Introduction

Protease inhibitors are characterized by their functional specificity of inhibiting proteolysis and to date 93 families of protease inhibitors have been reported (Inagaki, 2015). Amidst which, five families of inhibitors such as Kazal, Kunitz, waprin, cystatin, and bradykinin-potentiating peptide are found in snake venoms (Brahma et al., 2015). Owing to their large abundance, Kunitz-type protease inhibitors are the best characterized proteins in snake venoms (Mourao and Schwartz, 2013).

Primary target of snake venom Kunitz-type protease inhibitors are blood coagulation cascade and factors controlling blood pressure (Yamazaki and Morita, 2007). Blood coagulation cascade is controlled by the synchronised action of serine proteases where preceding factor activates the succeeding one (Spronk et al., 2003). Majority of the Kunitz-type proteins inhibit coagulation factors such as Xa, XIIa, activated protein C and thrombin which thereby promote bleeding (Inagaki, 2015). Kallikreins, a sub group of serine proteases is also targeted by Kunitz-type proteins. Kallikrein produces bradykinin; a potent vasodilator and inhibition of kallikrein thereby up regulate blood pressure (Earl et al., 2012).

Most of the studies with Kunitz-type proteins are practically confined to inhibit serine proteases. However, recently Chou and group reported the inhibitory effect of two Kunitz-type proteins; protease inhibitor-like protein-2 (PILP-2) and PILP-3 on MMP-2 activity. PILPs, which were cloned using the cDNA library of Bungarus multicinctus inhibited the MMP-2 activity with PILP-3 being more potent than PILP-2 (Chou et al., 2010). Another Kunitz-type protein identified from cDNA of Pseudechis australis is Pr-mulgin 1 which also showed to inhibit MMP-2 activity (Inagaki et al., 2012).
Further, catalytic domain structure of SVMPs is topologically similar to that of MMPs. SVMPs and MMPs being phylogenetically related, belongs to metzincin family of proteins (Takeda, 2016). SVMPs which are abundant in viperid snake venoms are considered as the key toxins in inducing profound local tissue damages such as hemorrhage and myonecrosis upon envenomation (Hernandez et al., 2011; Paes Leme et al., 2012). In similar lines, *E. carinatus* is an Asian endemic viperid snake and upon its envenomation inflicts extensive local tissue damage. These effects are so persistent and continuous that leads to amputation of bitten parts of victims. This large quantum of tissue damage is due to the high load of metalloproteases present in its venom (Nanjaraj Urs et al., 2015a; Nanjaraj Urs et al., 2015b). Hence, inhibition of SVMPs would be of great importance to counteract the associated pathological effects.

Considering the similarity between MMPs and SVMPs, effect of Kunitz-type protein(s) on SVMPs remain elusive. Further, proteomic analysis of Indian cobra *N. naja* venom has revealed that protease inhibitors are one of the predominant toxins (Dutta et al., 2017; Sintiprungrat et al., 2016). So far, two Kunitz-type proteins with trypsin and chymotrypsin inhibitory activity have been reported from *N. naja* venom (Shafqat et al., 1990a; Shafqat et al., 1990b). None of the two inhibitors were tested for the inhibitory activity against MMPs or SVMPs. With these, present work aimed at screening and purification of a protease inhibitor from the venom of *N. naja* which can inhibit the metalloprotease activity of *E. carinatus* venom. Here, we are reporting for the first time a Kunitz-type protease inhibitor from *N. naja* venom with anti-hemorrhagic and anti-myonecrotic effect.
Materials

Venom

Lyophilized *E. carinatus* and *N. naja* venoms were purchased from Irula Snake-Catchers Co-operative Society Ltd., (Chennai, India). Required amount of venom was re-dissolved in saline and centrifuged at 6000 g for 10 min to remove debris. Protein content of crude venom was determined according to the method of (Lowry et al., 1951) using bovine serum albumin (BSA) as standard. Aliquots were kept at freezing temperature until further use.

Chemicals and reagents

Sephadex G-75, CM-Sephadex C-50, DEAE-Sephadex A-50 gels, gelatin (type A) from porcine skin, collagen (type I) from calf skin, fibrinogen from bovine plasma, bovine trypsin, α- chymotrypsin from bovine pancreas, Na-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE), Na-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA), N-Benzoyl-L-tyrosine ethyl ester (BTEE), *Escherichia coli* [lyophilized cells of strain W (ATCC 9637)] and Amicon ultra centrifugal filters were purchased from Sigma Aldrich (St. Louis, USA). 14C-oleic acid and scintillation cocktail (Ultima Gold) was obtained from Perkin Elmer Life Sciences Inc. (Boston, MA, USA). Reagents for electrophoresis were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Medium range molecular weight markers (code: PPMWM) were purchased from Genei (Bangalore, India). Creatine kinase assay kit was purchased from AGAPPE, (Ernakulam, India). All the other chemicals and reagents used in this study were of analytical grade.

Animals

Swiss Albino mice (either gender; 25-30 g) were obtained from Central Animal Facility, University of Mysore (UOM), Mysuru, India. Animal care and
handling were in compliance with National Regulations for Animal Research and the experiments were performed according to the protocols reviewed by the Institutional Animal Ethical Committee. (No: UOM/IAEC/11/2013).

