Inhibition of local effects of Indian Daboia/Vipera russelli venom by the methanolic extract of grape (Vitis vinifera L.) seeds

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Although anti-venom therapy is available for the treatment of fatal bite by snakes, it offers less or no protection against the local effects such as dermo- and myonecrosis, edema, hemorrhage and inflammation at the bitten region. The viper species are known for their violent local effects and such effects have been commonly treated with plant extracts without any scientific validation in rural India. In this investigation, the methanolic extract of grapes (Vitis vinifera L.) seed was studied against the Indian Daboia/Vipera russelli venom-induced local effects. The extract abolished the proteolytic and hyaluronidase activities and also efficiently neutralized the hemorrhage, edema-inducing and myonecrotic properties of the venom. In addition, the extract also inhibited partially the pro-coagulant activity of the venom and abolished the degradation of Aα and Bβ chains of human fibrinogen. Thus, the extract possesses potent anti-snake venom property, especially against the local effects of viper bites.

Keywords: Hyaluronidase, Local effects, Metalloprotease, Neutralization, Snake venom, Proteolytic activity, Hemorrhagic activity, Edema-inducing activity, Myonecrotic activity, Fibrinogenolytic activity, Pro-coagulant activity

Daboia russelli is one of the predominant poisonous snakes of Indian subcontinent and is endemic in different states of India. The problem is particularly severe in the eastern zone of India. The death toll from D. russelli bite is highest in the Burdwan district of West Bengal, where about 1300 deaths occur every year. In humans, D. russelli bite causes severe local tissue destruction, more frequently the necrosis results in an irreversible loss of tissue and requires amputation of the affected limb. In addition, systemic alterations such as coagulopathy and effects on multiple organs give rise to serious complications such as cardiovascular shock, pulmonary bleeding and hemorrhage in the central nervous system. Hemorrhagic syndrome is one of the most serious consequences of D. russelli bite. The venom comprises a complex mixture of enzymatic and non-enzymatic proteins, peptides and small organic compounds, such as citrate, nucleosides and acetylcholine. The envenomation involves subcutaneous or intramuscular injection of venom into the prey/human victims. The pathology of snakebite includes both local and systemic effects such as neurotoxicity (pre/post synaptic), myotoxicity, cardiotoxicity, coagulant (pro/anti), hemostatic (activating/inhibiting), hemorrhagic, hemolytic and edema forming activities. The venom has been studied extensively for various active principles such as pro-coagulant factors, ATPase, phospholipase A2, trypsin inhibitors, daboioxin-like factor, platelet aggregation inhibitors, hemorrhagins, neurotoxic peptide and a heat stable protein drct-1.

The most effective and accepted therapy for snakebite patient is immediate administration of specific/polyvalent anti-venoms following envenomation. Generally, the anti-venoms do not provide enough protection against venom-induced local tissue damage and often associated with risk of anaphylaxis and serum reactions. Further, the anti-venom development is time-consuming, expensive and requires ideal storage condition, and therefore, alternative therapies are in demand. Over the years, several attempts have been made for the development of the snake venom antagonists, especially from plant sources and the anti-venom activity has been confirmed in some plant extracts and their isolates.
Earlier, we have reported that the methanolic extract of grape fruit (Vitis vinifera L.) seeds inhibits the enzymatic (casenolytic, hyaluronolytic and fibrinogenolytic) and pharmacological (edema-inducing, hemorrhage and myonecrosis and pro-coagulation) activities of the Indian saw-scaled viper (Echis carinatus) venom. The present study has examined the effectiveness of methanolic extract of grape fruit seed against the Indian D. russelli venom.

Materials and Methods

Hyaluronic acid, casein and fibrinogen were from Sigma Chemicals Co, St. Louis, MO, USA. All other chemicals used were of analytical grade. D. russelli venom was purchased from Hindustan Snake Park, Kolkatta, India.

