## INTRODUCTION

Snakebite is a major health hazard in many regions of the world especially in the tropical and sub tropical countries (Chippaux, 1998). In Asia, as a whole, there may be five million snakebites every year, of which almost 1, 25,000 are fatal bites. The annual snakebite in Indian subcontinent alone is more than 2, 00, 000 and 35, 000 – 50, 000 of them have turned out to be fatal (Brunda and Sashidhar, 2007).

*Daboia russellii* (*DR*) has a vast distribution which extends somewhat discontinuously across most of southern Asia and into the islands of Indonesia. It occurs in many parts of Pakistan, more or less throughout India, Bangladesh, Sri Lanka, Bhutan and some parts of Nepal. Further east, it is found in Taiwan and in parts of Burma, Thailand, and China and in eastern Java and some of the Lesser Sunda Islands (Wuster, 1998). *DR* is the predominant poisonous snake of the Indian sub continent and endemic through out the country. The venom has been studied extensively for various active principles such as pro-coagulant factors (Schffman et al., 1969), ATPase (Kini and Gowda, 1982), phospholipase $A_2$ (Vishwanath et al., 1987; Kasturi and Gowda, 1989, 1992; Chakrabarty et al., 2002; Tsai et al., 2007; Maity et al., 2007), trypsin inhibitors (Jayanthi and Gowda, 1990), Daboiatoxin like factor (Maung-Maung-Thawin et al., 1995), platelet aggregation inhibitors (Prasad et al., 1996), hemorrhagins (Chakrabarty et al., 1993, 2000; Kole et al., 2000; Mukherjee, 2008; Chen et al., 2008), neurotoxic peptide (Shelke et al., 2002; Kumar and Gowda, 2006) , cytotoxin (Choudhury et al., 2006), a heat stable protein toxin drct-1 (Gomes et al., 2007), L-amino oxidase (Mandal and Bhattacharyya, 2008) and reprotoxin (Kumar et al., 2008).

Primarily, the *DR* bite disrupts hemostasis causing bleeding disorders such as co-agulopathy and cardio-respiratory failure, nephropathy, pituitary gland dysfunction and conjunctiva of organs such as lungs, kidneys, testis, heart, brain etc. Although renal failure is secondary to co-agulopathy, it appears to be the principal cause of death, while, hemorrhage is one of the major manifestations of *DR* bites. In addition to the fatal

systemic toxicity, *DR* bite cause glaring local effects such as severe edema, extensive hemorrhage and tissue destruction at the bite site.

Extracellular matrix (ECM) degrading zinc dependent hemorrhagic metalloprotease and hyaluronidase are considered to be the key factors responsible for the venom induced local toxicity. However, myotoxic $PLA_2$s of snake venoms and sphingomyelinase-D of spider venoms also cause local toxicity (Kasturi and Gowda 1989; Futrell, 1992). Metalloproteases degrade collagen and other ECM proteins including adhesive glycoprotein and hyaluronidase degrade the hyaluronan. In the recent past, a zinc dependent hemorrhagic metalloprotease complex (HC) was isolated and characterized and its role in the local toxicity was studied from the *DR* venom (Ushanandhini thesis, 2006). However, no study has been focused so far regarding the hyaluronidase enzyme and its possible role in venom induced pharmacological effects of *DR* venom.

Hyaluronan (HA) is a negatively charged, high molecular weight, non-sulphated, linear, acidic glycosaminoglycan (GAG) and found in the ECM of tissues and organs. It is composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine (β 1, 3) linked through (β 1, 4) glycosidic linkage. The negative charge binds metal ions and holds water molecules and provided the viscoelasticity to the ECM. HA interact with a variety of proteins and thus provides the mechanical support and proper defined structure to the ECM.

Hyaluronidase activity converts the long chain, linear megadalton hyaluronan into fragments of varied molecular size at the site of envenomation. The activity not only destroyed the structural integrity of ECM that results in severe morbidity (local toxicity) but also found to increase the diffusion rate of systemic toxins in to circulation that were otherwise diffused much slowly and this was achieved by reducing the viscosity or by increased permeability of ECM (Tu and Hendon, 1983; Girish et al., 2002, 2004(b); Girish and Kemparaju, 2006).

Further, hyaluronidase inhibition not only reduced the local tissue damage, but also prevents the rate of diffusion of systemic toxins and thus increased the survival time of the experimental mice (Girish and Kemparaju, 2006). Inhibition of hyaluronidase finds immense therapeutic value, especially in the treatment and management of venom induced local toxicity. This is highly significant in view of the existing anti-venom therapy being not effective or less effective against local tissue destruction. Thus the victim experience continued local toxicity (local tissue destruction) even after neutralization of systemic toxicity. Therefore, venom researchers are in quest of alternate therapy and the use of natural hyaluronidase inhibitors especially that are derived from the plant sources and synthetic inhibitors. Sodium cromoglycate and Sodium auro-thiomalate, specific inhibitors reduced the local tissue damage and prolong the survival time of mice injected with *Naja kaouthia* and *Calloselasma rhodostoma* venoms (Yingprasertchai et al., 2003). Similarly, Aristolochic acid effectively inhibited the hyaluronan degradation and increased the survival time of mice injected with *Naja naja* venom (Girish and Kemparaju, 2006). Therefore, in the present study an attempt was made to isolate and characterization of hyaluronidase (s) from *DR* venom and beneficial effects of its inhibition by gallic acid (GA) in the management of snakebite has been addressed.

## MATERIALS AND METHODS

Sephadex G-75, CM-Sephadex C-25, stains all, bovine testicular hyaluronidase, chondroitin sulphate (A, B and C), *N*-acetyl–D-glucosamine, *N*-acetyl-D-galactosamine, chondroitin sulphate D were from ICN chemicals (USA). Different molecular forms of chitosan were from Fluka (USA). VRV-PL-VIII myotoxin, a myotoxic phospholipase $A_2$ from Indian *DR* venom (purchased from Irula Snake Catchers, Chennai, India) was isolated according to the method of Kasturi and Gowda (1989). HC, a hemorrhagic metalloprotease from Indian *DR* venom (purchased from Hindustan snake park, Kolkata, India) was isolated according to the method of Ushanadini thesis submitted to University

of Mysore, Mysore (2006). All other chemicals used were of analytical grade. Further materials are described in Chapter II.

**Sephadex G-75 column chromatography**

The lyophilized *DR* venom 200 mg in 1.0 ml of 150 mM NaCl was applied on to a Sephadex G-75 column (1.5 X 116 cm) that was equilibrated with 150 mM NaCl. The column was eluted using 150 mM NaCl with a flow rate of 20 ml/h and 2 ml fractions were collected. Protein elution was monitored at 280 nm using Shimadzu spectrophotometer (1601A). Alternate tubes were assayed for hyaluronidase activity using hyaluronic acid as substrate. Fractions having enzyme activity were pooled and concentrated.

**CM-Sephadex-C-25 column chromatography**

Hyaluronidase fraction recovered from the previous step (65 mg in 1 ml of equilibrating buffer) was loaded onto a CM-Sephadex C-25 column (1.5 X 60 cm) that was equilibrated with 0.2 M sodium acetate buffer containing 50 mM NaCl (pH 5.0). Column was eluted stepwise using 0.2 M sodium acetate buffer of different pH (5.0-6.5) containing NaCl of 50-300 mM. Fractionation was carried out at room temperature at a flow rate of 15 ml/hr and 1.5 ml fractions were collected. Alternate tubes were assayed for hyaluronidase activity. Fractions with hyaluronidase activity were pooled and concentrated. Hyaluronidase peaks were designated as DRHyal-I and DRHyal-II respectively based on the sequence of their elution.

**Protein estimation**

Protein estimation was done as described in Chapter-II.

**Assay of hyaluronidase activity and inhibition by GA**

Hyaluronidase activity of DRHyal-II was assayed according to the method described in chapter-II. To determine the type of inhibition, DRHyal-II was pre-incubated with different concentrations of GA for 15 min. Reactions were started by adding different concentration of hyaluronan (50-400 μgs) in 300 μl of 0.2 M sodium acetate buffer pH 5.5 containing 150 mM NaCl at $37^0$C for 150 min. The change in absorbance was monitored at 585 nm. Activity was expressed as μ moles of *N*-acetyl-D- glucosamine released/2h 30 min at $37^0$C. Specific activity is expressed as units/min/mg protein.

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE was carried out according to the method of Laemmli (1970) under both reduced and non-reduced conditions. Resolving gel (10%) was prepared by mixing 1.67 ml of monomeric acrylamide solution (30g acrylamide, 0.8g N'-N' bisacrylamide made up to 100 ml with distilled water), 1.25 ml of 4X separation gel buffer (1.5 M Tris-HCl buffer pH 8.8), 0.1 ml of 10% SDS, 0.1 ml of 10% ammonium per sulfate (APS) and distilled water and made up to the volume of 5 ml. TEMED (20 μl) was added to the mixture. The contents were poured into a vertical slab gel plate to form 1 mm thick gel slab. Stacking gel (4.5%) was prepared by mixing 0.75 ml monomeric acrylamide solution, 1.25 ml 4X stacking gel buffer (0.5 M Tris-HCl buffer pH 6.8), 0.1 ml of 10% SDS, 0.1 ml of 10% APS and 2.89 ml of distilled water. TEMED (20 μl) was added to the mixture. The contents were overlaid on top of the resolving gel.

Molecular weight markers (14.4-200 kDa) and venom (75 μg) and of purified DRHyal-I (10 μg) and DRHyal-II (10 μg) samples, each prepared independently with equal volumes of reducing and non-reducing sample buffers (4% SDS, 2% Glycerol, with and without 10% β-mercaptoethanol in 0.125 M Tris-HCl buffer pH 6.8) and kept in boiling water bath for 3 to 5 min. The samples were then cooled to room temperature and a suitable amount of bromophenol blue was added as a tracking dye. The samples were loaded into each well and electrophoresis was carried out using Tris-Glycine buffer (0.25

M Tris and 0.192 M Glycine pH 8.3 containing 0.1% SDS) at a constant current of 100 volts for 2hr. The gels were stained for protein with 0.1% (w/v) Coomassie brilliant blue R-250 and destained using Acetic acid: ethanol: water (ratio of 30: 10: 60).