**Methods**

**Hemorrhagic activity**

Experimental animals were randomly divided into different groups (n=3) and hemorrhagic activity was assessed according to method of (Kondo et al., 1960) with slight modifications. Inhibition studies were carried out by mixing *E. carinatus* venom with *N. naja* or *B. caeruleus* venoms and injected immediately by intradermal route (i.d). Briefly, various concentrations of crude *N. naja* or *B. caeruleus* venom was mixed with constant 4 µg of *E. carinatus* venom, taken in a constant volume of 30 µL saline was immediately injected by an i.d route on the back of mice. Saline and 4 µg of *E. carinatus* venom injected animals served as negative and positive controls respectively. Animals were sacrificed after three hours by administering pentabarbitone (30 mg/kg body weight). Dorsal patch of the skin was taken for observation of hemorrhagic spots. Area of hemorrhagic spot was measured using graph sheet and inhibition of hemorrhagic activity was measured in terms of decreased area of hemorrhage in comparison to *E. carinatus* venom injected hemorrhagic spot. Skin tissues were processed for histopathological studies.

**Sephadex G-75 column chromatography**

Lyophilized *N. naja* venom (80 mg in 1 mL of equilibration buffer) was loaded on to a Sephadex G-75 column (1.0 × 135 cm) equilibrated with phosphate buffer (10 mmol/L pH 7.0). The column was eluted using equilibration buffer with a flow rate of 20 mL/h and 2 mL fractions were collected. Protein elution was monitored at 280 nm using a Thermo Scientific UV-Vis spectrophotometer (Biomate-
Individual peaks were pooled separately and assayed for anti-hemorrhagic activity. The pooled sample was dialyzed against 50 mmol/L sodium acetate buffer pH 5.5 for subsequent purification.

**CM-Sephadex C-50 column chromatography**

The peak exhibiting anti-hemorrhagic activity (22.5 mg in 0.5 mL of equilibrating buffer) from the Sephadex G-75 column was loaded onto a CM-Sephadex C-50 column (1.5 × 30 cm), equilibrated in 50 mmol/L sodium acetate buffer pH 5.5. Subfractions were then eluted stepwise using equilibration buffer followed by 50 mmol/L phosphate buffers of pH 6.5 & 7.5 containing 0.1 & 0.15 mol/L NaCl respectively. Final elution was carried out with 50 mmol/L Tris-HCl buffer pH 8.5 containing 0.2 mol/L NaCl. Fractionation was carried out at a flow rate of 20 mL/h and 2.0 mL fractions were collected. Individual peaks were pooled separately and assayed for anti-hemorrhagic activity. The pooled sample was dialyzed against 50 mmol/L phosphate buffer pH 8.0 for further purification.

**DEAE-Sephadex A-50 column chromatography**

Peak exhibiting anti-hemorrhagic activity (2.5 mg in 0.2 mL of equilibrating buffer) from the CM-Sephadex C-50 column was loaded onto a DEAE-Sephadex A-50 column (1.2 × 20 cm), equilibrated in 50 mmol/L phosphate buffer (pH 8.0). Subfractions were then eluted stepwise using 50 mmol/L phosphate buffers of different pH (8.0-6.0) and sodium acetate buffer of pH 5.0. Fractionation was carried out at a flow rate of 10 mL/h and 1.0 mL fractions were collected. Individual peaks were pooled separately and assayed for anti-hemorrhagic activity. Pooled sample was dialyzed using Amicon ultra centrifugal filters (MWCO 10 kDa) against double distilled water.
Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method of (Laemmli, 1970) and 10% gels were used. Molecular mass standards (98–16 kDa) were used to monitor the electrophoretic profile of purified fraction(s). After electrophoresis, gels were stained with 0.25% coomassie brilliant blue R-250.

Reversed phase HPLC (RP-HPLC)

Purified protein (20 µg) was subjected to RP-HPLC using Sunfire™ C18 (5 µm, 0.46 × 25 cm, pore size- 100Å) column, pre-equilibrated with 0.1% TFA in water. The column was eluted using linear gradient from solution A (0.1% TFA in water) to 100% solution B (0.1% TFA in acetonitrile) for 60 min. The protein was eluted at a flow rate of 0.5 ml/min and monitored at 280 nm.

Myonecrotic activity

Myotoxic activity was carried out according to the method (Gutierrez et al., 1990). Constant dose of E. carinatus venom (5 µg) were injected intramuscularly (i.m) to the right thigh muscle of experimental animals (n=3) in a fixed volume of 30 µL of saline. For inhibition studies, DEAE P4 (purified anti-hemorrhagic protein; NNAh) in a dose dependent manner was co-injected with E. carinatus venom. Saline and E. carinatus venom injected animals served as negative and positive controls respectively. Three hours later, animals were anaesthetized; blood was drawn by cardiac puncture and processed to obtain serum. Part of muscle which received injections was processed for histopathological studies. Serum (1:20 v/v with saline) was used for determination of creatine kinase activity according to manufacturer's protocol and activity was expressed as U/L.
**Gelatinolytic activity**

Gelatinolytic activity was carried out by employing substrate gel electrophoresis according to the method of (Bee et al., 2001) with slight modifications. Briefly, 0.2% gelatin was incorporated into 10% SDS resolving gel and loaded with constant 5 µg of *E. carinatus* venom pre-incubated for 5 min with various dose of NNAh. All samples were loaded under non reducing conditions and electrophoresis was carried out at a constant voltage of 100V at room temperature. After electrophoresis, SDS was removed by washing the gel successively with 2.5% and 0.5% triton X-100 and distilled water until the removal of froth. The gel was incubated with 50 mmol/L Tris-HCl buffer (pH 7.6) at 37°C for 24 h and the gel was stained with coomassie brilliant blue and destained. Clear zones against dark blue back ground indicate the hydrolysis of gelatin due to enzyme activity. Inhibition offered by NNAh was quantitatively measured using NIH image J software (Bethesda, USA).

