Chapter 2

BIOCHEMICAL AND PHARMACOLOGICAL CHARACTERIZATION OF INDIAN KING COBRA (*OPHIOPHAGUS HANNAH*) VENOM

Chapter 2

Introduction

Snake venom is a lethal and a complex mixture of enzymatic and non-enzymatic proteins, peptides and small organic compounds. Snakes use their venom to immobilize, kill and digest the prey in addition use for the defence. Snakebite is a serious threat that remains as one of the major public health problem particularly in the tropical and sub-tropical countries. Though the actual incidence of snakebite is difficult to estimate, there are about 5 million people affected worldwide each year (Chippaux, 1998). In Asia alone, it contributes to approximately 100,000 deaths (Kasturiratne, 2008). In the Indian subcontinent, annual snakebite is more than 2, 00,000 and 35,000 to 50,000 of them have turned out to be fatal (Brunda et al, 2007). However, the exact contribution of individual snake species to overall snakebite morbidity and mortality is difficult to estimate, since the bitten snakes usually go unidentified (Norris et al, 2007).

Cobra (*Naja naja*), Krait (*Bungarus caerulus*), Russell`s viper (*Vipera/Daboia russelli*) and saw-scaled viper (*Echis carinatus*) are considered to be the major poisonous snakes of Indian sub-continent (also called as 'Big four') and their venom has been extensively characterized for biochemical and pharmacological properties. However, *Ophiophagus hannah* venom has been greatly ignored in the subcontinent. *Ophiophagus hannah*, commonly known as king cobra is the largest and most lethal snake belonging to the *Elapidae* family. A single bite of king cobra can deliver up to 400-500 mg of venom that is several folds higher than its lethal dose, so that one bite may contain up to 15000 LD_{50} mice doses (Broad et al, 1979). Gomes and co-workers (1999) isolated few low molecular weight non-enzymatic toxins from the Indian king cobra venom.

Although there are no reports of mortality and morbidity due to king cobra bite, confrontations with king cobras may always be expected due to encroachment and deforestation activities. This results in the overlapping of the king cobra habitat in to human habitat and thus there are expectable threats from king cobra and that India is

not prepared for the management of possible king cobra bites. Characterization of the whole venom in terms of its biochemical and pharmacological aspects has not been systematically done. Its active interference in the process of hemostasis which is one of the important manifestations of post snakebite symptoms has not been studied. Hence, this chapter aims at biochemical and pharmacological characterization of the Indian king cobra venom.

Materials and Methods

Materials

Lyophilized king cobra venom was obtained from Shri. Dipak Kumar Mitra, Hindustan Snake Park, Kolkata, India. Hyaluronic acid, Fat free Casein, Agarose, Alcian Blue, collagen type-I from bovine calf muscle was obtained from Sigma Chemicals Pvt. Ltd. St Louis, USA. Broad range protein molecular weight markers (Range 3.5 to 205 kDa) were obtained from Genei India Pvt. Ltd., Bangalore, India. Swiss Wistar albino mice were obtained from Central Animal House, Dept. of Studies in Zoology, University of Mysore, Mysore. Animal experiments were conducted based on the approval by the Animal Ethics Committee, CPCSEA, Chennai and permission from Central Animal House, Dept. of Zoology, University of Mysore, Mysore (No. MGZ/2518 (b)/ 2009-10). Animal care and handling were conducted in compliance with the National regulations for animal research. Fresh blood samples were collected from healthy human volunteers with the permission and clearance from Human Ethics Committee. All other chemicals used were of analytical grade.

Methods

SDS- Polyacrylamide gel electrophoresis

SDS-PAGE (10%) was carried out according to the method of Laemmli, (1970). 75µg of king cobra venom under non-reduced condition were loaded. Molecular weight standards from 3.5 to 205 kDa were used. Electrophoresis was carried out using Tris (25 mM), glycine (192 mM) and SDS (0.1%) for 2.5 h at 90V at room temperature. After electrophoresis, gels were stained with 0.1% Coomassie brilliant blue R-250 for

detection of the protein bands of the venom and destained with 25% ethanol in 8% acetic acid and water (30:10:60 v/v).