**pH and temperature kinetics and effect of NaCl on enzyme activity**

For determination of the optimum pH, 0.2 M buffers (sodium acetate [pH 4-6], sodium phosphate [pH 6-7] and Tris-HCl [pH 8-9] buffers) containing 150 mM NaCl were used. The temperature optimum was established by determining the activity at various temperatures (0-80$^0$ C) under standard assay conditions. Sodium acetate buffer (0.2 M, pH 5.5) containing various concentrations of NaCl (0-300 mM) were used to determine the effect of NaCl.

**Reversed phase –HPLC (RP-HPLC)**

RP-HPLC was performed using Vydac-$C_4$ column (5 μm, 0.21 x 25 cm) in Shimadzu 10 A*VP* HPLC system. The column had been pre-equilibrated with 0.1% TFA in water. It was eluted using linear gradient from solution A (0.1 % TFA in water) to 100 % solution B (0.1 %TFA in acetonitrile) for 40 min. The protein was eluted at a flow rate of 1 ml/min and monitored at 280 nm.

**Mass spectrometry**

The molecular mass was determined by mass spectrometry using Bruker Daltonics Matrix-Assisted Laser Desorption Ionization Time Of Flight (MALDI-TOF) machine in positive ionization mode. α-Cyano-4-hydroxycinnamic acid was used as MALDI matrix.

**Substrate specificity**

In a total reaction volume of 300 µl of 0.2 M sodium acetate buffer pH 5.5 containing 150 mM NaCl, 50 µg of different substrates and 10 µg of DRHyal-II were incubated separately for 1 h at $37^0$C. For activity determinations, the turbidometric method of Ferrente, (1956) was employed with chondroitin sulphate A-D and heparin and the decrease in O.D at 400 nm was recorded. The activity was expressed as turbidity reducing units. One unit of activity was expressed as the amount of enzyme required to hydrolyze 50% of the hyaluronan. *N*-acetyl-D-galactosamine was estimated by the method of Reissig et al., (1955) when chondroitin was used. One unit of activity was expressed as the amount of enzyme releasing 1 µmol of galactosamine in 2 h 30 min at $37^o$ C.

Chitosanase activity was assayed using different molecular forms of chitosans according to the method of Imato and Yagishita, (1971). One unit of activity was expressed as the amount of enzyme required to release 1µmol of glucosamine/h at $37^0$C. Similar experiments were performed for bovine testicular hyaluronidase using10 units of enzyme as control in all the cases.

**Spreading property of hyaluronidase**

Myotoxicity of VRV-PL-VIII myotoxin in the presence and in the absence of DRHyal-II was determined according to the method of Gutierrez et al., (1990) using four groups of mice (n=3). The following samples in a final volume of 50 µl saline were injected intramuscularly. The group I was injected with VRV-PL-VIII myotoxin alone. The group II includes five sub groups IIa, IIb, IIc, IId and IIe, they were injected with VRV-PL-VIII myotoxin and with different concentrations of DRHyal-II. The groups III and IV were injected with saline and DRHyal-II alone. Serum CK and LDH activities were determined according to the method described in chapter-II. For inhibition studies, DRHyal-II in presence of VRV-PL-VIII myotoxin was pre-incubated with different

concentrations of GA for 30 min at $37^0$C. In all the cases, lethal $dose_{25}$ ($LD_{25}$ dose) of VRV-PL-VIII myotoxin was injected unless otherwise indicated.

Hemorrhagic activity was determined according to the method of Kondo et al., (1960) using four groups of mice (n=3). The samples in a final volume of 50 µl saline were injected intradermally on the back of mice. The group I was injected with HC alone. The group II includes four sub groups IIa, IIb, IIc and IId, they were injected with HC and with different concentrations of DRHyal-II. The groups III and IV were injected with saline and DRHyal-II alone. Hemorrhagic activity was determined according to the method described in chapter-II. For inhibition studies, DRHyal-II in presence of HC was pre-incubated with different concentrations of GA for 30 min at 37 $^0$C. In all cases minimum hemorrhagic dose (2.5 µg) of HC toxin was injected unless otherwise indicated.

**Intrinsic fluorescence spectral studies**

The fluorescence emission of DRHyal-II in presence and in absence of GA was monitored using Shimadzu Spectrofluorophotometer RF-5301pc with quartz cuvette of 1 cm path length. The DRHyal-II was incubated with different concentrations of GA in 2 ml of 0.2 M Sodium acetate buffer, pH 5.5. The emission was recorded from 300 to 450 nm after excitation at 280 nm.

**Circular Dichroism spectral studies**

Far UV-CD spectra, DRHyal-II in a total reaction volume of 1 ml (0.2 M Sodium acetate buffer, pH 5.5) was obtained in absence or presence of GA ($IC_{50}$ concentration) using Jasco J715 spectropolarimeter. The spectra were recorded at room temperature between 190-250 nm using 1 cm path length quartz cuvette. The band width was 1nm and response time was 2 s. The final spectrum was a cumulative of three scans. The protein spectra were corrected by subtracting the blank solution containing 0.2 M Sodium acetate

buffer, pH 5.5. Secondary structure calculations from CD spectra obtained were determined by using k2d software (www.embl-heidelberg.de/-andrade/k2dfaq.html).

**Statistical analysis**

All the experiments were repeated for three independent observations. The data are presented as mean ± SD using SPSS (Version 11.5) software.

## RESULTS

### Purification of hyaluronidase isoforms

A two-step protocol was standardized for the isolation of isoforms of hyaluronidase DRHyal-I and DRHyal-II. The first step involved was the Sephadex G-75 gel filtration column chromatography, which fractionated *DR* venom into four peaks (Fig. 3.1a). Fractions with hyaluronidase activity (dotted line) were pooled and concentrated. About 99.7 % of the activity and 65 % of the protein loaded onto the column were recovered. The second step was the CM-Sephadex C-25 column chromatography, eight peaks were resolved (Fig. 3.1b). Of the eight peaks, fifth and seventh peaks showed the hyaluronidase activity. About 8.9 % of the activity and 0.7 % of the protein loaded were recovered in the fifth peak while, 32.3 % of the activity and 1.5 % of the protein loaded were recovered in the seventh peak. Based on the elution sequence in the CM-Sephadex C-25 column chromatography, the hyaluronidase isoforms resolved were designated as DRHyal-I and DRHyal-II respectively. The DRHyal-I and DRHyal-II were purified to the extent of about ~12.8 and ~21.6 folds respectively. A summary of the purification is given in table 3.1.

### Properties of DRHyal-I

The purified DRHyal-I (Fig. 3.2a) moved as a sharp band to the same extent in SDS-PAGE under both reduced and non-reduced conditions. Similar purity of DRHyal-I was also suggested by activity staining where a single translucent activity band was seen

(Fig. 3.2b). The apparent calculated mass of DRHyal-I was found to be 98 kDa in SDS-PAGE (Fig. 3.2a). It is optimally active at pH 5.5 and at $37^0$C (Fig. 3.3a). Activity was abolished below pH 4.0 and above pH 8.0 and at temperatures $>60^0$ C (Fig. 3.3b). DRHyal-I was found to be stable for more than fifteen days in assay buffer at $37^0$ C. Activity was greatly influenced by NaCl and optimum concentration was found to be 150 mM (Fig. 3.3c) while, concentration above 300 mM was inhibitory. Due to low yield of DRHyal-I (0.7%), this was not subjected for further biochemical and pathological investigations including the spreading activity. However, DRHyal-II was subjected for detailed investigation.

**Properties of DRHyal-II**

The purified DRHyal-II (Fig. 3.4a) moved as a sharp band to the same extent in SDS-PAGE under both reduced and non-reduced conditions. It did not show the translucent activity band in hyaluronan zymogram assay (Fig. 3.4b). DRHyal-II was eluted as a sharp peak with the retention time of 5 min (Fig. 3.5) in reversed phase HPLC on a $C_4$ column. It revealed a single symmetrical sharp peak at m/e of 28.3 in MALDI-TOF mass spectrometry (Fig. 3.6) while, the apparent calculated mass was found to be 31 kDa in SDS-PAGE (Fig. 3.4a). DRHyal-II also found to be optimally active at pH 5.5 and at $37^0$C (Fig. 3.7a). Activity was abolished below pH 4.0 and above pH 8.0 and at temperatures $>60^0$ C (Fig. 3.7b). It was found to be stable for more than fifteen days in assay buffer at $37^0$ C. Activity was greatly enhanced by NaCl and optimally active at 150 mM concentration (Fig. 3.7c) while, concentrations above 300 mM were inhibitory.

For the substrate specificity, the DRHyal-II was compared with the bovine testicular hyaluronidase, as the bovine enzyme is the most extensively studied of this class of enzymes. DRHyal-II was found to be highly specific for hyaluronan alone, showing no activity against other glycosaminoglycans (GAGs) or different forms of chitosan. In contrast, bovine testicular hyaluronidase cleaved several of the GAGs and chitosans tested (table. 3.2).

The DRHyal-II increased dose dependently the myotoxicity of VRV-PL-VIII myotoxin when co-injected and elevated levels of serum CK and LDH activities were seen and reached optimum at DRHyal-II concentration of VRV-PL-VIII myotoxin: DRHyal-II ratio of 1: 0.3 (w/w). At optimum concentration, CK and LDH activities increased about 2.4 and 1.6 folds respectively compared to the level when injected VRV-PL-VIII myotoxin alone, while DRHyal-II injected mice revealed the values that are similar to saline injected control mice (Fig. 3.8). Similarly, DRHyal-II when co-injected increased the hemorrhagic response of a HC (a hemorrhagic metalloenzyme complex from *DR* venom). At 1: 4 ratio (w/w) of HC and DRHyal-II, a violent hemorrhage was observed and there observed about 2.7 folds increased response (Fig. 3.9). The diameters of the hemorrhagic spots were measured and the quantitative results are presented in table 3.3.