**Collagenolytic activity**

Collagenolytic activity was carried out according to the method of (Shannon et al., 1989) with slight modifications. Briefly, 50 µg of collagen in a total reaction volume of 25 µL was incubated with constant 5 µg of *E. carinatus* venom for 2 h at 37°C in 50 mmol/L Tris-HCl buffer (pH 7.6) and the reaction was terminated by adding reducing sample buffer followed by boiling for 10 min. For inhibition studies various dose of NNAh was incubated with *E. carinatus* venom for 5 min prior to addition of collagen. Samples were loaded onto 7.5% SDS polyacrylamide gel and electrophoresis was carried out at a constant voltage of 100V at room temperature. Gel was stained with coomassie brilliant blue R-250 and destained.
**Phospholipase A2 activity**

Phospholipase A2 (PLA2) activity was measured using $^{14}$C-oleate labelled autoclaved *E. coli* cells as substrate according to the method of (Vishwanath et al., 1993) and $^{14}$C radiation was measured in Packard Scintillation analyzer (TRI-CARB 2100 TR). For inhibition studies, similar reactions were carried out after pre-incubating 10 μg of *E. carinatus* venom with various concentrations of NNAh for 5 min at 37°C. Concentration of NNAh selected for all the *in vitro* inhibition studies was based on its anti-hemorrhagic activity. Inhibition was expressed as percentage.

**Edema-inducing activity**

Edema-inducing activity of *E. carinatus* venom was determined according to the method of (Vishwanath et al., 1987). Experimental animals (n=3) were injected with a constant dose of *E. carinatus* venom (4 µg) in a total volume of 20 µL saline into intra plantar surface of right hind footpad and left footpad received 20 µL of saline/vehicle which served as control. For inhibition studies, NNAh in a dose dependent manner was co-injected with *E. carinatus* venom. After 45 min, mice were sacrificed and both hind limbs were removed at the ankle joint and weighed individually. The edema ratio was calculated using the formula, Edema ratio = (Weight of edematous leg/weight of normal leg) × 100.

**Histopathological studies**

Skin tissue as well as thigh muscle from hemorrhage and myonecrosis experimental groups respectively were excised after dissection and fixed in Bouin’s fixative solution overnight. Tissue samples were then treated with gradients of alcohol to dehydrate and final treatment was done using chloroform/alcohol mixture. Processed tissues were fixed in molten paraffin wax and 4 µ thick sections were taken using microtome (Leica, Solms, Germany). Slides were stained using haematoxylin
and eosin which were then observed under Axio Imager A2 bright field microscope with LED - Zeiss (Oberkochen, Germany) and photographed.

**Peptide mass fingerprinting and de-novo sequencing**

In gel tryptic digestion of NNAh was done according to the method of (Thiede et al., 2005). For peptide mass fingerprinting and de-novo sequencing, LC-MS/MS of the tryptic digests was carried out by loading the sample onto a Bruker nano-Advance HPLC coupled with ESI Q-TOF mass spectrometer. The MS/MS spectra obtained was searched against the Swiss-Prot database and confined to ‘bony vertebrates’ sequences using the Mascot database search engine (version 2.6). Iodoacetamide derivatives of cysteine and methionine oxidation were specified as fixed and variable modifications, respectively. Further, the de novo sequences of the peptides that were obtained from Mascot protein identification were subjected to a BLAST search in NCBI nr against a snake venom protein database (snakes, taxid: 8570).

**Caseinolytic activity**

Caseinolytic activity was assayed according to the method of (Satake et al., 1963). Briefly, casein (2%) in 0.2 mL of Tris-HCl buffer (pH-8.5) was incubated with constant 10 µg of *E. carinatus* venom at 37°C for 2.5 h. The reaction was stopped by adding 1.5 mL of 0.44 mmol/L trichloroacetic acid and allowed to stand for 30 min. The reaction mixture was centrifuged at 140 g for 15 min. An aliquot (1 mL) of supernatant was mixed with 2.5 mL of 0.4 mmol/L sodium carbonate and 0.5 mL of Folin Ciocalteau reagent (1:2 v/v). The reaction mixture was allowed to stand at room temperature for 30 min and the colour developed was measured at 660 nm. Activity was expressed as units and one unit is defined as the increase in absorbance by 0.01 OD at 660 nm/h at 37°C. For inhibition studies, *E. carinatus* venom was pre-incubated with various concentration of NNAh for 5 min and the similar protocol was
followed. The proteolytic activity of *E. carinatus* venom in the absence of NNAh was considered as 100%.