Swiss albino mice weighing 20-25 g were obtained from the Central Animal House facility, Department of Zoology, University of Mysore, Mysore, India. The animal care and handling were conducted in compliance with the National Regulations for Animal Research. The animal experiments were carried out after reviewing the protocols by the Animal Ethical Committee of the University of Mysore.

Preparation of seed methanolic extract

Fruit seeds (Vitis vinifera L.) collected from the local market, Mysore were washed thoroughly with water, shade-dried, grounded into coarse powder. The powder (5 g) was soaked in 20 ml of methanol for 30 min with constant stirring and the extract was filtered through Whatman filter paper grade I. The filtrate was dried at room temperature to evaporate methanol and dried sample was weighed (20 mg) and dissolved in minimum volume of dimethyl sulfoxide (DMSO) and diluted in distilled water (1: 9, v/v). DMSO and distilled water (1: 9, v/v) mixture did not induce any of the toxic effects in mice and served as control experiments. For inhibition studies, the venom sample was pre-incubated with various amounts of methanolic extract for 30 min at 37°C and the venom: extract ratio (w/w) was used unless otherwise indicated.

Proteolytic activity

The proteolytic activity was determined according to the previously described method using casein (2% in 0.2 M Tris-HCl buffer, pH 8.5) as substrate. The venom sample (100 µg) was incubated with 0.4 ml casein in a total volume of 1 ml for 2 h at 37°C. The reaction was terminated and the undigested casein was precipitated by the addition of 1.5 ml of 0.44 M trichloroacetic acid. The digested casein in the supernatant (1 ml) was measured using Folin-Ciocalteu’s reagent. One unit of activity was defined as the amount of venom sample required to cause an increase in optical density (OD) by 0.01 at 660 nm/min.

The proteolytic activity was also performed in SDS-PAGE zymogram as described previously. Briefly, casein was incorporated at a final concentration of 2% in the SDS-PAGE matrix (10%) and the venom sample (50 µg) prepared under non-reduced condition. The gel was rinsed in 2.7% Triton X-100 for 1 h after electrophoresis and incubated in a developing buffer (50 mM Tris, 40 mM NaCl and 200 mM CaCl₂·2H₂O) for 18 h and stained with 0.1% Coomassie brilliant blue R-250. The gel was analyzed for clear zones of substrate hydrolysis against a blue background.

Hyaluronidase activity

The hyaluronidase activity was assayed according to the previously described method. The venom sample (100 µg) was incubated with 50 µg of hyaluronic acid in 300 µl 0.2 M sodium acetate buffer, pH 5.5 containing 0.15 M NaCl at 37°C for 2 h. The reaction was terminated by adding 50 µl of potassium tetraborate and boiled for 3 min. The coloring reagent p-dimethyl aminobenzaldehyde (10% PDMAB) 1.5 ml (acetic acid: hydrochloric acid; 9: 1; v/v) was added and incubated for 30 min at 37°C. The absorbance was measured at 585 nm. Activity was expressed as n moles of N-acetyl glucosamine released/min/mg venom sample.

The SDS-PAGE zymogram was also performed as described previously. Briefly, hyaluronic acid was incorporated at a final concentration of 0.17 mg/ml in the SDS-PAGE matrix (10%). The venom sample (50 µg) prepared under non-reduced condition was electrophoresed. Thereafter, the gel was soaked consecutively 3-times in 50 ml of 0.15 M NaCl in sodium phosphate buffer (0.1 M, pH 5.5) containing 5%, 0.05% and 0% Tritan X-100 for 1 h. This was followed by equilibrating the gel in 0.1 M sodium formate buffer, pH 5.5 containing 0.15 M NaCl for 18 h at 37°C with constant agitation. The gel was washed in 0.015 M Tris-HCl buffer (pH 7.9) and placed in 0.1% stains-all solutions for 2 h and kept in the dark until photographed. The appearance of a translucent activity band against a dark blue background indicated the enzyme activity. The
working 1 ml of stock with 1 ml formamide, 4 ml isopropanol, 0.37 ml 1 M Tris-HCl buffer, pH 7.9 and de-ionized water to a volume of 20 ml. The molecular weight marker proteins run on one edge were stained with Coomassie brilliant blue R-250.