Proteolytic activity:

Proteolytic activity was assayed according to the method of Satake et al., (1963). Fat free casein (2% in 0.2 M Tris-HCl buffer pH 8.5) was incubated with king cobra venom of different concentration ranging from 0 to 150µg in 1ml of total volume at 37^0C for 2hr 30 mins. 1 ml of 0.44M Trichloroacetic acid was added to stop the reaction. The mixture was then centrifuged at 2500g for 10 min. Sodium carbonate (2.5ml, 0.4 M) and Folin-Ciocalteu's reagent (1:2 dilutions with water) were added to 1ml of the supernatant and the colour developed was read at 660 nm. One unit of enzyme activity was defined as the amount of the enzyme required to cause an increase in OD of 0.01 at 660 nm/min at 37^0C. The specific activity was expressed as units/min/mg of protein.

Hyaluronidase activity:

Substrate gel assay for hyaluronidase activity was performed as described by Cevallos et al., (1992) by incorporating hyaluronan 0.17 mg/ml into 10% SDS-PAGE. *O. hannah* venom sample (150µg) under non-reducing condition was loaded into the well. After electrophoresis, the gel was washed with Triton X-100 and soaked in 0.1M sodium phosphate buffer , pH 5.8 containing 0.15 M NaCl for 1 hr. This was followed by equilibrating the gel in 0.1M sodium acetate buffer pH 5.5 containing 0.15 M NaCl for 20 hr at 37^0C with constant agitation. The gel was then washed with 0.015 M Tris-HCl buffer pH 7.9 and placed in Alcian blue solution for 2hr and the gel was destained (water and acetic acid in the ratio 1:100).

Indirect hemolytic Activity:

Indirect hemolytic activity was determined according to the method of Boman and Kaletta, (1957) using washed human erythrocytes. Briefly, packed human erythrocytes, egg yolk and phosphate buffered saline (PBS; 10 mM, pH 7.2 with 0.9% NaCl) were mixed in the ratio 1:1: 8(v/v). 1 ml of this suspension was incubated independently with increasing concentrations of king cobra venom (0-25 µg) for 1 h at

37^0 C. The enzyme activity was arrested by adding 9 ml of ice cold PBS and centrifuged at 1000g for 10 min at 4^0 C. The amount of hemoglobin released in the supernatant was measured at 540 nm. Activity was expressed as percent of hemolysis against 100% lysis of cells due to addition of water taken as positive control of the experiment.

Plasma recalcification time:

The method of Quick et al., (1935) was followed. Normal human citrated plasma (0.2 ml) and different concentrations (1 to 10µg) of king cobra venom were taken in glass tubes and were mixed with Tris-HCl Buffer 10 mM, pH 7.4 to make up the final volume to 250µl and was incubated at 37^0C for 1 min. The clotting time was determined after adding 0.1 ml of 0.25 M $CaCl_2$. The clotting time of citrated plasma in the absence of venom sample formed the control of the experiment.

Fibrinogenolytic activity:

Fibrinogenolytic activity was tested according to Gao et al., (1998) by incubating venom sample (0.5µg to 8µg) with human plasminogen-free fibrinogen (50µg) in 50mM Tris-HCl buffer, 0.1M NaCl, pH 7.4. Both dose dependent and time dependent activities of the venom were analyzed. For dose dependent activity, the samples with increasing concentration (0-8 µg) were incubated for 120 min at 37^0C and for time dependent activity venom samples (2 µg) were incubated at 37^0C with different incubation time varying from 0 to 120 min. The reaction was stopped by adding 20µl of denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptaethanol. Samples were boiled for 5 min, the fibrinogen degradation products were then analyzed on a 10% SDS-PAGE.

Fibrinolytic activity by plate method:

The method described by Gene et al., (1989) was followed with some modification. A mixture consisting of 2 ml healthy human platelet poor plasma and 3 ml of 1.2% molten agarose (45^0C) in 10 mM Tris-HCl buffer containing 0.02% sodium azide, 70 mM $(NH_4)_2SO_4$, 90 mM NaCl, 0.70 mM $MgCl_2$ and 200 µl of 0.2 M $CaCl_2$

was poured into a 10 x 90 mm petridish and was allowed to solidify for 2 h at 27^0C. Increasing concentrations of king cobra venom (5-25μg) and one I.U. of urokinase in 10 μl of 10 mM Tris-HCl buffer pH 7.4 were placed independently on the surface of the gel matrix and were incubated for 15 h at 27^0C. The diameters of lysed zones were measured with millimeter scale and were photographed.