**Esterolytic activity**

Esterolytic activity of trypsin, chymotrypsin and *E. carinatus* venom was assayed using BAEE, BAPNA and BTEE as substrates according to the method of (Geiger and Kortmann, 1977) with slight modifications. BAEE and BAPNA hydrolysing activities of trypsin and *E. carinatus* venom was determined by incubating constant 5 µg of enzyme source in a reaction mixture of 0.5 mL containing 40 mmol/L Tris-HCl buffer (pH 7.8), 0.23 mmol/L (BAEE) or 0.7 mmol/L (BAPNA) and the reaction was monitored continuously for 5 min at 253 nm for BAEE and 405 nm for BAPNA respectively. For inhibition studies, trypsin and *E. carinatus* venom was pre-incubated with different concentration of NNAh for 5 min prior addition of substrates. BTEE hydrolysis by chymotrypsin and *E. carinatus* venom was studied by incubating constant 5 µg of enzyme source in a reaction mixture of 0.5 mL containing 40 mmol/L Tris-HCl buffer (pH 7.8), 0.58 mmol/L BTEE and the reaction was monitored continuously for 5 min at 256 nm. For inhibition studies, chymotrypsin and *E. carinatus* venom was pre-incubated with different concentration of NNAh for 5 min prior addition of substrate. Activity units were calculated by defining one unit as the increase in absorbance by 0.01 at respective wavelengths per minute.

**Data analysis**

Experiments were performed three times independently and the results are expressed as mean ± SD. Statistical analysis was carried out by Student’s *t*-test and by employing statistical package GraphPad Prism® (La Jolla, USA). The results were considered significant if *p* value is ≤ 0.05.
Results

Most cases of viperbite-induced local manifestations including hemorrhage and myotoxicity are persistent and progressive resulting in extensive tissue damage. Hemorrhage results from SVMPs mediated extracellular matrix (ECM) degradation followed by microvasculature damage. On the other hand certain Kunitz-type serine protease inhibitors found in snake venom are known to exert their action on MMPs. SVMPs being the close member of MMPs can also be a target for these inhibitors and which could further result in prevention of SVMPs-induced hemorrhage. Since Kunitz-type proteins are abundantly present in the venom of elapid species, searching for SVMPs inhibitors in the venom of elapidae seems practically feasible. Hence, present study is an attempt to screen and purify protease inhibitor from the venom of Indian elapid snakes such as *B. caeruleus* and *N. naja* which can also inhibit SVMP-induced local tissue damage such as hemorrhage and myonecrosis.

*N. naja venom but not B. caeruleus venom inhibited E. carinatus venom-induced hemorrhage*

Conventionally, inhibition studies are performed initially in *in vitro* set up followed by evaluation in animal models. Here, considering the interference of high protein content and subtle amount of proteases present in the venom of elapidae species in *in vitro* assays, inhibition studies were carried out directly using animal models. *E. carinatus* venom was selected to induce hemorrhage. While screening, *N. naja* venom dose dependently inhibited *E. carinatus* venom-induced hemorrhage whereas *B. caeruleus* venom failed to bring out inhibition [Figure 2.01 (A)]. When 4 µg of *E. carinatus* venom co-injected with different concentrations of *N. naja* venom (0.9 to 3.6 µg) intradermally to the skin of experimental animals, complete inhibition of *E. carinatus* venom-induced hemorrhage was observed at a concentration of 3.6 µg
of *N. naja* venom used for inhibition study [Figure 2.01 (B) (i)]. The inhibition offered by *N. naja* venom was quantitatively expressed [Figure 2.01 (B) (ii)]. Histopathological examination of *E. carinatus* venom alone injected group as well as *N. naja* venom co-injected with *E. carinatus* venom group also showed marked differences [Figure 2.01 (B) (iii)]. In view of the potent inhibitory potential of *N. naja* venom towards *E. carinatus* venom induced hemorrhage, we carried out fractionation of *N. naja* venom by combination of gel filtration and ion exchange chromatography to identify the active principle(s) exhibiting anti-hemorrhagic effect.

**Sephadex G-75 Peak 1 of *N. naja* venom inhibited *E. carinatus* venom-induced hemorrhage**

*N. naja* venom (240 mg) resolved into four peaks upon fractionation on a Sephadex G-75 column [Figure 2.02 (A) (i)]. Individual peaks were pooled separately and recovery of protein in each peak is represented in histogram [Figure 2.02 (A) (ii)]. Anti-hemorrhagic activity was evaluated in all the four peaks of Sephadex G-75 elution revealed, Peak 1 but not other fractions inhibited *E. carinatus* venom-induced hemorrhage. The response was dose dependent and complete inhibition of hemorrhage was achieved at a concentration of 2.25 µg of G-75 Peak 1 used for the study [Figure 2.02 (B) (i)]. Hemorrhagic spots were quantified and area of inhibition was plotted [Figure 2.02 (B) (ii)]. Histopathological studies revealed the restoration of the intactness in dermal layer and basement membrane of the skin sections in a dose dependent administration of G-75 Peak 1 along with *E. carinatus* venom in comparison to saline injected control [Figure 2.02 (B) (iii)].

**CM-Sephadex Peak 1 inhibited *E. carinatus* venom-induced hemorrhage**

Further purification of anti-hemorrhagic protein was carried out by sub-fractionating the G-75 Peak 1 (22.5 mg) of *N. naja* venom on a CM-Sephadex C-50
column which resolved into five peaks [Figure 2.03 (A) (i)]. Individual peaks were pooled separately and recovery of protein in each peak is represented in histogram [Figure 2.03 (A) (ii)]. Upon evaluating the anti-hemorrhagic activity in all the peaks eluted using CM sephadex column, Peak 1 showed dose dependent inhibition of *E. carinatus* venom-induced hemorrhage and complete inhibition was observed at a concentration of 1.8 µg [Figure 2.03 (B) (i)]. Quantification of *E. carinatus* venom-induced hemorrhage and its inhibition by CM-Sephadex Peak 1 is shown in Figure 2.03 (B) (ii). Further, histopathological examination of mice skin transverse sections confirmed the anti-hemorrhagic activity of CM-Sephadex Peak 1. Mice skin and tissue section injected alone with CM-Sephadex Peak 1 showed no hemorrhage or degradation of basement membrane and dermal layer [Figure 2.03 (B) (iii)].

