Local tissue damage induced by *Echis carinatus* venom: Neutralization by *Albizia lebbeck* seed aqueous extract in mice model

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**ABSTRACT**

**Background:** *Albizia lebbeck* L. (Fabaceae) is an Indian traditional medicinal plant used in treatment of snakebite and scorpion sting. The present study aims to determine its scientific basis. **Methods:** Pulverized seed aqueous extracts (cold and hot) was evaluated for neutralization of enzyme activities (hyaluronidases, proteases and PLA$_2$) of *Echis carinatus* S. (Viperidae) venom (ECV), *in vitro* and local tissue damage using murine model. **Results and discussion:** Cold extract contained proteins which resolved into three fractions on Sephadex G-50 (ALF-1, ALF-2, and ALF-3), while hot extract (ALHE) contained no proteins. ALF-1 contained proteins and did not inhibit any enzymes. ALF-2 and ALHE contained flavonoids, phenolics, saponins, and steroids. All four fractions were positive for peptides/amino acids. ALF-2 and ALHE demonstrated significant inhibition of hyaluronidase. Only ALHE showed potent inhibition of proteases, ECV induced hemorrhage, myotoxicity and pro-coagulant activity. ALF-3 inhibited PLA$_2$ activity and edema. In summary, hyaluronidases and proteases inhibitory phytochemicals present in *A. lebbeck* seed get extracted in both cold and hot water. The PLA$_2$ inhibiting peptides/amino acids get extracted largely in cold water. Therefore, this differential extraction method can be used to isolate specific inhibitors. **Conclusion:** The results support the significance of *A. lebbeck* extracts towards management of snakebite by the local traditional healers in India.

**KEYWORDS:** ECV hyaluronidase, ECV protease, ECV PLA$_2$, Folk medicine, Complementary medicine, Hemorrhage, Myotoxicity, Edema

**INTRODUCTION**

Snake venom is one of the unique secretions from venomous snakes, produced and stored in venom glands. It is an efficient offensive weapon in the armoury of snakes, principally used to immobilize/digest the prey$^1$. It causes grievous effects following bite to humans. Snakebite is medical emergency which causes serious impact on human health, often associated with permanent loss of tissue function and physical disability. Among various species of snakes, viperid bites are particularly associated with physical deformities$^2$. The heterogeneous group of target specific toxins including enzymes and peptides of *Echis carinatus* S. (Viperidae) venom (ECV) act on various tissues/organs$^3$. Based on the previous findings, the ECV induced local tissue manifestations arise due to the concerted action of the three major hydrolytic enzymes, hyaluronidases, proteases, and phospholipase A$_2$$^3$. In India, the polyvalent anti snake venom (ASV) is the only treatment available against snakebite. ASV offers least protection against the local tissue damage associated with viperid bites and also causes hypersensitive reactions$^{2,4,5}$. Therefore, plant based medicines appears to be a promising alternative and additional resource to manage the dreadful consequences of viperid snakebites alongside ASV.

Large populations of folk healers and Ayurveda system of medicine use plant preparations for treating snakebite complications, since time immemorial. Hence plant preparations and isolated compounds have gained importance in venom research as supportive therapeutic agents$^6$. Plant based medicines may interact directly with snake venom toxins to reduce the toxicity or provide optimal conditions to aid physiological response against venom toxicity by reducing the spreading of venom toxins$^5$. *A. lebbeck* L. (Fabaceae) is a potential folk medicinal plant used in Indian traditional medicine for the treatment of snakebite, scorpion sting, hemorrhage, gum inflammation, and related clinical conditions$^5$-$^{13}$. Based on the available reports$^5$-$^{13}$ and personal conversations with local folk healers of Mysuru and Hassan (Karnataka, Karnataka,
India), *A. lebbeck* seed aqueous extracts (both cold and hot) was selected to evaluate antidotal properties, particularly against ECV induced complications. The results demonstrate that *A. lebbeck* seed extracts effectively inhibited enzymatic toxins and the toxic symptoms induced by these toxins validating its use in folk medicine for management of snakebites.

