic acid (1) against the cytotoxic actions of *P. flavoviridis* venom on HUVEC.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>cell survival (%)</th>
</tr>
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<tbody>
<tr>
<td>Control (no treatment)</td>
<td>4</td>
<td>100 ± 5.4</td>
</tr>
<tr>
<td>RA (0.5 mg/ml) alone</td>
<td>4</td>
<td>100 ± 4.5</td>
</tr>
<tr>
<td>RA (0.5 mg/ml) + venom (0.14 mg/ml)</td>
<td>4</td>
<td>84.2 ± 5.2</td>
</tr>
<tr>
<td>RA (0.25 mg/ml) + venom (0.14 mg/ml)</td>
<td>4</td>
<td>82.3 ± 2.9</td>
</tr>
<tr>
<td>RA (0.125 mg/ml) + venom (0.14 mg/ml)</td>
<td>4</td>
<td>62.1 ± 12.4</td>
</tr>
<tr>
<td>RA (0.06 mg/ml) + venom (0.14 mg/ml)</td>
<td>4</td>
<td>39.0 ± 10.4</td>
</tr>
<tr>
<td>RA (0.03 mg/ml) + venom (0.14 mg/ml)</td>
<td>4</td>
<td>37.3 ± 9.4</td>
</tr>
</tbody>
</table>

Note: no viability on HUVEC with venom alone (0.14 mg/ml). RA; rosmarinic acid (1), venom; *P. flavoviridis* venom

**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 µg. When *P. elegans* venom was preincubated with RA (1) (0.5 mg/ml), the edema-forming was reduced approximately by two-third.

**Inhibition of venom lethal effect**

The lethal toxicity (LD$_{50}$) 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).

**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. 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 *B. arietans* or purified toxins (HTb, bilitoxin-2, HT-1 and Ac1-proteinase). Envenomation by snakebites often produces persistent hemorrhage due to considerable degradation of fibrinogen and other coagulation factors, thus preventing clot formation. The pathogenesis of venom-induced hemorrhage involves direct damage to endothelial cells in microvessels by hemorrhagic toxins. Snake venom metalloproteinases (especially snake venom metalloproteinase from *P. flavoviridis* venom) degrade the most important components of the basement membrane, such as laminin, type IV collagen and nidogen/entactin. 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 antifibrinogenolytic activity by inhibiting the digestion of the Aα-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. This is the first report of RA (1) that demonstrates the inhibitory mechanism for snake venom-induced hemorrhage and protection from snakebite envenomation. Further studies are needed to investigate post-administration of RA (1), and the effects of different methods of administration. After these issues are resolved, RA (1) could become a potent alternative antidote compound for snake envenomation.

**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. Snake venom inhibitors from plants may become helpful alternative or supplemental tools for the treatment of envenomings, as well as important leads for the synthesis of new drugs of medical interest.

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