**DEAE-Sephadex Peak 4 inhibited *E. carinatus* venom-induced hemorrhage**

Complete purification of anti-hemorrhagic protein was done by fractionation of CM-Sephadex Peak 1 (2.5 mg) on a DEAE-Sephadex A-50 column which resolved into four peaks [Figure 2.04 (A) (i)]. Individual peaks were pooled separately and recovery of protein in each peak is represented in histogram [Figure 2.04 (A) (ii)]. Anti-hemorrhagic activity in all the peaks was assessed; DEAE-Sephadex Peak 4 showed dose dependent inhibition of *E. carinatus* venom-induced hemorrhage and complete inhibition was observed at a concentration of 0.6 µg [Figure 2.04 (B) (i)]. Area of inhibition was quantified and plotted [Figure 2.04 (B) (ii)]. Histopathological examinations of mice skin transverse sections also confirmed the anti-hemorrhagic activity of DEAE Peak 4 where intact dermal layer and basement membrane can be observed comparable to saline injected animal. In contrast, disorganized dermal layer and basement membrane of skin was observed in *E. carinatus* venom-injected animal [Figure 2.04 (B) (iii)].
SDS PAGE and RP-HPLC

Homogeneity and molecular mass of DEAE Peak 4 was determined by SDS-PAGE under non-reducing condition. Results demonstrate the homogeneity of DEAE Peak 4 with the average molecular mass being around 44 kDa [Figure 2.05 (i)]. Sharp symmetrical chromatographic peak of DEAE Peak 4 in RP-HPLC provided an additional evidence for its homogeneity [Figure 2.05 (ii)]. Based on homogeneity and anti-hemorrhagic activity of DEAE Peak 4, it was named as NNAh (*Naja naja* Anti-hemorrhagic protein). Increase in fold anti-hemorrhagic activity in each chromatographic step and percentage yield in the peak of interest is provided in table 2.01 as comprehensive summary of protein purification. Under reducing conditions NNAh resolved as a single band which designates its single polypeptide structure.

**NNAh inhibited myonecrotic activity of *E. carinatus* venom**

Apart from hemorrhage, proteases present in *E. carinatus* venom also contribute to other pathological effects such as myonecrosis. Hence inhibitory potential of NNAh against *E. carinatus* venom-induced myotoxicity was evaluated. Upon co-injection of *E. carinatus* venom with various dose of NNAh to thigh muscle of animal showed concentration dependent inhibition of myonecrosis by NNAh which can be seen in histopathology [Figure 2.06 (A) (i)]. Further, evaluation of creatine kinase activity in serum of animals showed dose dependent reduction in the activity which was parallel to histopathology [Figure 2.06 (A) (ii)]. NNAh alone injected animal muscle section as well as serum creatine kinase activity was almost similar to that of saline injected control animal. Other than proteases, PLA$_2$s which are present in certain viperid snake venom can also contribute for muscle related pathologies for which they are termed as myotoxic PLA$_2$s. Inhibitory potential of NNAh against PLA$_2$ activity of *E. carinatus* was tested by carrying out both *in vitro* and *in vivo*
assays. Results obtained demonstrate that there was no inhibition offered by NNAh against PLA₂ activity of *E. carinatus* venom in *in vitro* experiments (Table 2.02). To acquire an additional confirmation, edema inducing activity of *E. carinatus* venom was assessed in presence of NNAh. In line with *in vitro* results, no inhibition of *E. carinatus* venom-induced edema was observed (Table 2.02).

**NNAh inhibited collagenolytic and gelatinolytic activities of *E. carinatus* venom**

SVMPs present in *E. carinatus* venom are the key factors which initially targets ECM proteins upon envenomation leading to destabilization of blood vessels which otherwise results in pathologies such as myonecrosis and hemorrhage. Collagen being the major component in the ECM is highly susceptible for degradation by SVMPs upon envenomation. Gelatin is a hydrolysate of collagen and hence selected to further evaluate the inhibition offered by NNAh against *E. carinatus* venom metalloprotease(s). Results obtained demonstrate dose dependent inhibition of gelatinolytic activity of *E. carinatus* venom by NNAh [Figure 2.06 (B) (i)]. Further, NNAh alone did not show any proteolytic activity. Intensity of the activity analysed by image J software aided in representing the inhibition quantitatively. Partial loss of β, α₁ and α₂ bands of collagen was evident in the presence of *E. carinatus* venom, whereas degraded products were not clearly observed in presence of NNAh suggesting inhibition of collagenolytic activity [Figure 2.06 (B) (ii)].