Platelet aggregation:

Turbidometric method of Born and Cross, (1963) was followed using a dual channel *Chrono-log* model 700-2 aggregometer (Havertown, USA). Briefly, 235 μl of PRP suspensions were maintained at 37^0C in a siliconized glass cuvettes, pre-incubated with different doses of king cobra venom for 3 min and the aggregation was initiated by adding the agonist Epinephrine, ADP and Thrombin (10 μM) in independent experiments. The aggregation was followed for 6 min with constant stirring at the speed of 1200 rpm. Aggregation induced by agonist alone was considered as 100% aggregation. The aggregation trace was the plot of light transmission between platelet rich plasma (PRP) and platelet poor plasma (PPP) base line, which represent 0% and 100% aggregation respectively.

Lethality:

LD_{50} of venom was determined according to the method of Meier and Theakston. Group of mice (8-10) was injected with king cobra venom followed by 24 hrs observation for the survival time. Venom was injected (*i.p*) into test group of mice (n = 6). Groups of mice (n = 6) into which the corresponding volumes of saline alone was injected separately served as experimental controls. Documentation of survival time was done on constant observation. % survival value was calculated as number of mice died x 100/number of mice used for the experiment (n).

Myotoxicity:

Myotoxicity was determined according to the method of Gutierrez et al., (1990) using groups of mice (n=3). Group I was injected with lethal dose$_{25}$ (LD_{25} dose) of king cobra venom alone. Group II was injected with saline alone. Mice were anesthetized by sodium barbiturate inhalation after 3 h and the abdominal cavities were opened to draw

the blood from vena cava. After clotting, the 1: 25 diluted serum was assayed for creatine phosphokinase (CPK, EC no: 2.7.3.2) and lactate dehydrogenase (LDH, EC no: 1.1.1.28) levels using AGAPEE diagnostic kits. Activities were expressed as IU/L.

Edema:

King cobra venom (20µg) was injected into the right foot pads of group of mice (n=3). The left foot pads were injected with the corresponding volume of saline only that served as controls. The mice were sacrificed after 45 min and both the foot pads were cut off at the ankle joints and weighed individually. Edema ratio was calculated according to Vishwanath et al. The increase in weight of leg due to edema was calculated as the edema ratio, which equals the weight of edematous leg x 100/ weight of normal leg. Minimum edema dose (MED) was defined as the amount of venom protein required to cause an edema ratio of 120%.

Protein estimation by Lowry's method:

Protein concentration of king cobra venom was determined according to the method of Lowry et al., (1951) using bovine serum albumin as standard.

Statistical analysis:

All the quantitative values were presented as Mean ± SD.

Results

Electrophoresis of king cobra venom in SDS-PAGE under non-reduced and reduced condition showed distinct protein banding patterns that lie in the wide molecular mass range from 3.5 kD to 205 kD. High and low molecular mass proteins were found to be distinct in king cobra venom (Fig. 2.1).

The king cobra venom was studied for the proteolytic activity as well as the effect of protease specific inhibitors (Table 2.1). The venom showed proteolytic activitiy with a specific activity of 1.02 ± 0.08. The optimum pH for proteolytic activity was found to be 8.5. The venom was tested with specific protease inhibitors such as PMSF (para-methyl sulphonyl fluoride), EDTA and 1,10-phenanthrolein. The proteolytic activity was inhibited by PMSF while the rest of the two inhibitors did not

cause any significant inhibition suggesting that the protease component of the king cobra venom is a serine protease.

The king cobra venom was devoid of direct hemolytic activity (data not shown) but showed indirect hemolysis with varying % of lysis with the increase in concentration. The venom showed 100% lysis at a concentration of 25 µg when compared with lysis caused by H_2O that was taken as positive control (Fig. 2.2).