**MATERIALS AND METHODS**

**Chemicals**

Alcian blue, *Escherichia coli* [lyophilized cells of strain W (ATCC9637)], fatty acids, gelatin, n-acetyl glucosamine, and para-dimethylaminobenzaldehyde were procured from Sigma Chemicals (St. Louis, USA). Hyaluronic acid was purchased from Across Organics (New Jersey, USA). \(^{14}\)C-oleic acid was obtained from Perkin Elmer Life Sciences Inc. (Boston, USA). Scintillation cocktail (Ultima Gold) was obtained from Packard Bioscience Co. (Meriden, USA). Bovine serum albumin (BSA) and casein was purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). All other chemicals and reagents used in this project were of analytical grade.

**Snake venom**

Lyophilized powder of *E. carinatus* venom (ECV) was purchased from Irula Co-operative Society Ltd. (Chennai, India). Venom was dissolved in saline and centrifuged at 6000 \(g\) for 10 min to remove debris. The supernatant was stored as aliquots at -20\(^\circ\)C until further use. Protein content of the crude venom was determined by Lowry’s test and studies were carried out based on the protein content of ECV.

**Animals**

Swiss albino mice (20-30 g; 6-8 weeks; either gender) were obtained from Central Animal House Facility, University of Mysore (UOM), Mysuru (Karnataka, India). Mice were maintained in polypropylene cages with 12 h light/dark cycle. Mice care and handling were conducted in compliance with National Regulations for Animal Research and the experiments were carried out according to the protocols reviewed by the Institutional Animal Ethical Committee (IAEC), UOM, Mysuru (voucher specimen accession no. 0256).

**Preparation of seed extracts**

Seeds of *A. lebbeck* were collected in Manasagangotri campus, UOM, Mysuru during the month of February, 2013. The plant was identified and authenticated by Dr. Sharvani KA, Assistant Professor and Herbarium in-charge, Department of Botany, Yuvaraja’s College, UOM, Mysuru (voucher specimen accession no. 0256). The seeds were washed under running tap water, shade dried, and pulverized. Exactly 20 g of pulverized seed powder was subjected to cold (4\(^\circ\)C overnight) and hot ( Soxhtlet extraction; 100\(^\circ\)C 20 cycles) aqueous extraction. As the hot extract was clear and free from debris, only cold extract was subjected to centrifugation for 20 min at 6000 \(g\). The clear supernatant of cold extract was obtained after successive steps of centrifugation and filtration. Protein content of seed extracts were determined using Lowry’s and Biuret method.

**Partial purification of proteins/peptides from *A. lebbeck* cold aqueous seed extract (ALCE)**

ALCE (50 mg) was loaded onto Sephadex G-50 column (100 ml bed volume) pre-equilibrated with 0.15 M Tris-HCl buffer pH 7.4. Fractions were collected at the rate of 15 ml/hr (1.5 ml fractions). After collecting one and half bed volume fractions, about 50 ml of fraction was collected separately in a beaker. The absorbance of the obtained fractions was measured at 280 nm.

**Electrophoresis**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the published method. Various concentrations of *A. lebbeck* extracts were electrophoresed on 15% gel under non-reducing conditions at constant voltage of 100 V for 2 h. The gel was silver stained to visualize the bands.

**Phytochemical screening**

The *A. lebbeck* hot aqueous seed extract (ALHE), *A. lebbeck* fraction 1 (ALF-1), *A. lebbeck* fraction 2 (ALF-2), and *A. lebbeck* fraction 3 (ALF-3) were screened for peptides/amino acids and various phytochemicals such as alkaloids, anthraquinones, flavonoids, glycosides, phenolics, saponins, steroids, and tannins as described in the published method.