**Peptide mass fingerprinting and de-novo sequencing of NNAh**

Amino acid sequence of the protein is necessary to model the structure and evaluate the inhibitory mechanism. Hence, tryptic digestion and peptide mass fingerprinting of NNAh was carried out. BLAST search of the tryptic peptide sequences belonging to NNAh in the NCBI database exhibited significant similarity with Kunitz-type protease inhibitors from other species such as *Dendroaspis angusticeps*
(NCBI accession- P00980), *Daboia siamensis* (AFA90080), *N. naja* (P20229) and *Dendroaspis polylepis polylepis* (P00983) [Figure 2.07 (i)]. Further, MS/MS spectra and m/z ratio of tryptic digested peptides of NNAh are shown in Figure 2.07 (ii) and table 2.03 respectively. Accession numbers with the matched sequence of the proteins is provided in table 2.04.

**NNAh inhibited caseinolytic and esterolytic activities of *E. carinatus* venom**

Peptide mass fingerprinting revealed that NNAh exhibited homology with Kunitz-type proteins and rationally should inhibit serine protease activity; therefore, inhibitory potential of NNAh against trypsin, chymotrypsin and *E. carinatus* venom was assessed using casein and different synthetic substrates. Casein being common substrate for proteases was initially selected to evaluate the inhibitory potential of NNAh against *E. carinatus* venom proteolytic activity. Results obtained demonstrated that 2 µg of NNAh completely inhibited caseinolytic activity of *E. carinatus* venom [Figure 2.08 (A)]. BAEE and BAPNA are commonly used substrates to study the kinetics of trypsin. NNAh failed to inhibit BAEE and BAPNA hydrolysis by trypsin even at a highest tested concentration of 2 µg used for inhibition [Figure 2.08 (C) (i) & Figure 2.08 (D) (i)]. In contrast, NNAh strongly inhibited BTEE hydrolysis of chymotrypsin in a dose dependent manner [Figure 2.08 (B) (i)]. Mild inhibition of hydrolysis of chromogenic substrates by *E. carinatus* venom in presence of 2 µg of NNAh was observed [Figure 2.08 (B) (C) (D) (ii)]. Further, NNAh alone did not hydrolyse the chromogenic substrates. These results suggest that NNAh being a Kunitz-type protein strongly inhibited chymotrypsin whereas mildly inhibited esterolytic activities of *E. carinatus* venom.
**Discussion**

Snake venoms are complex mixtures of pharmacologically active proteins and peptides. Some of these proteins exhibit enzymatic properties, whereas others are considered non-enzymatic. Existing are thirteen well-recognized families of non-enzymatic proteins in snake venoms and one of the well studied family is protease inhibitors (McCleary and Kini, 2013). Though numerous protease inhibitors have been reported from snake venoms which frequently belong to family of Kunitz-type serine protease inhibitor, till date there are no reports showing the existence of SVMP inhibitor in the venom of snakes. However, inhibition of catalytic activity of MMPs by some of the Kunitz-type serine protease inhibitors cloned from the cDNA library of the snake venom gland is evident (Chou et al., 2010; Inagaki et al., 2012). Since MMPs and SVMPs shares common catalytic features and falls into the M12B clan of metalloprotease database (MEROPS), inhibition of SVMPs by these inhibitors seems biologically plausible. Further, *N. naja* and *B. caeruleus* venoms also contains Kunitz-type proteins which are found to be inhibitors of trypsin and chymotrypsin. Based on these facts, the present study was aimed at screening for an SVMP inhibitor from the venom of Indian elapid snakes *N. naja* and *B. caeruleus*.

Hemorrhage is the most frequently observed pathology associated with SVMPs. In order to study the anti-hemorrhagic activity of *N. naja* venom, we selected *E. carinatus* venom to induce hemorrhage due to its strong hemorrhagic and myonecrotic activities which are mostly attributed to metalloproteases (Nanjaraj Urs et al., 2015a). *N. naja* venom but not *B. caeruleus* venom dose dependently inhibited *E. carinatus* venom-induced hemorrhage (Figure 2.01). *N. naja* venom is extremely neurotoxic in nature whereas *E. carinatus* is hemotoxic. Exacerbated pathological effects are expected when two toxic venoms such as *E. carinatus* and *N. naja* are
injected together. Lethal toxicity (LT$_{50}$, i.p) of the N. naja venom and E. carinatus as determined by our previous study are 0.58 mg/kg and 2.21 mg/kg body weight respectively (Hiremath et al., 2016). Concentration of both venoms used in the study was lesser than the sub-lethal dose and hence no mortality was observed during the course of experiment and no neurotoxic symptoms were noticed. Anti-hemorrhagic activity of N. naja venom speculated the existence of SVMP inhibitor. Although earlier report indicates the synthesis of SVMP inhibitor(s) by Bothrops jararaca snake venom gland (Luna et al., 2013), present work is the first report on SVMP inhibition by N. naja venom.

Varied molecular mass of snake venom toxins encouraged us to fractionate the N. naja venom on size exclusion chromatography which resolved into four peaks [Figure 2.02 (A) (i)]. Among the peaks, Peak 1 inhibited E. carinatus venom-induced hemorrhage [Figure 2.02 (B) (i)]. Till date, protease inhibitors reported from snake venoms are of low molecular mass (50-67 amino acid residues) (Mourao and Schwartz, 2013). In contrast, anti-hemorrhagic protein in N. naja venom is probably a high-molecular mass in nature. Further, complete purification of anti-hemorrhagic protein was achieved by combination of cation and anion exchange columns. Anti-hemorrhagic activity observed in Peak 4 (named as NNAh) of the anion exchange elution was used to determine the molecular weight and homogeneity of the protein.

