Purification and characterization of an organ specific haemorrhagic toxin from Vipera russelli russelli (Russell's viper) venom

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A haemorrhagic toxin (VRR-12) from Vipera russelli russelli (Russell's viper) venom has been purified by ion-exchange chromatography on CM-Sephadex C-50 followed by size-exclusion HPLC to electrophoretically homogeneous state. It is a 12 kDa single polypeptide having 1 mole of Zn²⁺ ion. This toxin induces intense intestinal haemorrhage and to a lesser extent skeletal muscle haemorrhage in mice. It does not show detectable proteolytic and esterolytic activity with selected substrates under specified conditions, haemolytic and phospholipase activity. When VRR-12, preincubated with bivalent antiserum against Saw-scaled and Russell's viper venom or EDTA was injected, haemorrhagic activity was not reduced, on the otherhand preincubation with phenylmethyl sulphonyl fluoride reduced the activity markedly. Biodistribution studies with [³⁵S]VRR-12 show that haemorrhagic manifestation by this toxin is not a direct function of the fraction of the totally administered toxin distributed to that tissue.

Russell's viper envenomation is a WHO identified occupational hazard of paddy farmers of many South-East Asian countries including India. Envenomation by Vipera russelli russelli, the Indian subspecies of Russell's viper, leads to immediate swelling of the bitten limb, cardio-respiratory failure, coagulopathy, nephrotoxicity, pituitary gland dysfunction and spontaneous haemorrhage from the site of the bite, previous unhealed wounds, conjunctiva and organs such as lung, kidney, heart, brain, etc. Although renal failure secondary to coagulopathy is the principal cause of death, haemorrhage is a major manifestation of Russell's viper envenoming. Viper bite does not produce haemorrhage equally in all human organs. The crude Russell's viper venom contains a number of haemorrhagic toxins (haemorrhagins) and the differing levels of haemorrhage from different organs is probably due to varying proportions of the toxins, their potency and site specificities. Application of antivenin is still the only mode of treatment for snake bite victims where success varies from case to case.

The objective of the present study is to isolate the major haemorrhagins of Vipera russelli russelli venom to find their organ specificities, mechanisms of action and possible means for neutralization. Since the crude venom contains far too many active substances, the haemorrhagins need to be examined in isolation. Earlier one such haemorrhagin was purified which was found to be acting predominantly on lung, conjunctiva and brain tissues in mice. Here we report the purification of another haemorrhagin from the same venom having specificity towards intestine and muscle in the same animal.

Materials and Methods

Reagents

Dry pooled desiccated Vipera russelli russelli (VRR) venom was purchased from a licensed local supplier (Mr. Dipak Mitra, Calcutta Snake Park). CM-Sephadex C-50, bovine serum albumin, haemoglobin, laminin, collagen (type III, acid soluble), benzoyl arginine ethyl ester (BAEE), benzoyl arginine-p-nitroanilide (BAPNA), 1,10-phenanthroline, dithiotheitol (DTT), antipain, aprotinin, leupeptine, bestatin, chymostatin, iodoacetic acid, iodoacetamide, N-tosylphenylalanine chloromethyl ketone (TPCK), phenylmethylsulfonyl fluoride (PMSF), low molecular weight protein markers (6.5-66 kDa) and reagents used in SDS-polyacrylamide gel electrophoresis were purchased from Sigma Chemical Co., USA. All other reagents were of analytical grade. Inbred Balb/c mice were obtained from central animal house of our Institute. Lyophilized bivalent anti-snake venom serum (Saw-scaled viper and Russell's viper) was a gift from Serum Institute of India, Pune.