The venom was found to show procoagulant activity when tested on citrated human plasma in the presence of calcium chloride. It reduced the clotting time in a dose dependent manner. The clotting time was significantly reduced from 3 min 45 sec (control) to 24 sec at a concentration of 10 µg (Fig. 2.3).

The zymogram assay for hyaluronidase activity was performed on a 10 % SDS-PAGE by incorporating 1.7 mg hyaluronan into the resolving gel. King cobra venom showed translucent activity band (Fig. 2.4) at a range of around 60 kDa. The single translucent activity band that appeared suggested the presence of single hyaluronidase enzyme in king cobra venom.

King cobra venom degraded human fibrinogen. The effect was found to be both dose (Fig. 2.5A) and time (Fig. 2.5B) dependent. The venom specifically degraded the Bβ chain, while the Aα and γ-chain remained resistant to cleavage when incubated for 2h. In time dependent assay, the Bβ chain experienced complete degradation in about 5 min of incubation period when used 2µg of venom incubated at 37^0C.

The fibrinolytic activity of king cobra venom was tested by plate method. The plasma clot degradation increased with increase in concentration of the venom that was confirmed by the opaque zones formed on the gel matrix as a result of venom proteolytic activity. The venom concentrations 5, 10, 15 20 and 25 µg produced activity zones of 3 ± 0.8 mm, 5 ± 0.6 mm, 6 ± 0.6 mm, 8 ± 0.4 and 10 ± 0.8 mm in a semi quantitative agarose gel plate method while, the urokinase produced the activity zone of 7 ± 0.6 mm for 1 I.U which served as the positive control (Fig. 2.6). This suggested that king cobra venom has plasmin like activity.

Further, king cobra venom interfered in human platelet aggregation function. It showed inhibitory activity on platelet aggregation in a dose dependent manner. 15 µg of king cobra venom was found to cause up to 70% inhibition of platelet aggregation induced by collagen (Fig. 2.7) suggesting its active interference in collagen induced human platelet aggregation process.

The king cobra venom showed edema inducing activity. It significantly increased edema ratio from 122 ± 1.6 (experimental control) to 164 ± 3.2 at a venom concentration of 20 µl. The lethal$_{50}$ dose (LD$_{50}$) of king cobra venom was found to be 2.63 ± 0.33 mg/kg body weight. Further, the venom showed myotoxic effects in mice models. It significantly increased the serum levels of Creatine Phosphokinase from 292.5 ± 14 to 1014 ± 52 IU/L and Lactate dehydrogenase from 1126 ± 34 to 4022 ± 40 IU/L.

Discussion

The present study is an attempt to characterize and study the biochemical and pharmacological aspects of king cobra venom. Various inhibitory and degradative activities revealed by the king cobra venom in the current study clearly deciphers its intervention in multiple human physiological and biochemical pathways that lead to various deleterious effects.

The venom was found to be significant in terms of its enzymatic components such as protease, phospholipase and hyaluronidase in exhibiting the activities. The venom protease component was found to be a serine protease based on its activity in the presence of protease specific inhibitors such as PMSF, EDTA and 1,10-Phenanthrolein. PMSF alone could completely inhibit the activity of venom protease. Hence, as expected, the venom was found to be devoid of hemorrhagic activity. However, protease activity when tested on human fibrinogen gave remarkable result and is noteworthy since it specifically cleaved the Bβ-chain alone leaving the Aα and γ-chain unhampered. This offers scope for a detailed characterization of the β-fibrinogenase component of this venom. The venom also showed fibrinolytic activity in a dose dependent manner which further adjoin to the deleterious effects of this venom. The