Hemorrhagins present in snake venoms are predominantly metalloproteases which essentially act on ECM components and leads to uncontrolled hemorrhage and myotoxicity. Myofibrillar compartments rich in proteins are susceptible for hydrolysis by metalloproteases present in the venom of E. carinatus which further leads to damage in the intactness of myocytes eventually resulting in the leakage of cell contents into the surrounding environment (Nanjaraj Urs et al., 2015b). Further,
Elevated levels of creatine kinase activity in serum of patients as a consequence of myonecrosis. NNAh dose dependently inhibited myonecrotic activity of *E. carinatus* venom which was evident by histopathology of injected muscle tissue and creatine kinase activity in serum of treated animals [Figure 2.06 (A) (i) & (ii)]. Extensive hydrolysis of collagen, a major structural protein of the ECM is the hallmark of viper-bite. This biochemical process disturbs the mechanical stability of the vessel walls leading to the extravasation of circulating cells resulting in uncontrolled hemorrhage and hypoxia (Gutierrez et al., 2016). NNAh dose dependently inhibited gelatinolytic and collagenolytic activities of *E. carinatus* venom providing additional evidence for the anti-hemorrhagic and anti-myonecrotic effects [Figure 2.06 (B) (i) & (ii)].

Other than proteases, several PLA$_2$s also have strong myonecrotic properties. Though small in mass, PLA$_2$s possess pharmacological site along with the active site. These toxins have different tissue targets, which is determined by the pharmacological site and two of the most frequently induced pathological effects of venom PLA$_2$s are neurotoxicity and myotoxicity (Kini, 2003). NNAh did not inhibit the PLA$_2$ activity of *E. carinatus* venom (Table 2.02) suggesting that inhibition of pathological effects such as hemorrhage and myonecrosis by NNAh is due to inhibition of metalloprotease(s) activity.

Peptide mass fingerprinting has been evolved as a major technique to determine the sequences of protein particularly in scenarios where protein yield is very less and complete N-terminal sequence determination is not possible due to requirement of large protein quantities (Thiede et al., 2005). BLAST search of the tryptic peptide sequences of NNAh in the NCBI database demonstrated significant
match with Kunitz-type serine protease inhibitors (Figure 2.07). As mentioned earlier, Kunitz-type proteins such as NNAh present in snake venoms mainly disturbs hemostasis and blood pressure which further facilitates bleeding and rapid diffusion of toxins respectively (Inagaki, 2015). According to the nomenclature and activity, Kunitz-type serine protease inhibitors are proteins with serine protease inhibitory activity and so far reported inhibitors are of small molecular mass in the range of 6-15 kDa. However, a few large molecular mass Kunitz-type inhibitors like NNAh are also reported (Vonk et al., 2013).

Kunitz-type protease inhibitors with positively charged residues at P1 position of the scissile bond tend to inhibit trypsin, while those with hydrophobic residues at P1 position tend to inhibit chymotrypsin (Laskowski and Kato, 1980). Upon evaluation of NNAh inhibitory potency towards trypsin and chymotrypsin, it significantly inhibited chymotrypsin activity in a dose dependent manner [Figure 2.08 (B) (i)]. Majority of the chymotrypsin inhibitors reported so far from snake venoms exhibits the inhibition due to the presence of strong hydrophobic residue at P1 position (Millers et al., 2009). Even in case of NNAh, this would appear to be likely reason for the dual inhibition of chymotrypsin as well as metalloprotease. For instance, BaP1, a metalloprotease isolated from the venom of Bothrops asper has shown to be greatly inhibited by the presence of hydrophobic thiazole group at P1 position in the peptide inhibitor (Lingott et al., 2009). Though chymotrypsin was inhibited strongly by NNAh, mild inhibition of esterolytic activity of E. carinatus venom was observed [Figure 2.08 B, C, D (ii)]. The probable reason would be the resistance of snake venom serine proteases (SVSPs) to natural inhibitors. Majority of SVSPs are insensitive to natural inhibitors such as anti-thrombin-III and aprotinin. For example, TSV-PA, plasminogen activator from Trimeresurus stejnegeri venom is not
inhibited by endogenous serine protease inhibitors (Serrano and Maroun, 2005). Together these results suggest that NNAh being a Kunitz-type protein inhibited chymotrypsin activity as well as metalloprotease activities of *E. carinatus* venom. Speculated presence of hydrophobic residue at P1 position may be the reason for the observed dual inhibition of chymotrypsin and metalloprotease(s) by NNAh. Further, complete sequence of the protein is required to study the mechanism of inhibition in detail. However, strong inhibition offered by NNAh against hemorrhage and myonecrosis can become a lead for the drug design to counteract the local tissue damage upon snake-bite.