Purification of haemorrhagin

Dry VRR venom (60 mg) was allowed to dissolve in 1 ml of 20 mM K-phosphate buffer, pH 7.4 at 4°C overnight. Tissue debris and other insoluble materials
were separated by low speed centrifugation. The clear yellowish supernatant containing about 42 mg of protein was fractionated on a CM-Sephadex C-50 (25 x 1.5 cm) column pre-equilibrated with the same buffer at a flow rate of 15 ml/hr at 4°C. The unabsorbed fractions were removed by washing with three column volume of the buffer. Bound proteins were eluted by a linear gradient of 0-0.5 M NaCl in the same buffer. Fraction size was 1.5 ml. All the protein peaks were tested for haemorrhagic activity. The second absorbed peak showed muscle haemorrhage, which was pooled and concentrated by dialyzing against sucrose (yield 4.5 mg). This was further purified by passing through Waters Protein Pak 60 size exclusion (SE)-HPLC column (7.5 x 300 mm). HPLC was done isocratically using the same phosphate buffer supplemented with 0.1 M NaCl at a flow rate of 0.5 ml/min at 25°C. In this chromatographic separation the major fraction, but not the others, showed haemorrhagic activity. This toxin, showing a single band in SDS-PAGE corresponding to 12 kDa, was named as VRR-12. All protein fractions were monitored at 280 nm and protein concentration was determined using Bio-Rad protein assay dye reagent (catalogue no: 500-0006). Final yield was 0.34 mg.

Assessment of haemorrhagic activity and intestinal loop assay

RVV and the chromatographic fractions were tested for haemorrhagic activity in Balb/c mice. Samples in 100 µl of 0.85% PBS were injected through intradermal (i.d.), intraperitoneal (i.p.) and intravenous (i.v.) routes. The skin haemorrhagic activity of the whole venom and its fractions was tested. In control experiments, 100 µl of normal PBS was substituted for venom or its fractions. The amount of whole venom or its fractions producing a haemorrhagic spot of 10 mm mean diameter was found by interpolation and is called minimum haemorrhagic dose (MHD). When animals died before development of haemorrhagic spot, they were subjected to autopsy.

For intestinal loop assay, the peritoneum was opened after light ether anaesthesia of fasted mice and two loops, each approximately 2 cm in length and 1.5 cm apart, were made by ligation in the small intestine. VRR-12 (0.5 µg) was injected in one loop and same volume of 0.85% saline in the other. The peritoneum and abdominal skin were then stitched with surgical cat gut string after insertion of the intestine into the abdomen. After 6 hr of incubation the mice were sacrificed and loops dissected out. The intestinal content along with fluid were taken out, centrifuged, the supernatant was lysed with distilled water and centrifuged once again. Amount of haemoglobin in the loop fluid content was measured spectrophotometrically at 540 nm.

Proteolytic and esterolytic activity

To detect proteolytic activity, protein substrates like bovine serum albumin, ovalbumin, haemoglobin, laminin and collagen were incubated with VRR-12 in presence of 20 mM K-phosphate buffer, pH 7.4 at 37° or 25°C for 1-24 hr at protein:toxin ratio of 10:1 (wt:wt). The samples were then resolved in 15% or 4% (for laminin) SDS-PAGE. The bands were visualized following staining with 0.1% Coomassie brilliant blue RC-250. Esterolytic activity of VRR-12 was assayed using BAPNA and BAEE as substrates. The ester (100 µl, 10 mg/ml in dimethyl sulphoxide) was added to 800 µl of 20 mM Tris-HCl buffer, pH 8.5 and was incubated at 37° or 25°C with 10-100 µg of the toxin for 10 min-24 hr. The reaction was stopped by the addition of 100 µl of 10% acetic acid. The absorbance change was monitored at 410 nm for BAPNA and at 252 nm for BAEE.

Haemolytic and phospholipase activity

Haemolytic activity was tested by incubating 10 ng-10 µg/ml of VRR-12 in 1% human or mice erythrocyte suspension in 0.85% saline at 37°C for 1 hr. Distilled water and 0.85% saline were used as positive and negative control respectively. Lysis was measured by monitoring absorbance at 540 nm.

The presence of phospholipase A (PLA) activity was tested using egg yolk as substrate. A suspension was made with 9 ml egg yolk, 2.51 ml of 2% NaCl, 1.49 ml of 0.5% EDTA, 4.44 ml of 1.0% CaCl2 2.0 ml of 50 mM Tris-HCl buffer, pH 7.5 and 0.56 ml of 0.85% saline. VRR-12 (50 µg) in 200 µl buffer was added to 2 ml of egg yolk suspension, was mixed well and incubated at 37°C for 1 hr, then placed in a boiling water bath and time required to coagulate the suspension was noted. 5 µg of crude RVV and 0.85% saline were used as positive and negative controls respectively.