**Hyaluronidase activity**

Hyaluronidase activity was determined using hyaluronic acid as substrate. For inhibition studies, hyaluronidase activity was determined after pre-incubating 100 μg ECV with various concentrations of *A. lebbeck* extracts (ALE – includes ALCE, ALHE, ALF-1, ALF-2, and ALF-3) (1:10 - 1:100 w/v) for 15 min at 37\(^\circ\)C with appropriate controls. One unit of enzyme activity was defined as the amount of enzyme required to increase an absorbance of 0.01 at 585 nm/ h at 37\(^\circ\)C.
Hyaluronidase activity by substrate gel assay was performed according to the published procedure\(^9\). For inhibition studies, similar experiments were carried out with 100 µg ECV, following pre-incubation with ALF-2 and ALHE (1:100; w/w) for 15 min at 37°C with appropriate controls. The activity was visualized as a clear zone of hydrolysis against greenish blue background. Zones of hydrolysis were measured using graph sheet and the results were expressed in mm\(^2\).

**Protease activity**

Protease activity was determined using casein as substrate\(^20\). For inhibition studies, 25 µg ECV was separately pre-incubated with various concentrations of ALE (1:1 - 1:100 w/w) at 37°C for 15 min prior to assay, keeping appropriate controls. One unit of enzyme activity was defined as the amount of enzyme required to increase an absorbance of 0.01 at 660 nm/ h at 37°C.

Gelatinolytic activity of ECV by substrate gel assay was carried out according to the published protocol with slight modifications\(^20\). For inhibition studies, 10 µg of ECV was pre-incubated with ALHE (1:100; w/w) at 37°C for 15 min prior to assay, keeping appropriate controls. The activity was visualized as a clear zone of hydrolysis against dark blue background. Zones of hydrolysis were measured using graph sheet and the results were expressed in mm\(^2\).

**Re-calcification time**

Plasma re-calcification time was determined according to the published method\(^21\). For inhibition studies, 1 µg ECV was pre-incubated with various concentrations of ALHE (1:1 - 1:100 w/w) at 37°C for 15 min prior to assay, with appropriate controls.

**Phospholipase A\(_2\) activity**

PLA\(_2\) activity was measured using \(^1^4\)C-oleate labeled autoclaved E. coli cells as substrate using liquid scintillation counter\(^22\). For inhibition studies, 30 µg of ECV was pre-incubated with ALE (1:100 w/w) and various concentrations of ALF-3 (1:10 - 1:100 w/w) at 37°C for 15 min prior to assay with appropriate controls. Results were expressed as percent activity.

**Hemorrhagic activity**

Hemorrhagic activity was assayed according to the published method\(^23\). Inhibition studies were carried out by co-injecting (simultaneous) various concentrations of ALHE (1:10 - 1:50 w/w). Saline, 3 µg ECV, and ALHE alone injected (i.d.), respectively, served as negative, positive, and extract controls respectively. Inhibition of hemorrhagic activity was observed as a decreased area of hemorrhagic spot in comparison to venom injected hemorrhagic spot. Hemorrhagic spot was measured using graph sheet and the results were expressed in mm\(^2\).

**Myotoxicity**

Myotoxic activity was measured according to our previously published method\(^23\). Inhibition studies were carried out by co-injecting (simultaneous) various concentrations of ALHE (1:10 - 1:50 w/w) to thigh muscle. Saline, 5 µg ECV, and extracts alone injected (i.m) served as negative, positive, and extract controls respectively.

**Edema**

Edema inducing activity of ECV was determined according to the published protocol\(^24\). For inhibition studies, 3 µg ECV was pre-incubated with different concentrations of ALF-3 (1:10 - 1:50 w/w) prior to assay, keeping appropriate controls. Results were expressed as percentage edema ratio.

**Statistical analysis**

The results of experiments were expressed as mean ± SD (n = 3). Statistical analysis was carried out using Student’s \(t\)-test. The comparison between the groups was considered significant if \(p \leq 0.01\). Data were analyzed using the statistical package Graph Pad Prism\(^6\) (La Jolla, USA).