Gallic acid (GA), 3, 4, 5-trihydroxy benzoic acid an abundant polyphenol present in the seeds of *V. vinifera* L. inhibited the activity of DRHyal-II. GA was assessed for its purity before the inhibition studies. It eluted as a sharp symmetric peak on a C18 column (Fig. 3.10a) and (Fig. 3.10b) indicate the structure of GA. The GA caused DRHyal-II inhibition. The inhibition was found to be dose dependent (Fig. 3.11a) and the activity was abolished at 0.8 mM and the $IC_{50}$ value determined was 0.4 mM. In addition, GA also abolished the activity of *DR* venom dose dependently. The activity of *DR* venom was abolished at 20 mM (Fig. 3.11b). The $IC_{50}$ value determined was found to be 9 mM. GA inhibited DRHyal-II uncompetitively as revealed by Line Weaver-Burk plot (Fig. 3.12). GA revealed a weak fluorescence emission maximum between 340 to 450 nm, while DRHyal-II showed prominent emission maximum between 320 to 330 nm when excited independently at 280 nm. In presence of GA, the DRHyal-II showed much enhanced fluorescence emission and further, the fluorescence emission maximum was drastically shifted from 320 to 330 nm to 370 to 380 nm. The enhanced fluorescence emission was found to be dose dependent (Fig. 3.13).

Further, the interaction between DRHyal-II and GA was substantiated by circular dichroism (CD) interaction studies. Far UV-CD spectrum of DRHyal-II showed the characteristic prominent single large negative band between 190 to 220 nm. In presence of $IC_{50}$ concentration of GA, the negative band absorbance was diminished and the peak height was drastically reduced compared to the native DRHyal-II band (Fig. 3.14). Change in the percentage of secondary structures of DRHayl-II upon interaction with GA is summarized in table 3.4.

Further, the GA abolished the spreading property of DRHyal-II. The group of mice that received VRV-PL-VIII myotoxin ($LD_{25}$ dose) along with the DRHyal-II (VRV-PL-VIII myotoxin: DRHyal-II ratio 1: 0.3, w/w) that was pre-incubated with GA recorded the serum CK and LDH activities similar to VRV-PL-VIII myotoxin injected control mice. The serum CK and LDH activities of groups of mice that were injected independently with DRHyal-II (40 µg) and 26 mM of GA alone were in good agreement with the values of saline injected control mice (Fig. 3.15). Further, the inhibition of spreading property of DRHyal-II was supported using the HC and GA. The increased hemorrhagic response of HC in presence of DRHyal-II was inhibited when used DRHyal-II that was preincubated with GA (Fig. 3.16). The diameters of the hemorrhagic spots were measured and the results are presented in table 3.5.

**DISCUSSION**

The pathology of *DR* venom induced local tissue destruction and the spreading effect of locally acting enzymes and toxins are currently gaining importance. The *DR* venom induced local and systemic toxic effects are due to the synergistic effects of several toxins. Extra cellular matrix (ECM) degrading hyaluronidase(s) and hemorrhagic metalloprotease(s) are collectively referred to as “spreading factors” and these two groups of hydrolytic enzymes appears to be the major local tissue destructing agents (Xu et al., 1982; Baramova et al., 1989; Maruyama et al., 1992; Anai et al., 2002; Girish et al., 2002; Girish and Kemparaju, 2006).

Successive two-step fractionation of Indian *DR* venom through gel filtration on a Sephadex G-75 column and cation exchange chromatography on a CM-Sephadex C-25 column resulted in purification of two isoforms of hyaluronidases the DRHyal-I and DRHyal-II. DRHyal-I was purified to the extent of 12.8 folds while; DRHyal-II was purified to the extent of 21.6 folds. The final protein and activity yield of DRHyal-I was 0.7% and 8.9% while; DRHyal-II was 1.5% and 32.2% respectively.

The purity of DRHyal-I and DRHyal-II was adjudged by SDS-PAGE. Appearance of single band under both reduced and non-reduced conditions suggests the monomeric nature. The apparent molecular mass of DRHyal-I and DRHyal-II was found to be 98 kDa and 31 kDa in SDS-PAGE respectively. The DRHyal-I showed single translucent activity band in hyaluronan zymogram while, DRHyal-II did not, suggesting that DRHyal-I was insensitive while DRHyal-II was sensitive to SDS. Further, the purity of DRHyal-II was ratified by the appearance of single symmetric peak in reversed phase HPLC on a C4 column and also by MALDI-TOF mass spectrometry. The molecular mass of DRHyal-II determined by MALDI-TOF was found to be 28.3 kDa. The determined molecular mass of both DRHyal-I and DRHyal-II is well within the reported range of 28 kDa to 110 kDa for several hyaluronidases studied from the venoms of snakes, bees, scorpions, stonefish, lizards and mammalian enzymes (Xu et al., 1982; Tu and Hendon, 1983; Kemeny et al., 1984; Ramanaiah et al., 1990; Poh et al., 1992; Frost et al., 1996; Kudo and Tu, 2001; Girish and Kemparaju, 2006).

Based on the catalytic mechanism, hyaluronidases are classified into three groups type-I, type-II and type-III (Menzel and Farr, 1998). Snake venom hyaluronidases are belong to type-I enzymes, however; hyaluronidases are also loosely classified as acid active (active between pH 3 and 4) or neutral active (active between pH 5 and 6) enzymes (Frost et al., 1996). Being optimally active at pH 5.5, both DRHyal-I and DRHyal-II belongs to the neutral active class of enzymes. Since DRHyal-I is a high molecular weight enzyme, it appears to share the properties with the reported high molecular mass venom hyaluronidases, in addition, the yield of DRHyal-I was very low, and hence no

detailed investigation was undertaken on DRHyal-I. In contrast, DRHyal-II being a low molecular mass enzyme and low molecular mass hyaluronidases are less studied from snake venoms and since the yield was little high, DRHyal-II was used for further investigations. DRHyal-II exhibited absolute specificity for hyaluronan, compared to bovine testicular hyaluronidase, which degraded relatively a broad spectrum of substrates such as chondroitin, chondroitin sulphates A, C and D and different molecular forms of chitosans.

Unlike other enzymes and toxins of snake venoms, hyaluronidases are least toxic or most likely they lack visible toxicity and were considered as simple spreading factors. Nevertheless, the spreading property is now gained impact due to its toxicity enhancing property (Girish et al., 2002; Girish and Kemparaju, 2006). The spreading property is basically due to degradation of the glycosaminoglycans (GAGs) especially, the hyaluronan present in the ECM of tissues. This not only increases the permeability of systemic toxins into circulation there by increasing the toxicity but also leads to morbidity due to the damage caused at the envenomed region. Thus, inhibition of spreading property has dual benefits, minimizing or prevention of local tissue damage and an eventual increased survival rate due to retarded rate of toxins diffusion. Inhibition of spreading property has been addressed using GA and a myotoxic $PLA_2$ the VRV-PL-VIII myotoxin and hemorrhagic metalloproteases complex the HC, both were the isolated components from ***DR*** venom. The increased myotoxicity as evidenced by increased levels of cytoplasmic marker enzymes CK and LDH activities of VRV-PL-VIII myotoxin in presence of DRHyal-II and normal response of CK and LDH levels due to GA inhibition of DRHyal-II suggests the inhibition of spreading activity and associated enhanced/potentiated toxicity. Similar effect was observed with the hemorrhage induced by the HC.

Inhibition of spreading property, perhaps directly influence the local toxicity of snakebite, especially the severe edema, hemorrhage, myonecrosis and continued tissue destruction during Viper bites. This has been also evidenced by the inhibition of

hemorrhagic activity of *DR* venom (please see page number, 113). However, GA might also inhibit the factors that induce hemorrhage). Inhibition of spreading property gained much impact due to the inability of anti-venom therapy to offer protection against local toxicity. GA effectively inhibited DRHyal-II than *DR* venom as evidenced by the $IC_{50}$ values. In *DR* venom multiple toxins may be competing simultaneously hence higher concentration of GA is required to achieve complete inhibition. GA appears to bind DRHyal-II to the site other than the substrate-binding site as suggested by uncompetitive type of inhibition.

In this study, our findings clearly suggested that, the inhibition of hyaluronidase activity by GA is due to its specific binding to DRHyal-II. The binding of GA resulted in significant changes in the structure of DRHyal-II, suggesting the formation of DRHyal-II -GA complex. This was evident from the GA induced increased fluorescence emission of DRHyal-II. This was due to the possible exposure of more number of tryptophan residues that were hidden in the folded structure of native DRHyal-II. Further, the interaction between DRHyal-II and GA was confirmed by CD studies. The α-helical and β-sheet content of DRHyal-II vary in presence and in absence of GA. The α-helical content increased while the β-sheet content decreased upon binding GA. Presence of high percentage of β-sheet over α-helix suggests the high stability of DRHyal-II as high content of β-sheet known to augment the stability. GA appears to alter both secondary and as well as the tertiary structure of DRHyal-II. A drastic variation in the fluorescence emission response, and α-helical and β-sheet contents in presence and in absence of GA, strongly suggests the change in the tertiary and secondary structures respectively.

In conclusion, hyaluronidases play a key role in the pathophysiology of both local and systemic complications of snake envenomation and hence, it is a highly promising target not only in the management of local toxicity but perhaps in the management of systemic toxicity of snakebites as well. Also this study offers scope for the development of potent generics of GA that might contribute for the better management of snakebite pathology.

**Table. 3.1: Summary of the purification of DRHyal-I and DRHyal-II from *DR* venom.**

| **Procedure** | **Total protein (mg)** | **Protein recovery (%)** | **Specific activity (Units/mg/min)** | **Total activity (Units)** | **Activity yield (%)** | **Fold purity** |
|---|---|---|---|---|---|---|
| **Sephadex G-75 column chromatography** | | | | | | |
| Whole venom | 200 | 100 | 0.39 | 78.0 | 100 | 1 |
| Sephadex G-75 column | 65 | 32.5 | 1.2 | 78.0 | 99.7 | 3.0 |
| **CM-Sephadex C-25 column chromatography** | | | | | | |
| CM-Sephadex C-25 column Peak -V (DRHyal-I) | 1.4 | 0.7 | 5 | 7 | 8.9 | 12.8 |
| CM-Sephadex C-25column Peak-VII (DRHyal-II) | 3 | 1.5 | 8.44 | 25.3 | 32.3 | 21.6 |

- One unit of activity is expressed as the μmoles of *N*-acetyl-D-glucosamine released /min/mg of protein at $37^{O}$ C.