Fractionation of crude venom by SE-HPLC

Crude venom (5 mg) dissolved in 1 ml of 20 mM K-phosphate buffer, pH 7.4 was centrifuged and passed through a Spartan 3 Nylon filter (pore size
0.45 μ, Schleicher & Schuell). The sample (100 μl) was applied to a Waters Protein Pak 60 SE-HPLC column under conditions as described earlier. After void volume (Vo = 5.54 ml, measured from elution of alcohol dehydrogenase) fractions of 0.5 ml were collected up to 13 ml which is little further of elution volume of tyroptophan (12.38 ml). Several overlapping peaks appeared.

**Spectroscopic methods**

Detection and estimation of metal ions associated with VRR-12 was carried out in an atomic absorption spectrophotometer (Perkin-Elmar, model 2380) at the University Science Instrumentation Centre, Jadavpur University, Calcutta. The purified protein was exhaustively dialysed against glass distilled (2×) Millipore water (filter size 0.45 μ) and the final dialysate served as control. The optical absorbances were measured using a Hitachi 3200U spectrophotometer. Fluorescence spectrum was scanned using a Hitachi F4020 spectrofluorimeter.

**Tissue distribution of ^125I VRR-12**

VRR-12 (0.5 mg) was radiolabelled with 5 μl Na[^125]I (0.67 mCi) in 1 ml of 0.5 M K-phosphate buffer, pH 7.4 containing 2% chloramine-T. Excess[^125]I was removed from labelled protein by passing through a Sephadex G-50 spin column^6. Radioactivity was monitored by a well type gamma counter (Electronic Corporation of India, model LV 4755). The radioanalytical purity of the labelled VRR-12 was checked by thin layer chromatography on silica gel coated glass slide (7.5 x 2.5 cm) using acetone as solvent. The labelled toxin produced a single spot parallel to unlabelled VRR-12. The chromatogram was then cut into 0.5 cm portion and each part was read for radioactivity. About 90% activity was concentrated on the spot produced by ^125I VRR-12. Anaesthetized male Balb/c mice (20-25 g) were administered 100 μl of ^125I VRR-12 i.v. The animals were sacrificed by i.v. infusion of air at 5, 10, 30 and 60 min after injection of the radiolabelled toxin. The radioactivity of liver, lung, heart, muscle, small intestine, large intestine, kidney and blood was measured after washing the organs in normal saline and soaking on filter paper. Radioactivity was expressed as % of dose per 100 g of tissue.

**Results**

**Purification of VRR-12**

Chromatography of crude VRR venom on CM-Sephadex C-50 at pH 7.4 produced two peaks (I and II) in the unabsorbed region and one major (III) and several minor (IV, V and other) peaks in the absorbed region (Fig. 1A). All the peaks were tested for dermal haemorrhage activity in mice for 18 hr. Fraction IV (0.5 μg) did not produce significant dermal haemorrhage but the animals died when administered i.p. or i.v. at the same dose within 30 min and 18 hr respectively. Intraperitoneal injection of 0.5 μg of the toxin produced retardation in movement, watery secretions oozing from urinary opening and limbs were swollen after 6 hr post injection. Haemorrhage on peritoneum (outer side that adjunct to endodermal region), marked vasodilatation of mesenteric blood vessels and vessels supplying to the skin and other peritoneal organs, discoloration of liver and red patches on kidney capsule were observed at the dying condition.

This haemorrhagic fraction was further purified by passing through Waters Protein Pak-60 SE-HPLC column where at least six components were separated. The first major fraction corresponding to elution volume (Ve) 7.9 ml showed toxicity as described above while other fractions were not haemorrhagic. This fraction showed a single band in SDS-PAGE (Fig. 1B, marked by arrow). Intramuscular injection of 0.05 μg of the purified toxin produced swelling of limbs, gradual loss of its walkability after 3 hr post injection but did not show significant dermal haemorrhage, making it impossible to measure MHD. After dissection it can be shown that the thigh muscles become reddish in colour and some watery secretion oozes from muscle. It was reported that 0.5 μg of crude venom produced a haemorrhagic spot of 3-4 mm in diameter when animals died of severe lung haemorrhage^1. LD₅₀ of the toxin was found to be 100 μg/kg body weight of mice after 24 hr when injected i.p.