**RESULTS AND DISCUSSIONS**

The development of modern medicine has resulted in the increased average lifespan and overall health status of humans\(^25\). However, prevention of deaths due to venomous snakebite and its associated local tissue damage remains a challenge. In India, the polyvalent anti snake venom (ASV) is the only treatment available against snakebite. ASV offers least protection against the local tissue damage associated with viperid bites and also causes hypersensitive reactions\(^2,4,5\). Therefore, plant based medicines are gaining importance alongside ASV in complementary/alternative therapeutics for envenomation associated local tissue damage\(^2,5\).

Aqueous extracts of A. lebbeck, a folk medicinal plant is used in Ayurveda for the treatment of snakebite, scorpion sting, and their related complications\(^7-13\). However, the usage of seeds/extracts for treatment has not been scientifically evaluated. Therefore, the present work is an attempt to examine the scientific validity and applicability of A. lebbeck seeds in treating venom induced local manifestations.

As a preliminary test the inhibition of hyaluronidase, protease, and phospholipase A\(_2\) activity was determined using cold and hot extracts of A. lebbeck seed (50 µg). Both the extracts demonstrated significant inhibition of the enzyme activities (data not shown).
Following which the extracts were screened for the presence of protein contents using 15% SDS-PAGE. Protein bands were only observed in the cold extract but not in the hot extract (Fig. 1A). However, both the extracts were positive for the presence of peptides/amino acids by ninhydrin test (Table 1). Since A. lebbeck cold extract (ALCE) showed the presence of 5-6 major protein bands it was subjected for gel filtration on Sephadex G-50 column and it resolved into two major peaks, ALF-1 and ALF-2 [Fig. 1B]. Additionally, a volume of 50 ml eluent was collected since ALCE was found to be positive for peptides/amino acids which would elute later due to their low molecular weight and labeled as ALF-3. On 15% SDS-PAGE, ALF-1 gave similar banding pattern to that of crude ALCE. ALF-2 and ALF-3 did not show any protein bands [Fig. 1A], but were positive for peptide/amino acids test indicating the absence of proteins but the presence of low molecular weight peptides/amino acids. Additionally when all four samples (ALF-1, ALF-2, ALF-3, and ALHE) were screened for phytochemicals, it was evident that ALF-2 and ALHE had similar contents i.e. presence of flavonoids, phenolics, saponins and steroids. Alkaloids, anthraquinones, glycosides, and tannins were absent in all the samples tested (Table 1). Further, following fractionation the preliminary studies demonstrated that ALF-1, the protein rich fraction failed to inhibit ECV enzymes. Only ALF-2, ALF-3, and ALHE had inhibition potential against the toxic enzymes of ECV such as hyaluronidase, protease, and PLA₂. Therefore, these fractions were tested against the ECV induced array of local tissue manifestations such as hemorrhage, myotoxicity, hemorrhagic edema, and other complications.