venom showed active inhibition of platelet aggregation process induced by collagen in a dose dependent manner. This makes way for a detailed and systematic investigation of possible anti-platelet property or receptor mediated inhibitory activities of the isolated component(s) of king cobra venom. The venom showed edema inducing activity similar to the activity of viper venoms. However, the % of edema was moderate. The lethal dose of the venom though was found to be less when compared to the lethal doses of venoms of cobra family, since king cobras inject large quantities of venom in a single bite, the volume of venom itself contribute for high doses. The venom was also found to increase the serum levels of both CPK and LDH, indicating the myotoxic effects of the venom.
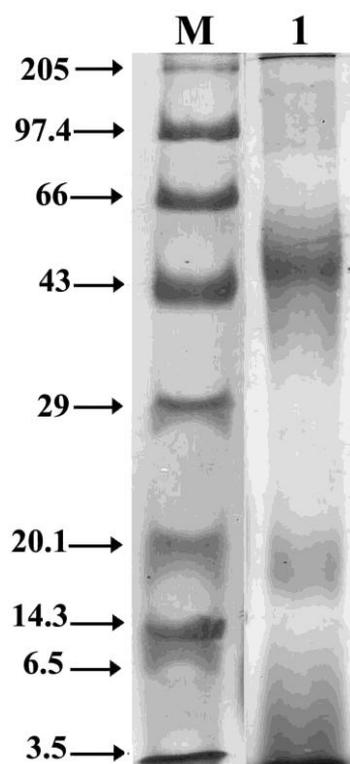
Fig. 2.1: Protein banding pattern of king cobra venom: Lane M represents protein Mol. wt markers; Rabbit muscle myosin (205), phosphorylase B (97.4), Bovine serum albimin (66), ovalbumin (43), carbonic anhydrase (29), soyabean trypsin inhibitor (20.1), lysozyme (14.3), aprotinin (6.5) and insulin (3.5). Lane 1 represents protein banding pattern of king cobra venom under non reduced condition.

Protease Inhibitors	Activity in Units
Venom alone	1.02 ± 0.08
Venom+PMSF	0.04 ± 0.06
Venom+EDTA	0.98 ± 0.08
Venom+1,10-Phenanthrolein	1.0 ± 0.08