Specific activity of the toxin and fold purification during its isolation could not be ascertained because of the limitation of its assay system (described below). The test animals died after administration of the crude venom or the semipurified toxin in the intestinal loop before development of intestinal haemorrhage, usually within 10-15 min as compared to 6 hr adapted in this study.

**Purity and molecular weight**

The final preparation of the purified toxin showed a single band of about 12 kDa in 12.5% SDS-PAGE with reference to standard marker proteins e.g. soya-bean trypsin inhibitor (20 kDa), myoglobin (17 kDa),...
cytochrome c (12.5 kDa) and aprotinin (6.5 kDa), irrespective of treating the sample with 2-mercaptoethanol. Silver staining of the gel in place of Coomassie blue staining did not reveal further protein bands. The same sample when rechromatographed on the same pre-calibrated Waters Protein Pak-60 SE-HPLC column (fractionation range 1-20 kDa), eluted as a single symmetrical peak of identical elution volume (Ve = 7.9 ± 0.2 ml) as that of its original pooled fraction (upper inset, Fig. 1B). The following

Fig. 1 — (A): Chromatogram of crude VRR venom on CM-Sephadex C-50. [Detailed description has been provided in the text. Unbound fractions were eluted by washing with equilibrating buffer and the bound fractions were eluted by a salt gradient (0-0.5 M NaCl). The five major components in the chromatogram have been marked as I-V. The bar representing fraction IV was pooled and was concentrated. (B): SE-HPLC of fraction IV in Protein Pak 60 column. [The only fraction marked by an arrow showed haemorrhagic activity similar to fraction IV of the previous chromatogram. The bar represents the pooled fraction of VRR-12. In all cases protein was estimated from A280 and haemorrhagic activity by several ways as described in the text. Upper inset: Rechromatogram of pooled fraction of VRR-12 in the same SE-HPLC column. Lower inset: Plot of log M, versus elution volumes (Ve) of molecular weight markers. ↓ indicates the void volume (Vo) and ↑ indicates the position of VRR-12 in the plate]
marker proteins were used for calibration of the column: alcohol dehydrogenase (150 kDa, for void volume determination, elution volume Ve, 5.54 ml), soyabean trypsin inhibitor (20 kDa, 5.64 ml), myoglobin (17 kDa, 6.77 ml), cytochrome c (12.5 kDa, 7.52 ml), aprotinin (6.5 kDa, 9.26 ml) and tryptophan (for determination of desalting zone, 12.38 ml) where a linear dependence of log Mr versus Ve was observed (lower inset, Fig. 1B). The purified toxin was eluted at 7.90 ml corresponding to Mr of 10.0 kDa. It was designated as VRR-12 (12 stands for Mr) because of the ease of identification of the toxin in SDS-PAGE profile of the crude venom.

Spectral properties

VRR-12 shows ultraviolet absorption spectra in 240-320 nm near UV region typical of globular proteins having absorption maxima at 275.6 nm. No absorption was detected in the visible wavelength region. Exciting at 280 nm VRR-12 shows a fluorescence emission maxima at 345.5 nm. A red shift of emission maxima from 345.5 to 346.5 nm was observed after excitation at 295 nm (tryptophan fluorescence only) with 60% quenching of fluorescence intensity. This suggested a relative abundance of tryptophan residues of the molecule over tyrosines and solvent exposure of the aromatic residues (em. max of tryptophan in aqueous buffer is 350.5 nm after excitation at 295 nm).

Metal ion and carbohydrate content

Atomic absorption spectrophotometry showed that VRR 12 contains 1 mole/mole of Zn$^{2+}$. Formation of zinc free toxin after treatment with EDTA was confirmed by atomic absorption spectroscopy when the metal content was reduced to 0.06 mole/mole. No Ca$^{2+}$ or Mg$^{2+}$ could be detected. No carbohydrate was detected in VRR-12 by the phenol-sulphuric acid method of Dubois et al. after SDS-PAGE having ovalbumin as control.