Hemorrhagic activity
The hemorrhagic activity was assayed according to the previously described method. Groups of 5 mice each were injected intradermally with twice the minimum hemorrhagic dose (MHD) of venom sample (10 µg) in 50 µl saline. After 3 h, the mice were anesthetized by diethyl ether inhalation, a dorsal patch of the skin was removed and the inner surface was observed for the hemorrhage. The MHD was calculated as the amount of venom sample that produced a hemorrhagic spot of 10 mm.

Edema-inducing activity
The edema inducing activity was assayed according to the previously described method. Groups of 5 mice were injected in the right footpads with six-times the minimum edema dose (MED) of venom sample (6 µg) in 20 µl saline. The left footpads received saline and served as controls. The mice were anesthetized by diethyl ether inhalation; the legs were removed at the ankle joint after 1 h. Increase in weight due to edema was calculated as edema ratio, which equals the weight of edematous leg x 100/weight of control leg. The MED was defined as the amount of venom sample required to cause an edema ratio of 120%.

Myonecrotic activity
The myonecrotic activity was assayed according to the previously described method. Half the LD50 value of venom sample (1.25 mg/kg body wt) in 50 µl saline was injected intramuscularly into the right thigh of three groups of mice (n = 5). The group receiving 50 µl saline alone served as a control experiment. The mice were anesthetized after 3 h by diethyl ether inhalation. The thigh muscle tissues dissected out from the site of venom sample injection were fixed in Bouin’s solution and dehydrated by processing through different grades of alcohol and chloroform: alcohol mixture. The processed tissues were embedded in paraffin wax, cut in to a 4 µm thick sections, which were stained with hematoxylin-eosin for microscopic observations (Leitz Wetzlar Germany type-307-148,002 microscope) and photographs were taken using Photometrics colorsnap CF camera (Leitz Diaplan Germany, Roper Scientific Photometrics type- A014872002) attached to the microscope.

Coagulant and fibrinogenolytic activities
The plasma coagulation activity was assayed according to the previously described method. The normal human citrated plasma (200 µl) was incubated with venom sample (1 µg) and the clotting time was recorded against a light source. Fibrinogenolytic activity was assayed according to the previously described method. The human plasma fibrinogen (50 µg) was incubated with the venom sample (10 µg) in 40 µl of 5 mM Tris HCl buffer (pH 7.4) containing 10 mM NaCl. The reaction was terminated after 6 h by adding 20 µl denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol and samples were analyzed on 10% SDS-PAGE as previously described method. The protein estimation was carried out according to the previously described method using bovine serum albumin (BSA) as a standard.

Statistical analysis
All the experiments were repeated for five independent observations. The data were presented as mean ± S.E.M.

Results
The toxicity studies D. russelli venom in mouse models revealed the minimum hemorrhagic dose (MHD) to be 5 ± 0.03 µg, and the 2 x MHD caused the hemorrhagic area of 20 ± 0.05 mm, whereas the minimum edema dose (MED) was 1 µg and the 6 x MED caused an edema ratio of 170 ± 2%. The venom was pro-coagulant in nature and caused the rapid clotting of human plasma when added directly. It degraded the human fibrinogen with Aα-chain degraded preferentially over the Bβ-chain, while the γ-chain was resistant and remained intact, irrespective of incubation period and concentrations of venom sample used. The venom caused an extensive dermo- and myonecrosis at the injected site. It hydrolyzed casein and also hyaluronic acid with the specific activity of 1.1 ± 0.05 and 1.25 ± 0.04 units/min/mg, respectively.