Table 2.1: Proteolytic activity of King Cobra venom with protease specific inhibitors; PMSF, EDTA and 1, 10-Phenanthrolein, 5 mM concentrations each.
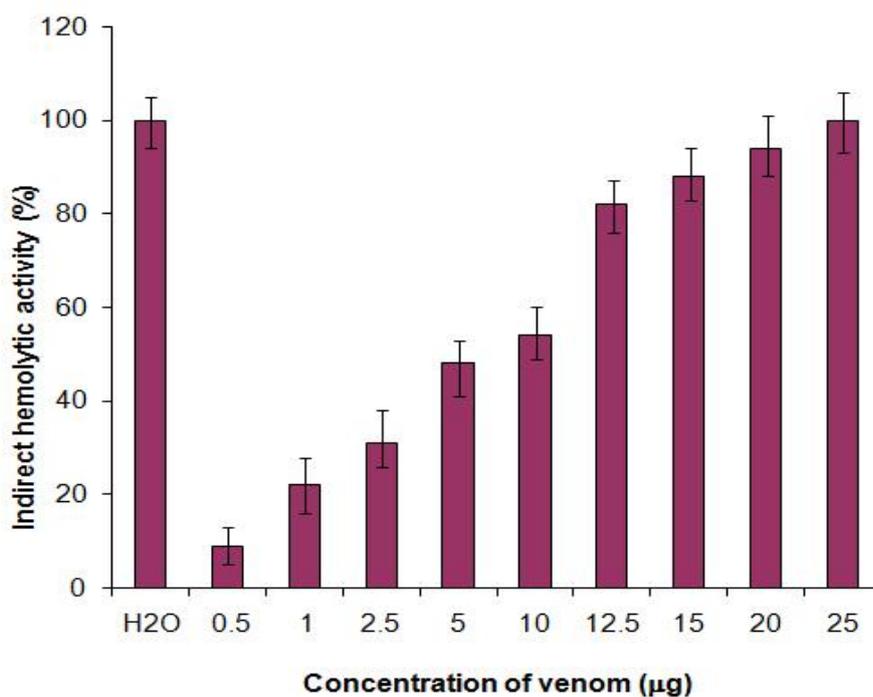
Fig. 2.2: Indirect Hemolytic activity: H_2O represents 100% lysis of erythrocytes with water, graph showing percentage lysis of washed erythrocytes when treated with increasing concentrations of king cobra venom.
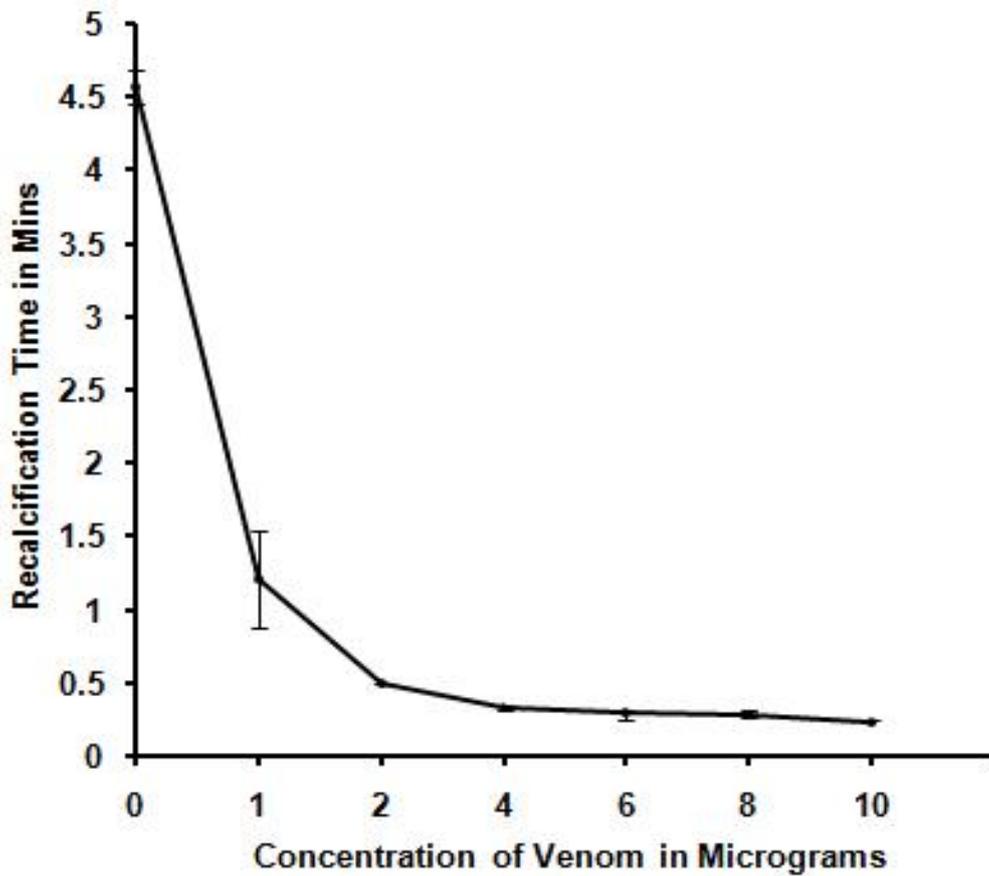
Fig. 2.3: Recalcification time of citrated human plasma: Effect of venom showing decreased clotting time when 0.2 ml of citrated human plasma was incubated with increasing concentrations of venom ranging from 0 to 10 μg for 5 min at 37^{0}C. Clotting was initiated by adding 0.25M $CaCl_2$ and time recorded in min.