**Table. 3.2: Substrate specificity of DRHyal-II and Bovine testicular hyaluronidase.**

| **Substrate** | **DRHyal-II** | **Bovine testicular hyaluronidase** |
|---|---|---|
| Hyaluronan | + | + |
| Chondroitin | -- | + |
| Chondroitin sulphate A | -- | + |
| Chondroitin sulphate B | -- | -- |
| Chondroitin sulphate C | -- | + |
| Chondroitin sulphate D | -- | + |
| Heparin | -- | -- |
| Chitosan (high molecular weight) | -- | + |
| Chitosan (medium molecular weight) | -- | + |
| Chitosan (low molecular weight) | -- | + |

+ hydrolyzed.

-- No hydrolysis.

**Table. 3.3: Hemorrhagic activity of HC in presence and absence of DRHyal-II (Quantitative data).**

| **Groups (n=3),** w/w | **Hemorrhagic spot ($mm^2$)** |
|---|---|
| HC (2.5 µg) alone | 10 ± 1.5 |
| HC (2.5 µg) + DRHyal-II (2.5 µg), 1 : 1 | 14 ± 3 |
| HC (2.5 µg) + DRHyal-II (5 µg), 1: 2 | 20 ± 2 |
| HC (2.5 µg) + DRHyal-II (7.5 µg), 1 : 3 | 25 ± 2 |
| HC (2.5 µg) + DRHyal-II (10 µg), 1 : 4 | 27 ± 3 |
| Saline | 0 |
| DRHyal-II (10 µg) alone | 0 |

MHD of HC (2.5 µg) and different concentrations of DRHyal-II were injected intradermally into the back of mice (n=3) in a total volume of 50 µl saline. After 3 h, mice were anesthetized and sacrificed. The dorsal patch of the skin surface was removed and the diameters of hemorrhagic spots were measured in $mm^2$. The values represent as mean ± SEM (n = 3).

**Table. 3.4: Effect of GA on the secondary structures of DRHyal-II.**

| **Types of secondary structures** | **DRHyal-II** | **DRHyal-II + GA ($IC_{50}$)** |
|---|---|---|
| α-Helix (%) | 1.76 | 11.56 |
| β-sheet (%) | 47.42 | 28.35 |

**Table. 3.5: Inhibition of hemorrhagic activity of HC in presence and absence of DRHyal-II by GA (Quantitative data).**

| **Groups (n=3), w/w** | **GA (mM)** | **Hemorrhagic spot ($mm^2$)** |
|---|---|---|
| HC (2.5 μg) + DRHyal-II (5 μg), 1: 2 | ------- | 20 ± 2 |
| HC (2.5 μg) + DRHyal-II (5 μg), 1: 2 | 3 | 19 ± 3 |
| HC (2.5 μg) + DRHyal-II (5 μg), 1: 2 | 6 | 14 ± 1.5 |
| HC (2.5 μg) + DRHyal-II (5 μg), 1: 2 | 12 | 10 ± 2 |
| HC (2.5 μg) + DRHyal-II (5 μg), 1: 2 | 18 | 4 ± 1 |
| HC (2.5 μg) + DRHyal-II (5 μg), 1: 2 | 24 | 0 |
| Saline | | 0 |
| GA (24 mM) alone | | 0 |

MHD of HC (2.5 μg) plus DRHyal-II (5 μg) were pre-incubated with the different concentrations of GA for 30 min at $37^0$ C and injected intradermally into the back of mice (n=3) in a total volume of 50 μl saline. After 3 h, mice were anesthetized and sacrificed. The dorsal patch of the skin surface was removed and the diameter of hemorrhagic spots was measured in $mm^2$. The values represented as mean ± SEM (n=3).

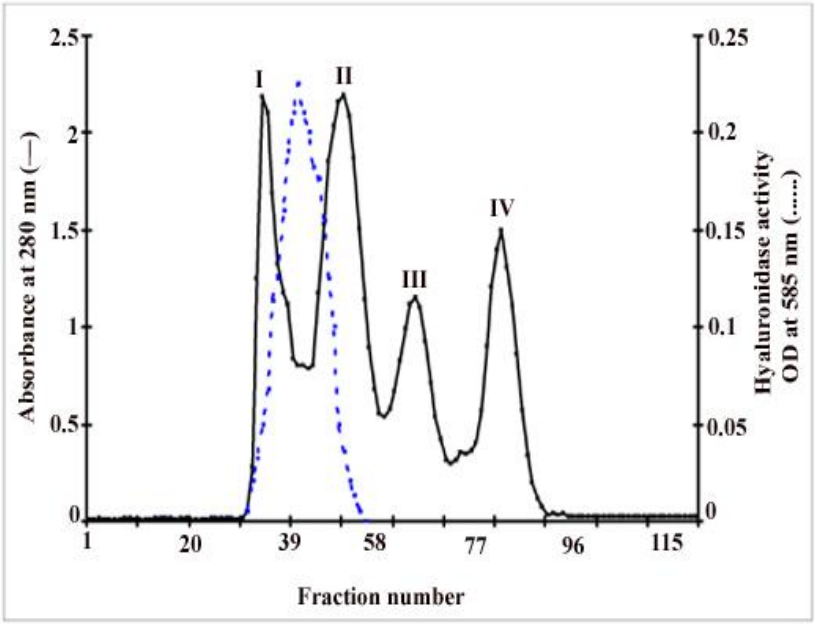


**Fig. 3.1a: Elution profile from Sephadex G-75 column chromatography.** The column (1.5 X 116 cm) was equilibrated and eluted with 150 mM NaCl at a flow rate of 20 ml/hr and 2 ml fractions were collected. Protein elution was monitored at 280 nm (—). Alternate fractions were assayed for hyaluronidase activity at 585 nm (----). Fractions having the hyaluronidase activity (dotted line) were pooled, concentrated and applied onto CM–Sephadex C-25 column for further fractionation. Chromatogram is cumulative of three independent trials.

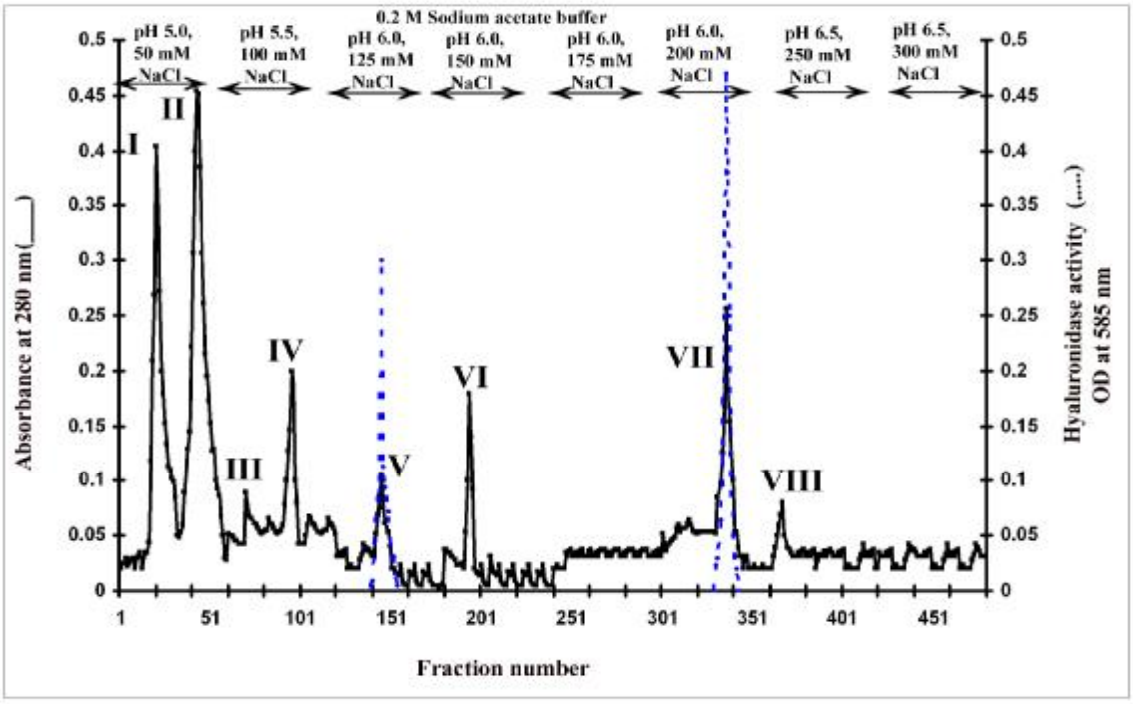


**Fig. 3.1b: Elution profile from CM-Sephadex C-25 column chromatography.** The column (1.5 X 60 cm) was equilibrated with 0.2 M sodium acetate buffer (pH 5.0) containing 50 mM NaCl. Sub fractions were eluted stepwise using 0.2 M sodium acetate buffers of different pH (5-6.5) containing NaCl of varied molarities (50-300 mM). Protein elution was monitored at 280 nm (—). Alternate fractions were assayed for hyaluronidase activity at 585 nm (----). Fractions having the hyaluronidase activity (dotted line) were pooled and concentrated. Chromatogram is cumulative of three independent trials.

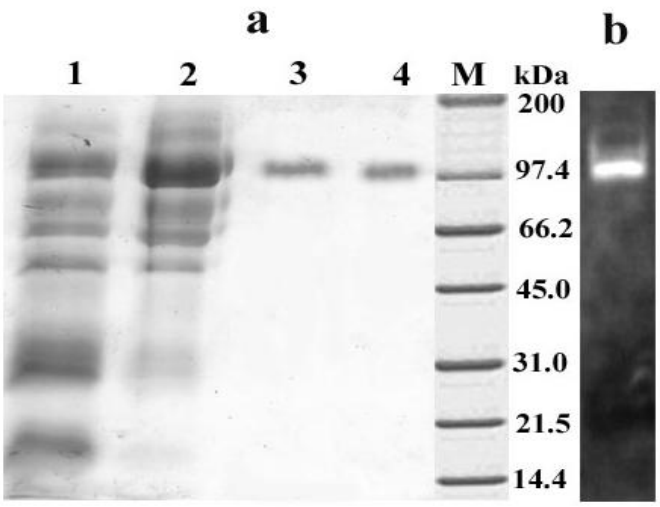


**Fig. 3.2a: SDS-PAGE pattern of purified hyaluronidase DRHyal-I.** Samples resolved by SDS-PAGE (10%) containing 75 µg of *DR* venom (lane 1), 50 µg of Sephadex G-75 column fraction (lane 2), 10 µg of DRHyal-I under non-reduced (lane 3) and reduced (lane 4) conditions. M represents the molecular weight markers in kDa [from top to bottom: myosin-H-chain (200.0), phosphorylase b (97.4), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), soya bean trypsin inhibitor (21.5) and lysozyme (14.4)].