Snake venom hyaluronidases (SVHYs), popularly known as ‘spreading factors’ act on the major ECM glycosaminoglycan - hyaluronic acid, resulting in loss of ECM integrity. Damaged ECM allows increased diffusion of venom toxins, leading to both systemic complications and local tissue damage. Inhibition of SVHYs does directly reduce the spreading of toxins from bitten site. In addition, the potentiating effect of SVHYs towards snake venom proteases and PLA₂ mediated tissue damage will be minimized. In this direction, SVHYs inhibition studies by seed extracts were performed. Only ALF-2 and ALHE inhibited the hyaluronidase activity of ECV dose-dependently, maximum inhibition was observed at venom to extract ratio of 1:100 w/w (68%; \( p < 0.0001 \) and 77%; \( p < 0.0001 \) respectively) [Fig. 2A]. This was further confirmed by substrate gel assay. ALF-2 and ALHE showed inhibition of ECV hyaluronidase activity in zymography upon pre-incubation for 15 min with 100 µg ECV, prior to electrophoresis at the venom to extract ratio of 1:100 w/w (90%; \( p < 0.0001 \)) [Fig. 2B].
only ALHE significantly inhibited proteolytic activity of ECV. The inhibition was concentration dependent and significant inhibition was observed at a venom to extract ratio of 1:100 w/w (62%; \( p < 0.0001 \)) \[\text{Fig. 2C}\]. ECV is a rich source of proteases, which are responsible for hemostatic and local tissue manifestations due to their actions on ECM and hemostatic system\(^{26}\). Majority of proteolytic activity of ECV is contributed by metalloproteases (about 90%) and the rest is due to serine proteases (about 10%)\(^{23}\). Higher concentrations of metalloproteases in ECV are directly responsible for ECM disintegration and the resulting local tissue disturbances. The inhibitory potential of ALHE towards ECV proteases was further evaluated using ECM protein (gelatin); to determine the role of ALHE in protecting ECV induced anatomical and physiological alterations. ECV metalloproteases efficiently hydrolyzed gelatin in a dose-dependent response. ALHE significantly inhibited gelatinolytic activity of ECV at a venom to extract ratio of 1:100 w/w (60%; \( p < 0.0001 \)) \[\text{Fig. 2D}\]. Gelatinolytic activity is exhibited by proteases which act on ECM proteins (collagen, laminin, and fibronectin)\(^{27}\). Snake venom proteases acting on ECM and basement membrane proteins characterized to date are metalloproteases which cause hemorrhage and associated clinical complications. The onset of hemorrhage is due to destruction of basement membrane proteins and ECM surrounding the blood vessels, resulting in fragile blood vessels, which rupture easily. Hemorrhage induces a secondary event in the form of hypoxia, which leads to the onset of progressive local tissue damage and necrosis\(^{23,28}\). Inhibition of gelatinolytic activity by ALHE provides additional evidence supporting the use of \textit{A. lebbeck} in management of viper bites.

**Fig. 2:** A & B - Inhibition of hyaluronidase activity of ECV by ALF-2 and ALHE: A) Reaction mixture (0.5 ml) contained 50 µl of hyaluronic acid (1 µg/µl in 0.1 M sodium acetate buffer containing 0.15 M NaCl, pH 8.5) incubated with 100 µg ECV + different concentrations of ALF-2 and ALHE ranging from 1:1 – 1:100 w/w for 2.5 h at 37°C. B) Hyaluronic acid was incorporated into the SDS polyacrylamide resolving gel matrix (0.03%). ECV (100 µg) and ECV pre-incubated with ALF-2 and ALHE for 15 min was loaded onto separate wells. Lanes: ECV – 100 µg ECV; ALF-2 - 1:100, ECV (µg): ALF-2 (µg); ALHE - 1:100, ECV (µg): ALHE (µg). C & D - Inhibition of protease activity of ECV by ALHE: C) Reaction mixture (1 ml) contained 0.4 ml of casein (2% in 0.2 M Tris-HCl buffer, pH 8.5) incubated with 25 µg ECV + different concentrations of ALHE ranging from 1:1 – 1:100 w/w for 2.5 h at 37°C. D) Gelatin was incorporated into the SDS polyacrylamide resolving gel matrix (0.08%). ECV (10 µg) alone and ECV pre-incubated with ALHE for 15 min was loaded into separate wells. Lanes: ECV - 10 µg ECV; ALHE - 1:100, ECV (µg): ALHE (µg). Data represents mean ± SD (n = 3). *\( p < 0.01 \), **\( p < 0.001 \), and ***\( p < 0.0001 \) compared to ECV.
We further tested the inhibitory potential of ECV induced hemorrhagic activity inhibition by ALHE. Initially, pre-incubation experiments were conducted using different ratios of ECV and ALHE (w/w). Significant inhibition of hemorrhage was observed at the venom to extract ratio of 1:50 w/w. Based on the results of pre-incubation, co-injection (simultaneous) studies were performed at 1:50 w/w. The result demonstrated dose-dependent inhibition of hemorrhage upon co-injection at the tested dose (66%; \( p < 0.0001 \)) [Fig. 3A]. Potent inhibition of ECV induced hemorrhage by ALHE suggests its potential in preventing the onset of local tissue damage and spreading. Metalloproteases of ECV induce myotoxicity, which is secondary to hemorrhage, characterized by elevated serum creatine kinase (CK) and lactate dehydrogenase (LDH)\(^29\). These enzymes are abundantly present in the cytosolic compartment of skeletal muscles and are released into circulation following skeletal muscle damage as observed in many clinical conditions including snakebite\(^8\). ALHE inhibited the myotoxicity of ECV by significantly reducing serum CK (55%; \( p < 0.0001 \)) and LDH (50%; \( p = 0.0054 \)) levels at the venom to extract ratio of 1:50 w/w upon co-injection [Fig. 3B].