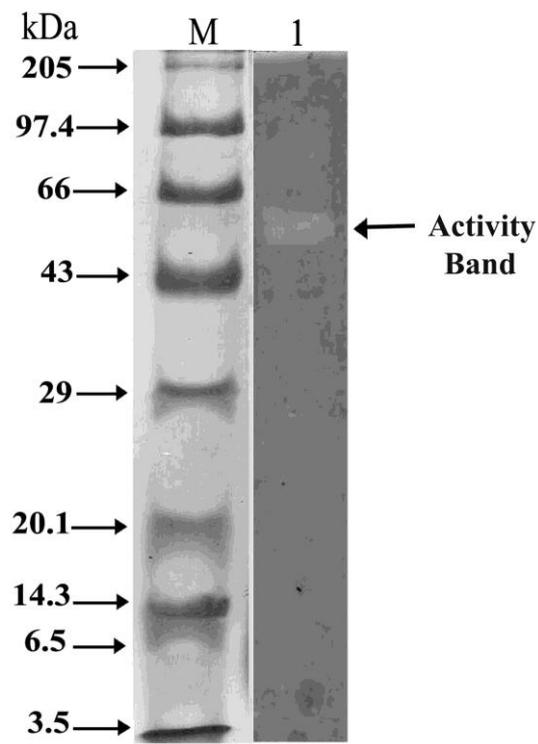
Fig. 2.4: Hyaluronan Zymogram: Lane M represents Protein Molecular weight markers and lane 1 showing hyaluronidase activity band of king cobra venom (150 µg) prepared under non-reduced condition, run on a 10% SDS-PAGE hyaluronan zymogram.
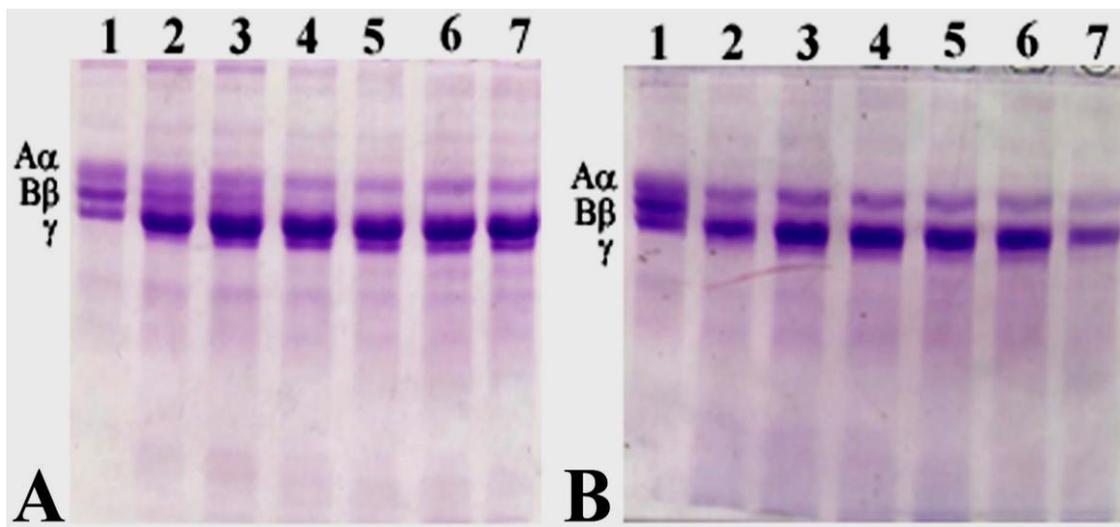
Fig. 2.5 Fibrinogenolytic activity: A: Lane 1 showing Fibrinogen (50μg) alone served as control, lane 2, 3, 4, 5, 6 and 7 containing fibrinogen (50 μg) + 0.5, 1, 2, 4, 6 and 8 μg of king cobra venom for 120 min and separated on 10% SDS-PAGE under reduced condition. **B:** Lane 1 containing fibrinogen (50 μg) alone, lane 2, 3, 4, 5, 6 and 7 + 5μg of king cobra venom incubated for 5, 10, 20, 40, 60 and 120 min respectively and run on 10% SDS-PAGE under reduced condition.