**3.2b: Pattern of enzyme activity (zymogram assay).** DRHyal-I (2 µg) resolved by SDS-PAGE (10%) containing 0.17 mg/ml of hyaluronic acid under non-reduced condition and electrophoresis was done as described in the methodology.

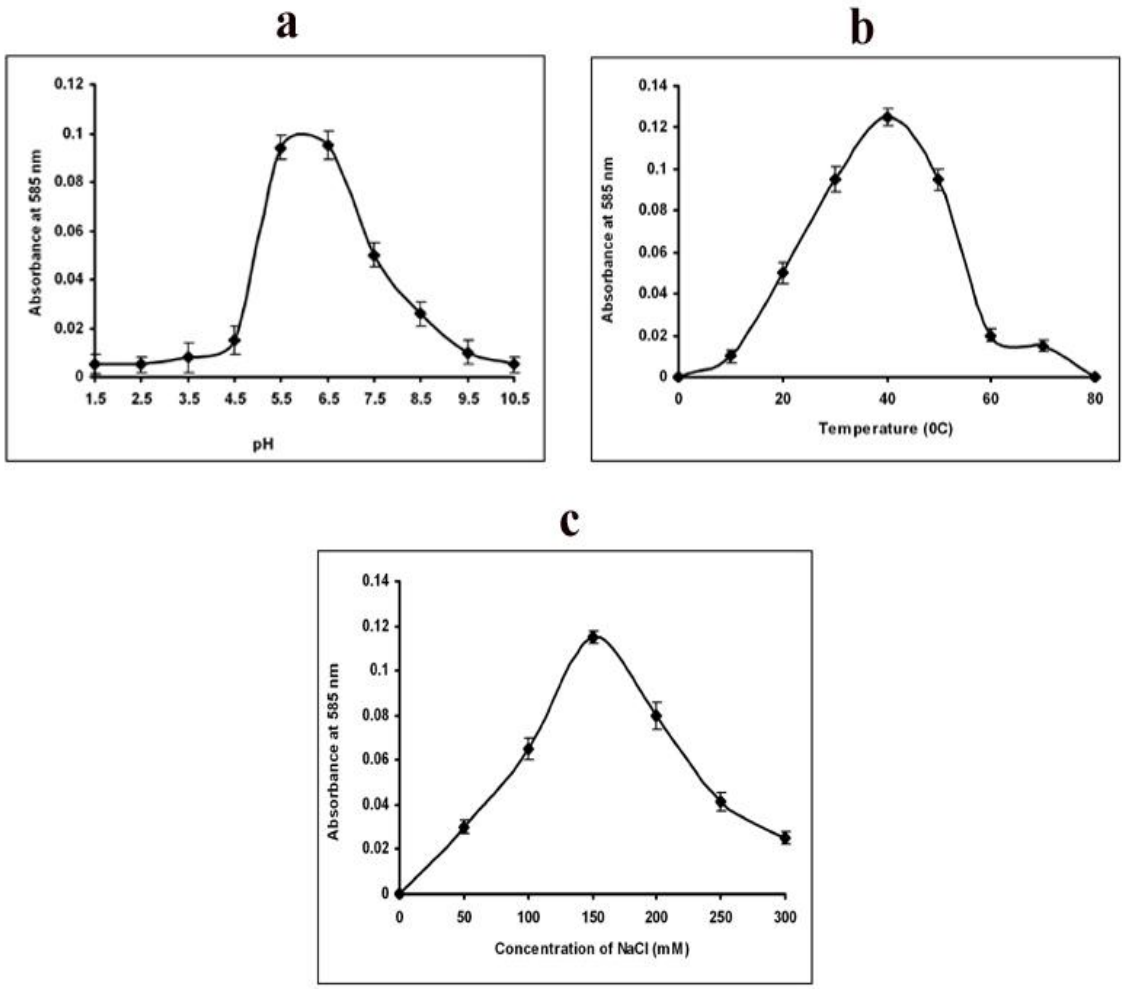


**Fig. 3.3**: **Effect of pH (a), temperature (b) and NaCl (c) profiles of DRHyal-I**

**a.** DRHyal-I (10 µg) was incubated independently with hyaluronic acid (50 µg) in a final reaction volume of 0.3 ml of 0.2 M buffers (sodium acetate [pH 4-6], sodium phosphate [pH 6-7] and Tris-HCl [pH 8-9] buffers) containing 150 mM NaCl for 2 h 30 min at $37^0$C. **b.** DRHyal-I (10 µg) was incubated independently with hyaluronic acid (50 µg) in a final reaction volume of 0.3 ml of sodium acetate buffer (0.2 M, pH 5.5) containing 150 mM NaCl for 2 h 30 at various temperatures (0-$80^0$ C). **c.** DRHyal-I (10 µg) was incubated independently with hyaluronic acid (50 µg) in a final reaction volume of 0.3 ml of Sodium acetate buffer (0.2 M, pH 5.5) containing various concentrations of NaCl (0-300 mM) were used. The hyaluronidase activity was carried out as described in the methodology section. The values represent as mean ± SEM (n = 3).

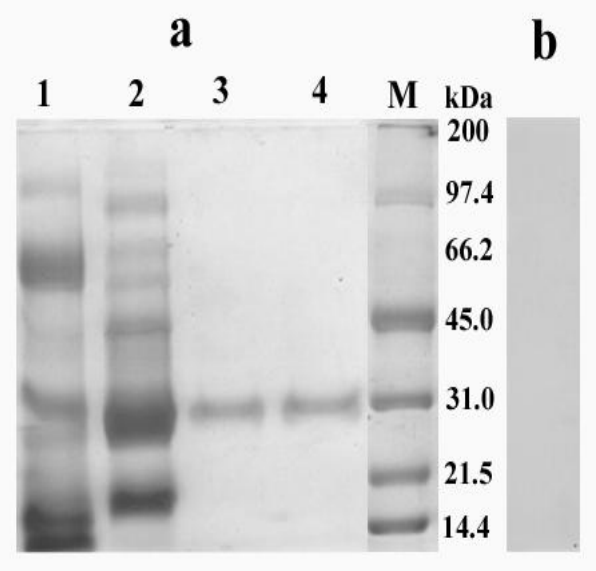


**Fig. 3.4a: SDS-PAGE pattern of purified hyaluronidase DRHyal-II.** Samples resolved by SDS-PAGE (10%) containing 75 µg *DR* venom (lane 1), 50 µg Sephadex G-75 column fraction (lane 2), 10 µg DRHyal-II under non-reduced (lane 3) and reduced (lane 4) conditions. M represents the molecular weight markers in kDa [from top to bottom: myosin-H-chain (200.0), phosphorylase b (97.4), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), soya bean trypsin inhibitor (21.5) and lysozyme (14.4)].

**3.4b: Pattern of enzyme activity (zymogram assay).** DRHyal-II (2 µg) resolved by SDS-PAGE (10%) containing 0.17 mg/ml of hyaluronic acid under non-reduced condition and electrophoresis was done as described in the methodology.

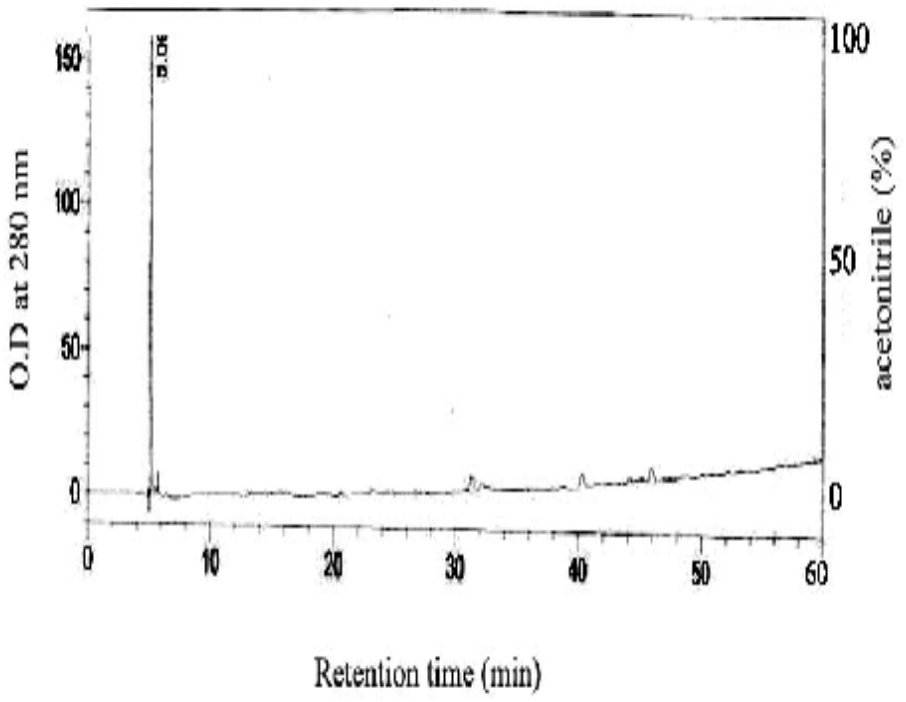


**Fig. 3.5: RP-HPLC profile of DRHyal-II.** DRHyal-II from the CM-Sephadex C-25 column was subjected to RP-HPLC using Vydac C4 column (5 µm, 0.21 x 25 cm), that had been equilibrated with 0.1% TFA in water. Protein was eluted using linear gradient from solution A (0.1% TFA in water) to 100% solution B (0.1% TFA in acetonitrile) over 60 min. Protein was eluted at a flow rate of 1 ml/min and monitored at 280 nm.