Proteolytic and esterolytic activity

No proteolysis of bovine serum albumin, ovalbumin, haemoglobin, collagen and laminin was observed in SDS-PAGE after incubation with VRR-12. Variable incubation conditions were applied to account for thermo lability of VRR-12. Collagen being a prominent basement membrane protein, collagenase activity of crude VRR venom was also investigated by incubating collagen with the crude toxin. No such activity could be detected (Fig. 2). To rule out the possibility that crude venom also contains collagenase inhibitor, attempts were made to separate these two activities by passing the crude toxin through a Protein Pak 60 SE-HPLC column. About 92% of the venom toxin components fall within its fractionation range. None of the fractions of the chromatogram showed neither collagenase activity (by demonstrating degradation of collagen in SDS-PAGE) nor inhibition of collagenase activity (by demonstrating inability of collagenase preincubated with the fractions from preventing degradation of collagen in SDS-PAGE) (Datta K unpublished observation).

No significant esterase activity of VRR-12 was detected using BAPNA or BAEE as substrates.

Haemolytic and phospholipase activity

Haemolytic activity of VRR-12 was tested by incubating human or mice erythrocyte suspension at 37°C. No significant haemolysis was detected over the negative control, using 0.9% saline as RBC diluant. Negligible phospholipase activity of VRR-12 on egg yolk suspension was observed in comparison to the activity present in crude VRR venom. Time required to coagulate the suspension was as follows: saline, 30 ± 5 sec; VRR-12 (50 µg), 35 ± 5 sec and crude venom (5 µg) , >300 sec.

Intestinal loop assay

Intestinal loop assay of 0.5 µg of VRR-12 after 6 hr has demonstrated profuse haemorrhage on small intestine of mice specially when it started from post
duodenal region. Absorbance of the fluid after lysis at 540 nm was usually close to 2.0 whereas a control set with saline produced absorbance of 0.320. The value described for saline replacing toxin did not necessarily indicate haemorrhage rather presence of optically absorbing materials. When the toxin was preincubated with metal chelators like EDTA, 1,10-phenanthroline, (DTT) at 5-10 mM for 15 min before application, no inhibition of haemorrhagic activity was observed. Similarly marginal or no inhibition of activity was observed with peptide inhibitors like antipain, aprotinin, leupeptine, bestatin, chymostatin at 5 mM which have broad specificity towards serine or cysteine proteases. A 10-20% inhibition of activity was observed with iodoacetic acid or iodoacetamide at pH 7.0 at 5 mM but inhibition was more (30-40%) with TPCK and TLCK under identical conditions of incubation. Haemorrhage was completely checked at 5 mM of PMSF. It has been tested that the carried over reagents had no toxicity on the animals. The inhibitory effect of bivalent antiserum (Saw scaled and Russell's viper) against haemorrhage was not satisfactory. Major results of haemorrhagic effect of 0.5 μg VRR-12 in test animals and its inhibition are given in Table 1. Results have been expressed after normalizing length of the loop.

Tissue distribution study

To investigate whether physical adherence of VRR-12 or a degraded fraction of the toxin to any organ caused specificity in haemorrhage, tissue distribution study was performed. Distribution of radioiodinated VRR-12 injected in mice (i.v.) showed maximum initial accumulation of the toxin in kidney followed by liver in 15 min. Within an hour the accumulation in kidney was reduced to 45%, but in liver about 70% of the radioactivity was retained. The distribution of the toxin in heart, blood, lung, muscle, small and large intestine was comparatively low and no significant variation was observed within an hour.

Discussion

Vipera russelli russelli venom contains at least 5-6 haemorrhagins of which one, VRR-12, of 12 kDa has been purified to homogeneity. This is possibly the first haemorrhagin with such a low molecular size. It is a single polypeptide metalloprotein containing one mole of Zn$^{2+}$ ion. Haemorrhagins from other venoms are also known to be Zn$^{2+}$ containing metalloproteins e.g. Crotalus atrox, Crotalus ruber ruber, Lachesis muta muta, Trimeresurus gramineus, etc. VRR-12 is a thermolabile toxin. Repeated freeze-thawing or incubation at 24°C for 24 hr completely inactivates the toxin. Thus compared to low molecular weight protease inhibitors that are extensively cross-linked by cystine bridges, VRR-12 does not appear to be stabilized by disulphide bridges or strong hydrophobic core structure. VRR-12 shows $M_r$ of 10 kDa in SE-HPLC but 12 kDa in SDS-PAGE. This discrepancy is possibly due to asymmetry or small size of the molecule. In this context we propose naming of the protein components of VRR-venom in accordance with their molecular weight as shown in SDS-PAGE. Thus, the toxin purified is called VRR-12. Similarly the toxin VRH-1 purified earlier from the same source has been renamed as VRR-22 according to its molecular weight.