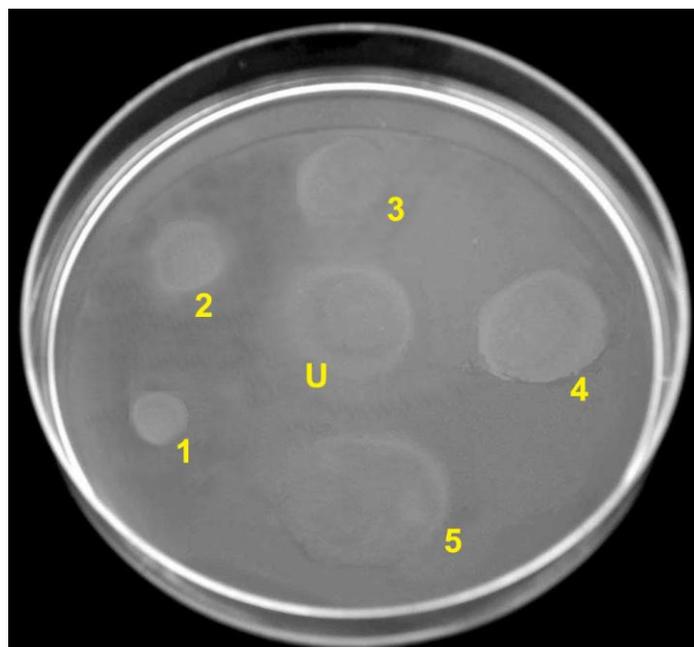
Fig. 2.6: Fibrinolytic activity of king cobra venom by plate method: King cobra venom (5 to 25 µg, numbered 1 to 5) showing increasing fibrinolytic activity zones. Activity zone of urokinase (U) was taken as positive control on the surface of the agarose gel incubated for 12 h at 27^{0}C.
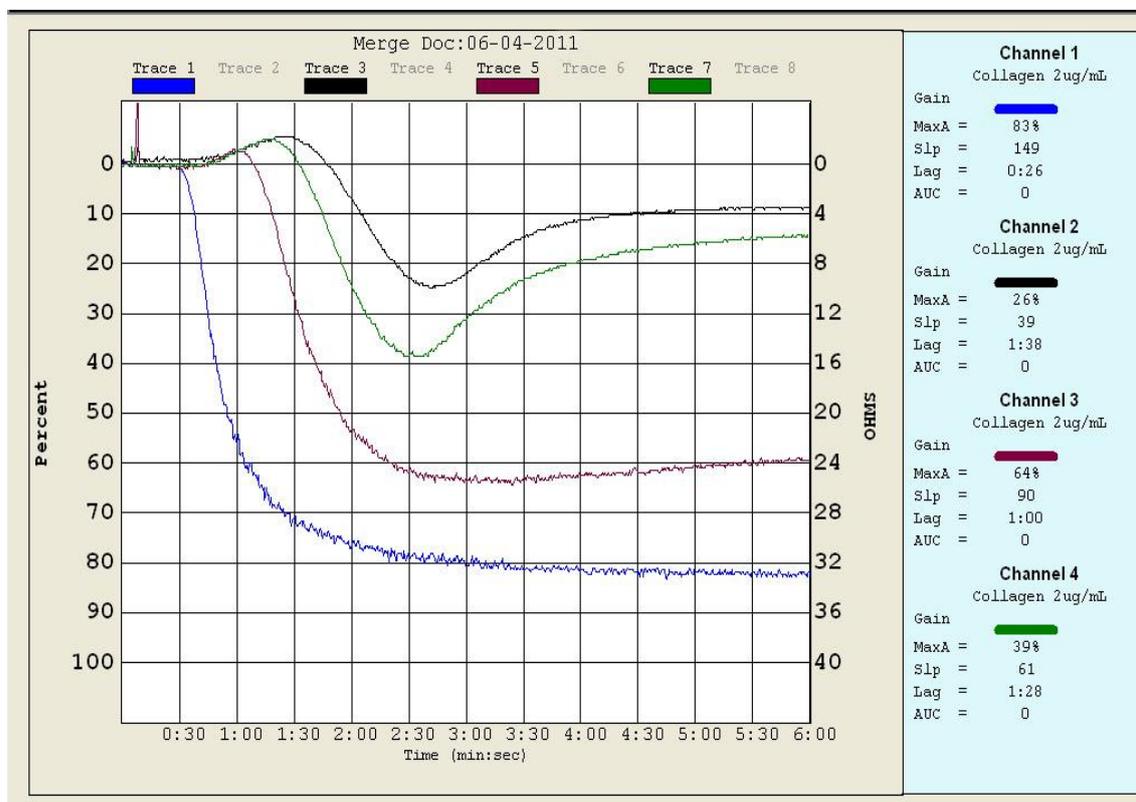
Fig. 2.7: Effect of *O. hannah* **venom on collagen induced human platelet aggregation:** Platelet aggregation trace showing inhibitory activity of *O. hannah* venom in increasing concentration (5 to 15 μg) on collagen induced human platelet aggregation process. Blue trace served as control (taken as 100% aggregation).