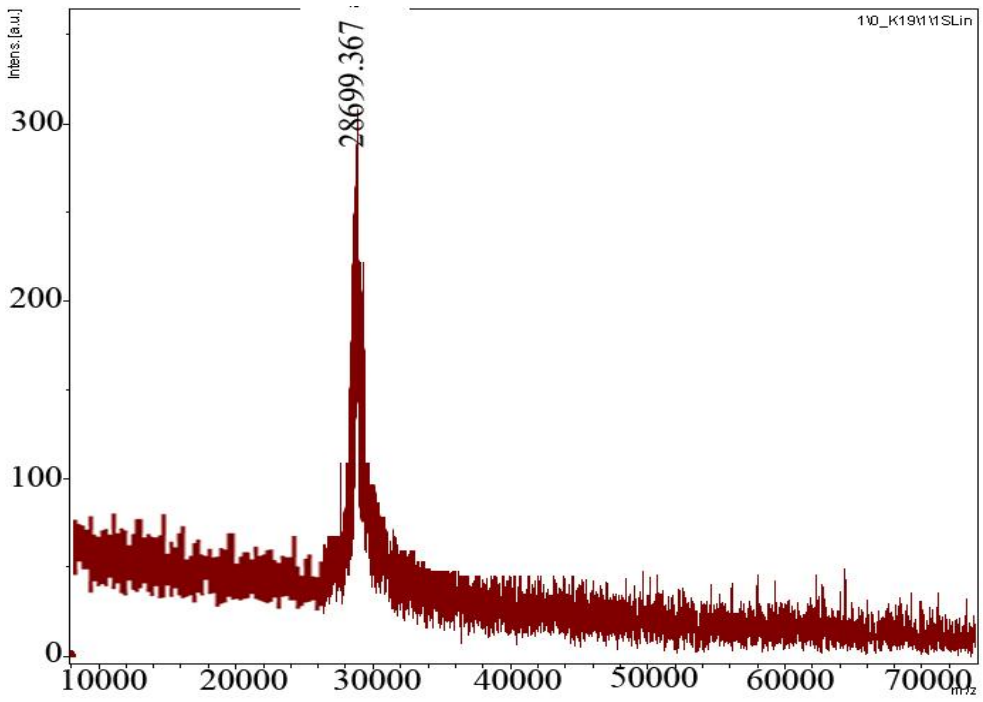


**Fig. 3.6: MALDI-TOF Mass spectrometry of DRHyal-II.** DRHyal-II from the CM-Sephadex C-25 column was subjected to Bruker Daltonics Matrix-Assisted Laser Desorption Ionization Time Of Flight (MALDI-TOF) machine in positive ionization mode. α-Cyano-4-hydroxycinnamic acid was used as MALDI matrix.

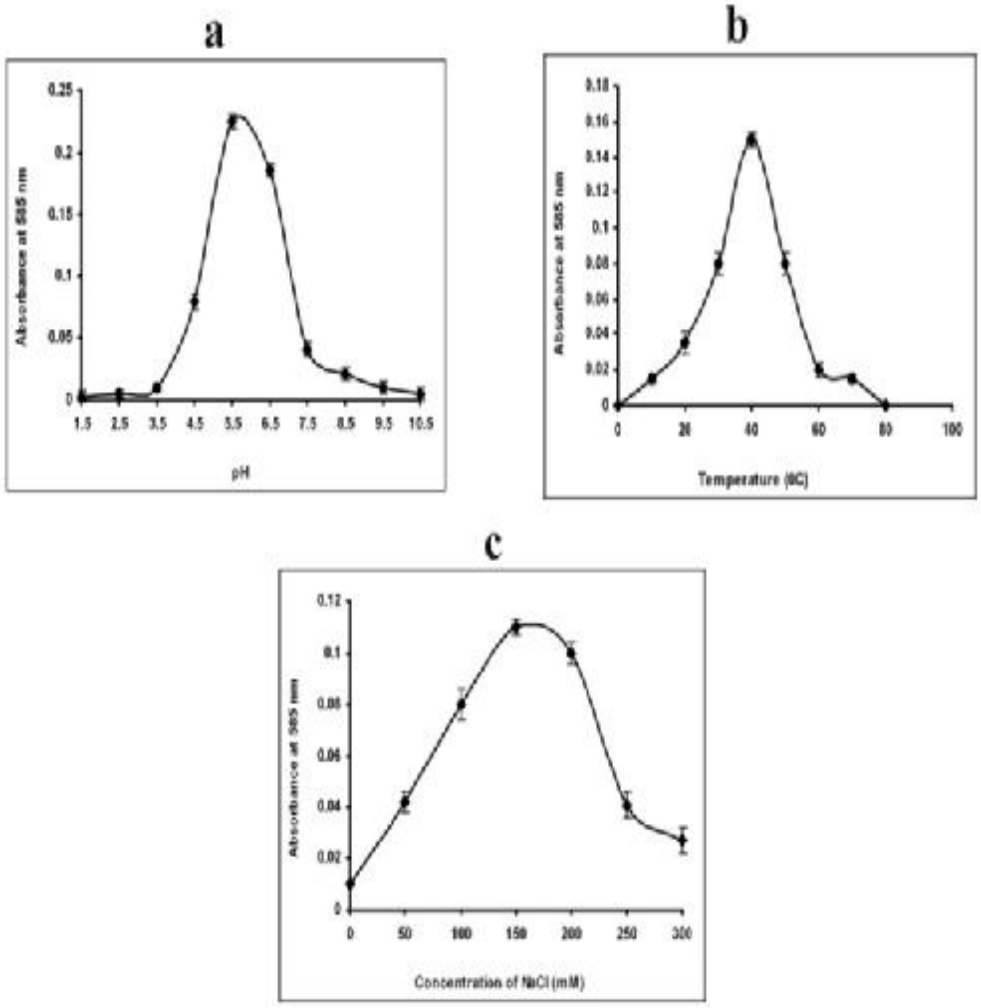


**Fig. 3.7**: **Effect of pH (a), temperature (b) and NaCl (c) profiles of DRHyal-II**

**a.** DRHyal-II (10 µg) was incubated independently with hyaluronic acid (50 µg) in a final reaction volume of 0.3 ml of 0.2 M buffers (sodium acetate [pH 4-6], sodium phosphate [pH 6-7] and Tris-HCl [pH 8-9] buffers) containing 150 mM NaCl for 2 h 30 min at $37^0$C. **b.** DRHyal-II (10 µg) was incubated independently with hyaluronic acid (50 µg) in a final reaction volume of 0.3 ml of sodium acetate buffer (0.2 M, pH 5.5) containing 150 mM NaCl for 2 h 30 at various temperatures (0-$80^0$ C). **c.** DRHyal-II (10 µg) was incubated independently with hyaluronic acid (50 µg) in a final reaction volume of 0.3 ml of Sodium acetate buffer (0.2 M, pH 5.5) containing various concentrations of NaCl (0-300 mM) were used. The hyaluronidase activity was carried out as described in the methodology section. The values represent as mean ± SEM (n = 3).

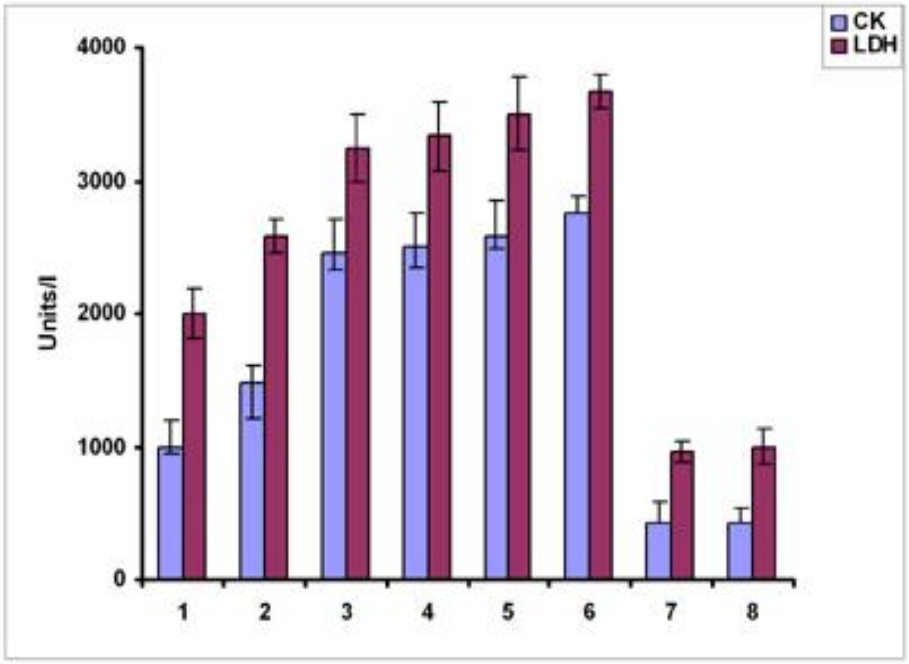


**Fig. 3.8: Dose dependence of DRHyal-II on the myotoxicity of VRV-PL-VIII myotoxin.** VRV-PL-VIII myotoxin ($LD_{25}$ dose) plus DRHyal-II in different ratios (myotoxin: DRHyal-II; 1: 0.15, 1: 0.30, 1: 0.45, 1: 0.60 and 1: 0.75) was injected in a final volume of 50 µl saline intramuscularly in to the right thigh of mice. Mice were anesthetized by ether inhalation, after 3 h blood was drawn from the abdominal vena cava and serum CK and LDH levels were determined as described in methodology section. Samples injected are VRV-PL-VIII ($LD_{25}$ dose) (1), VRV-PL-VIII ($LD_{25}$ dose) + DRHyal-II (w/w) 1: 0.15 (2), 1: 0.30 (3), 1: 0.45 (4), 1: 0.6 (5) and 1: 0.75 (6), saline (7) and DRHyal-II alone (40 µg) (8). The values represented as mean ± SEM (n=3).