There are suggestions that blood capillaries are ruptured due to degradation of basement membranes during haemorrhage. Most of the Viperidae snake venoms causing haemorrhage have been reported earlier to be Zn-metalloproteases. Haemorrhagins devoid of proteolytic activity is also known. However, a recent review indicates that all haemorrhagins show proteolytic activity but the correlation between proteolytic and haemorrhagic activity are still questionable. VRR-12 does not show any detectable proteolytic activity towards a number of proteins like BSA, ovalbumin, haemoglobin or prominent basement membrane proteins like laminin or collagen. VRR-12 does not show esterolytic, haemolytic or phospholytic activity but shows site specific haemorrhagic activity in muscle and particularly in small intestine in mice. It appears that VRR-12 does not act as Zn-metalloprotease to express haemorrhagic activity (because of the functionality of the apo-protein). On the other hand, inhibition by PMSF, a potent serine protease inhibitor, does not indicate that VRR-12 is a protease, as PMSF interacts per se with any activated serine or cysteine residues of proteins. Further utilization of several group

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<th>Toxin</th>
<th>Haemorrhagic activity</th>
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<tr>
<td>VRR-12</td>
<td>100 ± 16</td>
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<tr>
<td>VRR-12 + 5 mM EDTA</td>
<td>89 ± 10</td>
</tr>
<tr>
<td>VRR-12 + bivalent antiserum</td>
<td>85 ± 12</td>
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<tr>
<td>VRR-12 + 5 mM PMSF</td>
<td>20 ± 3</td>
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<td>Saline as control</td>
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Table 1—Haemorrhagic activity of VRR-12 in presence and absence of inhibitors as measured by intestinal loop assay

(Number of animals tested, n=6. The carried over reagents had no toxicity on the test animals)
specific reagents suggest that VRR-12 possibly acts through a serine residue.

How the haemorrhagins act is still obscure. Their actions may be receptor mediated or through metabolic intermediate or by other mechanisms. There are indications that the haemorrhagin, VRR-22 possibly acts through hitherto unidentified metabolic intermediate. In case of VRR-12 it is more likely to act regionally in the intestinal loop. The difference in composition of vascular wall in different organs might be a reason for the organ preference of the toxins. This has been further tested in a rather simple way. Muscle degradation assay of VRR-12 with tissues like abdominal muscles, liver, kidney, lung, heart and intestine has been done by incubating small pieces of the organs (1-2 mm thickness) with 0.25-0.5 μg of the toxin in 1 ml of PBS, pH 7.2 for 0-3 hr at 37°C. After centrifugation absorption change for each set at 280 nm has been followed and the result has been expressed per mg of wet weight of the tissue. In control experiments, saline replaces VRR-12. Proteins liberated are low and within experimental variations, no difference could be detected among the tissues (result not shown). Thus, it appears that haemorrhagic activity of VRR-12 is not due to direct interaction of the toxin with the target organs. It is worthwhile to note that crude VRR venom contains neither collagenase activity nor collagenase inhibitory activity (Fig. 2).

There are reports that antivenin when administered after snake bite often fails to neutralize the toxicity though preincubation of the two substances in vitro substantially neutralize the toxicity. In case of VRR-12, preincubation of the toxin with the bivalent antibody (Saw-scaled viper and Russell’s viper) cannot reduce its haemorrhagic potency to any significant extent at least in intestine of test animals. Thus, the antigenic determinant sites and pharmacological functional sites of this molecule appear to be different. Similar speculations have also been made for other toxins e.g. separation of enzymatic activity and pharmacological properties of phospholipases from VRR venom, N. naja naja venom and Echis carinatus venom.

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