The methanolic extract of grape seeds inhibited the proteolytic activity of the venom in a dose-dependent manner. At the venom: extract ratio of 1: 8, complete inhibition of proteolytic activity was observed (Fig. 1a). Inhibition was further confirmed by the casein
zymogram assay. Lack of translucent activity band in zymogram assay in presence of the extract and appearance in its absence against a blue background suggested the inhibition (Fig. 1b). Further, the extract also inhibited the hyaluronidase activity of the venom in a dose-dependent manner and the venom: extract ratio of 1: 2 caused the complete inhibition of the activity (Fig. 2a). Inhibition was also confirmed in the hyaluronidase zymogram assay (Fig. 2b). The extract effectively inhibited the hemorrhage induced by the venom in a dose-dependent manner and at the venom: extract ratio of 1: 25 neutralized the hemorrhage caused due to 2 x MHD (Fig. 3). Further, the extract completely abolished the hemorrhagic edema induced by the venom dose-dependently and at the venom: extract ratio of 1: 20, the edema ratio due to 6 x MED of 170 ± 2% neutralized to 117 ± 3.1% (Fig. 4).

The extract also inhibited the myonecrotic activity induced at the site of venom injection and caused complete inhibition of the myonecrosis at the venom: extract ratio of 1: 8. The longitudinal section of muscle tissues revealed extensive destruction of myocytes and disorganized myofibrils with the accumulation of polymorphonuclear leucocytes at the

Fig. 1—(a): Inhibition of proteolytic activity [D. russelli venom (100 µg) was pre-incubated with the increased amounts of the extract for 30 min at 37°C. The reaction was initiated by adding 0.4 ml of 2% casein in 0.2 M Tris HCl buffer (pH 8.5) and adjusted the reaction volume to 1 ml with the buffer and incubated for 2 h at 37°C. The activity was determined as described in the ‘Materials and Methods’. Values represent as mean ± S.E.M (n = 5); and (b): Inhibition of proteolytic activity in casein zymogram [D. russelli venom (50 µg) was pre-incubated with the extract for 30 min at 37°C and resolved by SDS-PAGE (10%) containing 2% casein under non-reduced condition and processed as described in the ‘Materials and Methods’. Lane 1, 50 µg of venom sample alone; lane 2, venom: extract ratio of 1: 8; lane M, molecular weight markers from top to bottom: myosin-H-chain (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (31.0 kDa), soyabean trypsin inhibitor (21.5 kDa) and lysozome (14.4 kDa)]

Fig. 2—(a): Inhibition of hyaluronidase activity [D. russelli venom (100 µg) was pre-incubated with the increased amounts of the extract for 30 min at 37°C and incubated with hyaluronic acid in a final reaction volume of 0.3 ml of 0.2 M sodium acetate buffer containing 0.15 M NaCl for 2 h at 37°C. The hyaluronidase activity was carried out as described in the ‘Materials and Methods’. Values represent mean ± S.E.M (n = 5); and (b): Inhibition of hyaluronidase activity in zymogram [D. russelli venom (50 µg) was pre-incubated with the extract for 30 min at 37°C and resolved by SDS-PAGE (10%) containing hyaluronic acid (0.17 mg/ml) under non-reduced condition and processed as described in the ‘Materials and Methods’. Lane 1, 50 µg of venom sample alone; lane 2, venom: extract ratio of 1: 2; lane M; molecular weight markers from top to bottom: myosin-H-chain (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (31.0 kDa), soyabean trypsin inhibitor (21.5) and lysozome (14.4 kDa)]
venom injected site (Fig. 5b), while in presence of the extract, the tissue sections revealed normal muscular striations (Fig. 5c), similar to the control section (Fig. 5a).