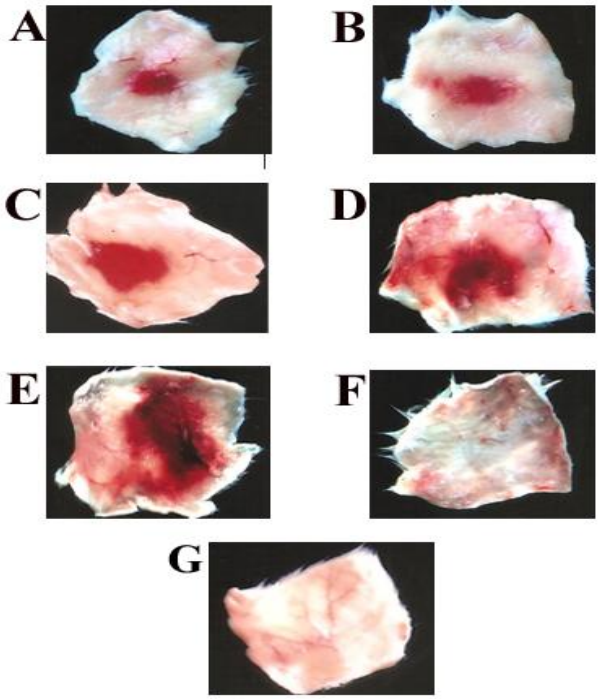


**Fig. 3.9: Hemorrhagic activity of HC in the presence of DRHyal-II.** MHD of HC (2.5 µg) and different concentrations of DRHyal-II were injected intradermally into the back of mice (n=3) in a total volume of 50 µl saline. After 3 h, mice were anesthetized and sacrificed. The dorsal patch of the skin surface was removed and the diameters of hemorrhagic spots were measured in $mm^2$. HC (2.5 µg) alone (A), HC + DRHyal-II (w/w); 1: 1 (B), 1: 2 (C), 1: 3 (D) and 1: 4 (E), saline (F) and DRHyal-II (10 µg) alone (G) were injected.

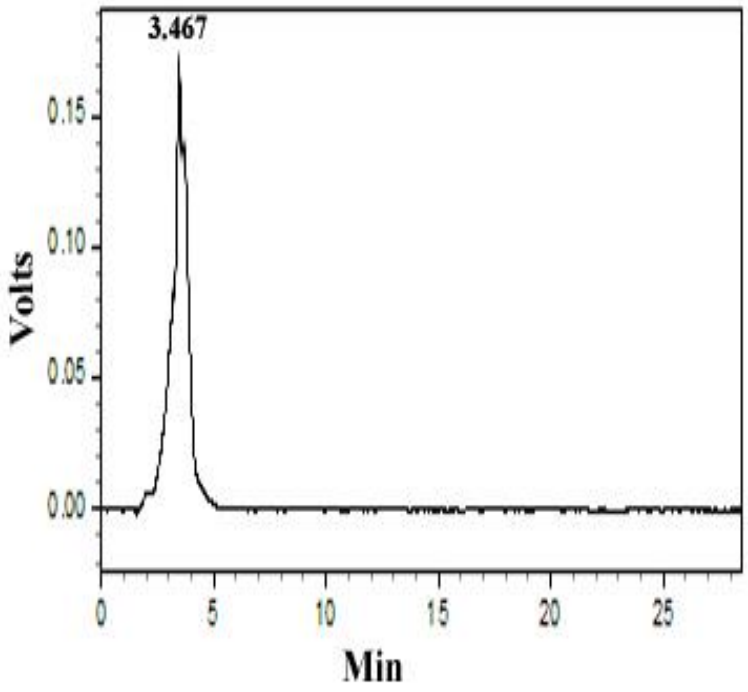


**Fig. 3.10a: RP-HPLC profile of gallic acid (GA).** GA (10 µg) in 20 µl of water was applied on to a Waters C-18 column. Column was eluted using water: methanol: acetic acid (70: 27: 3, v/v) solvent system.

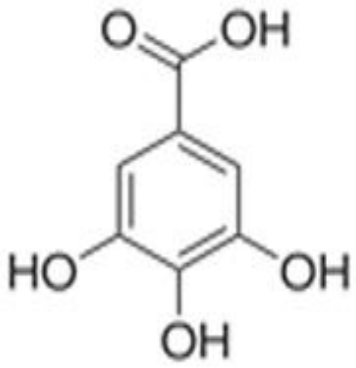


**Fig. 3.10b: Structure of gallic acid (GA).**

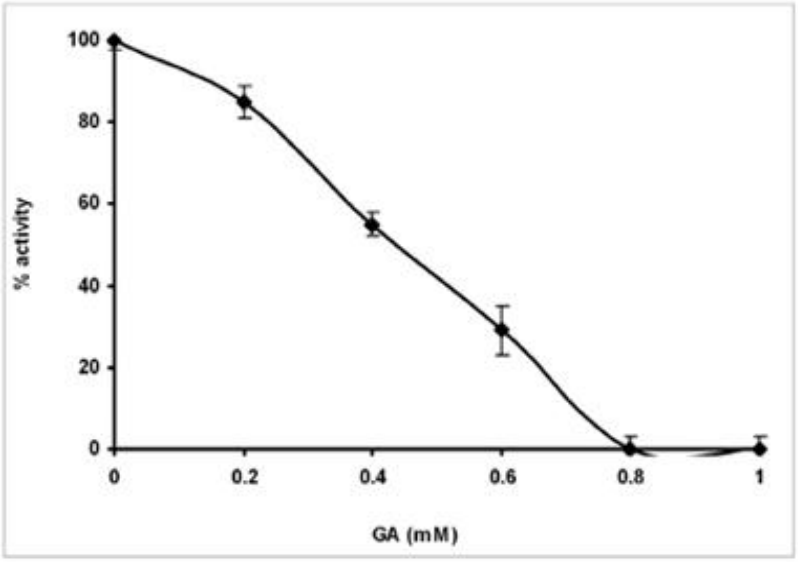


**Fig. 3.11a: Inhibition of hyaluronidase activity of DRHyal-II by GA**. DRHyal-II (10 µg) was pre-incubated for 15 min with different concentrations of GA (0.2, 0.4, 0.6, 0.8 and 1mM). The reaction was initiated by adding 50 µg of HA in 0.2 M sodium acetate buffer pH 5.5 with 150 mM NaCl and incubated for 2 h 30 min at $37^0$ C. The activity was determined as described in the methodology section. The values represented as mean ± SEM (n=3).

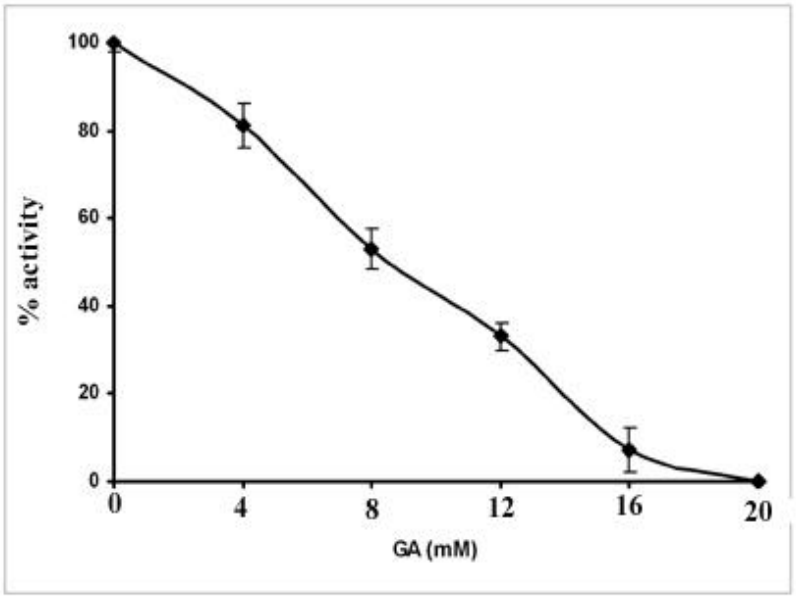


**Fig. 3.11b: Inhibition of hyaluronidase activity of *DR* venom by GA**. *DR* venom (100 µg) was pre-incubated for 15 min with different concentrations of GA (4, 8, 12, 16 and 20 mM). The reaction was initiated by adding 50 µg of HA in 0.2 M sodium acetate buffer pH 5.5 with 150 mM NaCl and incubated for 2 h 30 min at $37^0$ C. The activity was determined as described in the methodology section. The values represented as mean ± SEM (n=3).

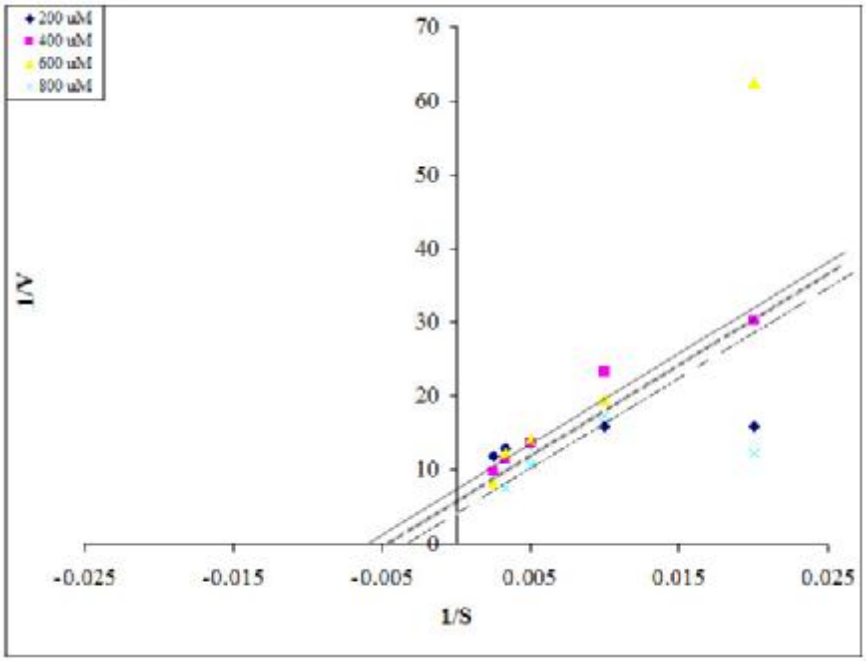


**Fig. 3.12: Line Weaver- Burk plot showing the uncompetitive nature of inhibition of DRHyal-II by GA**. The initial velocity is expressed as µ moles of *N*-acetyl-D-glucosamine released at 37 $^{0}$ C. Data represent average of three independent trails.