Apart from extensive tissue damage, ECV exerts its action towards various components of blood\(^31\), predominantly the coagulation factors and exhibits a strong pro-coagulant effect \textit{in vitro} [32 s vs. 300 s (normal)]. The pro-coagulant effect of ECV is due to prothrombin activating metalloproteases – ecarin and carinactivase\(^32-33\). The pro-coagulant effect of ECV was inhibited by ALHE at the venom to extract ratio of 1:100 w/w (50%; \( p = 0.0004 \)) and the clotting time was restored to 150 s [Fig. 4].

Snake venom PLA\(_2\)s are multi-toxic enzymes that act locally as well as systemically to induce wide range of pathophysiological alterations in the victim. PLA\(_2\)s release arachidonic acid from sn-2 position of membrane phospholipids including erythrocytes which induce plasma membrane lesions and direct hemolysis respectively. Arachidonic acid released is converted into prostaglandins, leukotrienes, and cysteinyl leukotrienes by cyclooxygenase and lipoxygenase, which are potent
inflammatory mediators, leading to edema. Further, lysophospholipids released by the action of PLA₂s can act as surfactants, which in turn rupture the erythrocyte membrane causing indirect hemolysis. Snake venom PLA₂s also affects neurons (at pre-synaptic or post-synaptic cleft) and skeletal muscle to cause neurotoxicity and myotoxicity respectively. Snake venom PLA₂s also exhibit hemostatic disturbances by hydrolyzing pro-coagulant phospholipids. In this direction inhibition of PLA₂ was carried out. Of all the fractions tested, significant inhibition of PLA₂ was demonstrated by ALF-3 (50%) and all other extracts inhibited PLA₂ but to lesser extent (30%, data not shown). Therefore, dose-dependent inhibition of PLA₂ by ALF-3 was performed. ALF-3 inhibited the PLA₂ activity dose-dependently and maximally at the venom to extract ratio of 1:100 w/w (55%; p < 0.0001) [Fig. 5A]; this was supported by the inhibition of hemorrhagic paw edema at 1:50 w/w in mice model (43%; p = 0.0001) [Fig. 5B].

ALF-1 did not inhibit any of the toxic enzymes of ECV, which rules out the role of intact proteins in venom neutralization. ALF-2 demonstrated significant inhibition of hyaluronidase. Similarly, ALHE showed potent inhibition of proteases and hyaluronidases. Since the phytochemical analysis revealed similar phytochemicals in ALF-2 and ALHE, it can be concluded that hyaluronidase inhibition is due to phytochemicals present in ALF-2 and ALHE. In addition, ALHE is the only fraction which also contains required concentrations of phytochemicals with protease inhibition capability. ALF-3 was the only fraction with potent PLA₂ inhibition potential, which indicates the role of small molecular weight peptides/amino acids present in the fraction. This observation is also supported by the partial inhibition of PLA₂ by other fractions which contained peptides/amino acids. Based on our results, we conclude that, hyaluronidases and proteases inhibitory phytochemicals present in A. lebbeck seed get extracted in both cold and hot water. On the contrary, the PLA₂s inhibiting peptides/amino acids get extracted largely in cold water. Therefore, this differential extraction method can be used to isolate and characterize specific inhibitors from A. lebbeck seeds. Over all our results support the applications of A. lebbeck extracts towards management of snake bite by traditional and Ayurvedic healers in India.
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CONFLICT OF INTEREST
The authors have no conflicts of interests to disclose.

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