The *D. russelli* venom at 1 µg concentration revealed the plasma clotting time of 31 ± 1.5 s, as compared to the control value of 190 ± 4 s. The seed extract prolonged the clotting time of the venom dose-dependently from 31 ± 1.5 s to a maximum of 87 ± 3.5 s (Fig. 6) at the venom: extract ratio of 1: 20. Further, the extract inhibited the fibrinogenolytic activity of the venom dose-dependently. At the venom: extract ratio of 1: 10, the preferential inhibition of Bβ chain degradation over Aα chain was observed, while at venom: extract ratio of 1: 30, inhibition of both Aα and Bβ chains degradation was achieved (Fig. 7).

**Discussion**

Envenomation by *D. russelli* venom is well characterized by its prominent local effects involving blistering, hemorrhage, edema, dermonecrosis, myonecrosis and inflammation. The bite causes the hemostatic disturbances, leading to bleeding disorders and damage of the vital organs. Although anti-venom therapy is effective in neutralizing the systemic toxicity, if administered in time, but it is less effective or ineffective against local tissue destruction, resulting in the continuous tissue destruction at the bite site even after the neutralization of systemic toxicity. Thus, there is a great demand for new alternative therapy.

The methanolic extract of grape seeds abolished both proteolytic and hyaluronidase activities, with the later inhibited more effectively. The hemorrhagic activity of the venom was also inhibited. The tissue destruction and hemorrhage is primarily due to the degradation of structural and adhesive components such as collagen, elastin, fibronectin and glycosaminoglycans of the extracellular matrix (ECM) of connective tissue, surrounding the blood vessels and capillaries by matrix-degrading venom hyaluronidases and metalloproteases. These two
groups of hydrolytic enzymes appear to be the principal agents responsible for tissue degradation at the site of bite.

The snake venom metalloproteases (SVMPs) are the Zn$^{2+}$-dependent hemorrhagic metalloproteases of the metzincin family enzymes which degrade the structural protein scaffold of the ECM. While hyaluronidases degrade the hyaluronic acid into small fragments, hyaluronic acid is a megadalton polymer and holds aggrecan monomers, metal ions and water molecules through ionic interaction. Thus, the degradation of ECM components not only destroys the mechanical support of the tissue, but also makes the ECM smooth and fragile, resulting in easy diffusion of systemic toxins into the circulating blood.$^{34,35}$

As the hemorrhagic metalloproteases are usually dependent on divalent metal ions for their activity, the metal ions appear to play role both in the catalysis and as well offer structural stability for the enzymes. The seed extract might chelate the metal ion or interact with a specific domain to inactivate the hemorrhagic protease and that could have led to inhibition of the hemorrhage. Similar results were also observed by different plant extracts and bioactive component(s)$^{18-22}$. Although inhibitory mechanisms are not well understood for hyaluronidases, there are reports suggesting the inhibition of the activity by the plant components.$^{36}$ Thus, it appears that inhibition of hemorrhagic metalloproteases and hyaluronidases could offer effective protection against tissue destruction at the bitten region. Inhibition of edema-inducing activity suggests the inhibition of inflammatory reactions, most likely phospholipase A$_2$ activity of the venom. Inhibition of myonecrotic activity is undoubtedly strengthened by the restoration of normal striated musculature in histopathological studies. Earlier, similar neutralization of myonecrotic activity by various plants extracts and their bioactive component(s) has been reported.$^{18-22}$

The D. russelli venom is rich in components that interact with the coagulation cascade and make the venom highly pro-coagulant. It is likely that inhibition of fibrinogenolytic activity by the extract is responsible for the observed inhibition of pro-coagulant activity of the venom. Snake venom fibrinogenases releasing fibrinopeptides A and or B from the N-terminal disulfide knot of A$\alpha$ or B$\beta$ chains are thrombin-like enzymes and are found to be responsible for the pro-coagulant activity while, those acting from the C-terminal end are responsible for the anti-coagulant activity.$^{37}$

**Conclusion**

The methanolic extract of grape seeds inhibited the local toxicity and as well as pro-coagulant activity of D. russelli venom. However, further studies are needed for the isolation of specific inhibitors for the efficient management of threatening local toxicity and the fatal systemic toxicity of D. russelli bite.

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