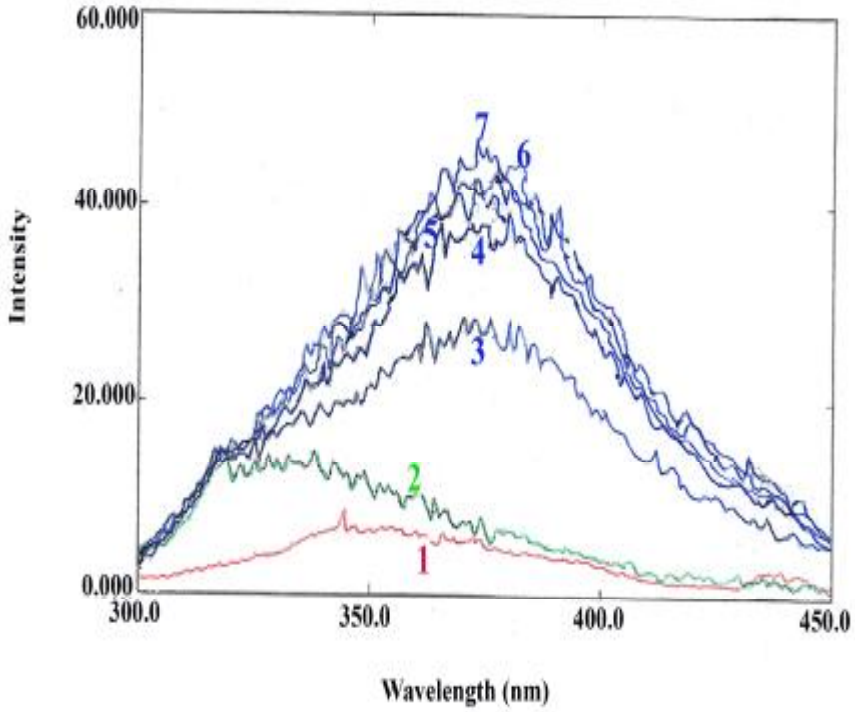


**Fig. 3.13: Fluorescence emission spectra of DRHyal-II in presence of GA.** DRHyal-II (20 μg) in 2 ml of 0.2 M sodium acetate buffer pH 5.5 containing 150 mM NaCl and with different concentrations of GA. GA alone 0.75 mM (1), DRHyal-II (20 μg) alone (2), DRHyal-II (20 μg) with 0.15 (3), 0.3 (4), 0.45 (5), 0.6 (6) and 0.75 (7) mM of GA. The fluorescence spectra were measured between 300-450 nm after excitation at 280 nm.

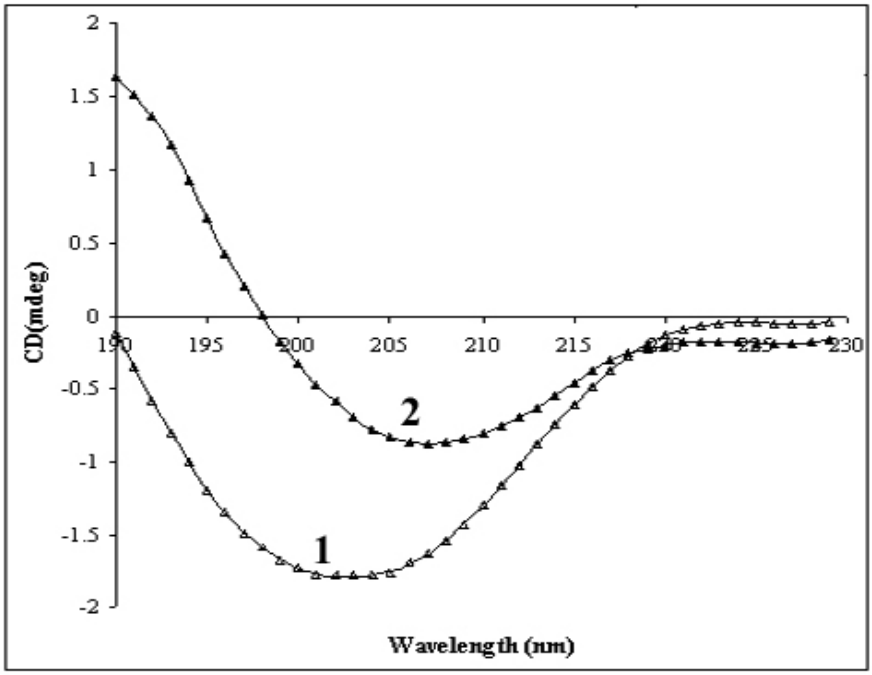


**Fig. 3.14: Far UV Circular Dichroism spectra of DRHyal-II.** Spectra in presence and absence of GA were recorded. The reaction mixture 1 ml contained DRHyal-II (200 µg) in 0.2 M sodium acetate buffer pH 5.5 containing 150 mM of NaCl and $IC_{50}$ concentration of GA (0.4 mM) was used. DRHyal-II (200 µg) alone (1) and DRHyal-II (200 µg) + GA (0.4 mM) (2) were performed. The spectra were measured between 190-250 nm on Jasco J715 spectropolarimeter.

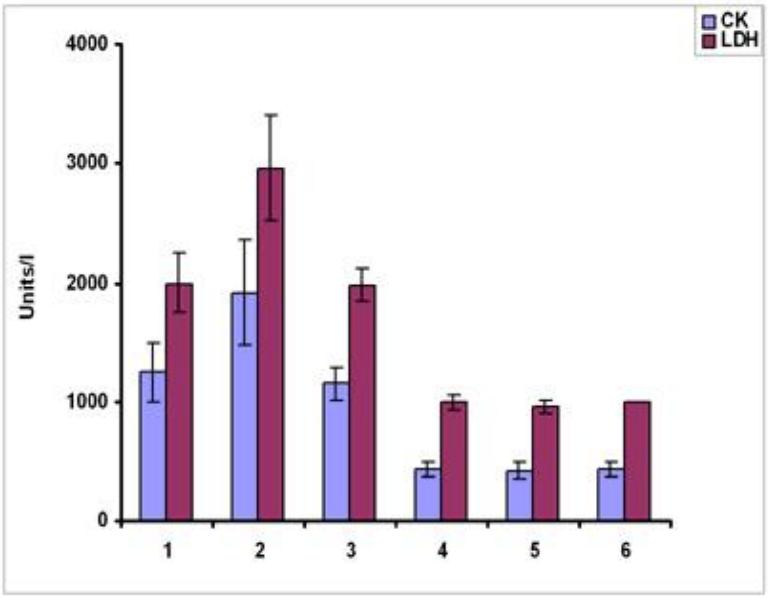


**Fig. 3.15: Inhibition of spreading property of DRHyal-II by GA.** VRV-PL-VIII myotoxin ($LD_{25}$ dose) plus DRHyal-II (40 µg) were pre-incubated with GA (26 mM) and then injected in a final volume of 50 µl saline intramuscularly in to the right thigh of mice. Mice were anesthetized by ether inhalation, after 3 h blood was drawn from the abdominal vena cava and serum CK and LDH levels were determined as described in methodology section. Samples injected are VRV-PL-VIII ($LD_{25}$ dose) (1), VRV-PL-VIII ($LD_{25}$ dose) plus DRHyal-II (w/w) 1: 0.30 (2), VRV-PL-VIII ($LD_{25}$ dose) plus DRHyal-II (w/w) 1: 0.30 were pre-incubated with GA (DRHyal-II: GA, 1: 4, w/w) (3), saline (4), DRHyal-II (40 µg) (5) and GA alone (26 mM) (6). The values represented as mean ± SEM (n=3).

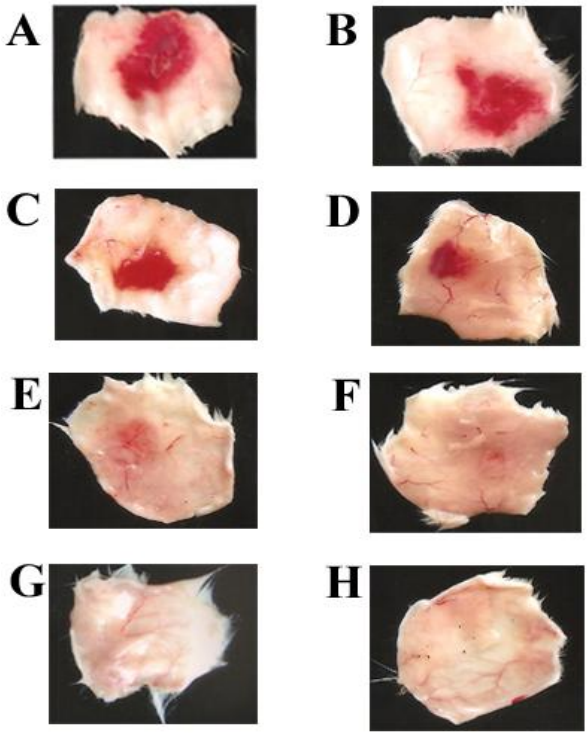


**Fig. 3.16: Inhibition of spreading activity of DRHyal-II by GA (hemorrhagic activity).** MHD of HC (2.5 µg) plus DRHyal-II (5 µg) were pre-incubated with the different concentrations of GA for 30 min at $37^0$ C and injected intradermally into the back of mice (n=3) in a total volume of 50 µl saline. After 3 h, mice were anesthetized and sacrificed. The dorsal patch of the skin surface was removed and the diameter of hemorrhagic spots was measured in $mm^2$. HC + DRHyal-II (w/w) (1: 2) (A), HC plus DRHyal-II (w/w) (1: 2) were pre-incubated with 3 (B), 6 (C), 12 (D), 18 (E) and 24 (F) mM of GA, saline (G) and GA alone (24 mM) (H).