**Conclusion**

Findings of the present study describe the purification of a Kunitz-type protease inhibitor from *N. naja* venom by combination of three successive chromatographic steps. Isolated protein shows inhibitory activity towards *E. carinatus* venom protease(s)-induced hemorrhage and named as NNAh. Further, NNAh resolved as 44 kDa protein in SDS-PAGE and single peak in RP-HPLC demonstrated its homogeneity. Collagen and gelatin hydrolysis by *E. carinatus* venom and its inhibition by NNAh provided the molecular basis for anti-hemorrhagic and anti-myonecrotic effects. Upon peptide mass fingerprinting analysis of NNAh, majority of hits were with Kunitz-family of proteins and strongly inhibited chymotrypsin but failed to inhibit trypsin activity. To our knowledge, NNAh is the first report on a high molecular mass anti-hemorrhagic protein from the snake venom. In future, the complete characterization of protein and detailed studies on mechanism of inhibition enlighten the possible therapeutic use of NNAh against SVMPs-induced local tissue damage.
Figure 2.01. Screening for inhibition of *E. carinatus* venom-induced hemorrhage by *B. caeruleus* and/or *N. naja* venom. Mice were co-injected intradermally with constant 4 µg of *E. carinatus* venom along with various doses of *B. caeruleus* or *N. naja* venom (0.9-3.6 µg) and further assay was performed as described in the methods section. **A:** (a) Saline, (b) *E. carinatus* venom alone, (c, d and e) *E. carinatus* venom with 0.9 µg, 1.8 µg and 3.6 µg of *B. caeruleus* venom respectively and (f) *B. caeruleus* venom (3.6 µg) alone. **B:** (i) Dorsal surface of the skin showing hemorrhagic spot (ii) Area of hemorrhagic spots (iii) Photomicrographs of mice skin transverse sections: (a) Saline, (b) *E. carinatus* venom alone, (c, d and e) *E. carinatus* venom with 0.9 µg, 1.8 µg and 3.6 µg *N. naja* venom respectively and (f) *N. naja* venom (3.6 µg) alone. The tissue sections were photographed at 10 X magnification. (a) Saline injected control section shows intact dermal layer (D), basement membrane (BM) and muscle (M) layer. (b) *E. carinatus* venom injected section shows damaged dermis, basement membrane and muscle layer. The dark arrow head point the damaged portion and the light arrow head point the reduction in damage due to inhibition. *** *p* <0.01 and ** *p* < 0.05 compared to *E. carinatus* venom-induced hemorrhage.
Figure 2.02: First step purification of protease inhibitor from *N. naja* venom and its anti-hemorrhagic property.

**A:** (i): **Elution profile of Sephadex G-75 column:** Crude *N. naja* venom (240 mg) was loaded onto pre-equilibrated Sephadex G-75 column. Elution was carried out as described in methods section. Protein elution was monitored at 280 nm (solid line) and anti-hemorrhagic activity was assayed after pooling the peaks (dotted line). P in the graph indicates peak numbers. (ii): **Recovery of protein (mg) in each peak.**

**B:** **Hemorrhagic activity of *E. carinatus* venom and its inhibition by G-75 Peak 1 of *N. naja* venom (G-75 P1);** (i) Dorsal surface of the skin showing hemorrhagic spot (ii) Area of hemorrhagic spots measured using graph sheet (iii) **Photomicrographs of mice skin transverse sections:** Mice were co-injected intradermally with constant 4 µg of *E. carinatus* venom along with various doses of G-75 P1 (0.75-2.25 µg) and further assay was performed as described in the methods section. (a) Saline, (b) *E. carinatus* venom alone, (c, d and e) *E. carinatus* venom with 0.75 µg, 1.5 µg, 2.25 µg of G-75 P1 respectively and (f) G-75 P1 (2.25 µg) alone. The dark arrow head point the damaged portion and the light arrow head point the reduction in damage due to inhibition. *** p<0.01 compared to *E. carinatus* venom-induced hemorrhage.
Figure 2.03: Second step purification of protease inhibitor from *N. naja* venom and its anti-hemorrhagic property.

A: (i); Elution profile of CM-Sephadex C-50 column: Peak 1 fraction from Sephadex G-75 chromatography was loaded onto CM-Sephadex C-50 column and the fractions were eluted as described in methods section. Protein elution was monitored at 280 nm (solid line) and anti-hemorrhagic activity was assayed after pooling the peaks (dotted line), P in the graph indicates peak numbers. (ii); Recovery of protein (mg) in each peak.

B: Hemorrhagic activity of *E. carinatus* venom and its inhibition by CM-Sephadex Peak 1 of *N. naja* venom (CMS P1); (i) Dorsal surface of the skin showing hemorrhagic spot (ii) Area of hemorrhagic spots measured using graph sheet (iii) Photomicrographs of mice skin transverse sections: Mice were co-injected intradermally with constant 4 µg of *E. carinatus* venom along with various doses of CMS P1 (0.6-1.8 µg) and further assay was performed as described in the methods section. (a) Saline, (b) *E. carinatus* venom alone, (c, d and e) *E. carinatus* venom with 0.6 µg, 1.2 µg, 1.8 µg of CMS P1 and (f) CMS P1 (1.8 µg) alone. The dark arrow head point the damaged portion and the light arrow head point the reduction in damage due to inhibition. *** *p* <0.01 and ** *p* < 0.05 compared to *E. carinatus* venom-induced hemorrhage.
Figure 2.04: Final step purification of protease inhibitor from *N. naja* venom and its anti-hemorrhagic property.

**A:** (i): *Elution profile of DEAE-Sephadex A-50 column:* Peak 1 fraction from CM-Sephadex C-50 column was loaded onto DEAE-Sephadex A-50 column and the fractionation was carried as described in methods section. Protein elution was monitored at 280 nm (solid line) and anti-hemorrhagic activity was assayed after pooling the peaks (dotted line). P in the graph indicates peak numbers. (ii): *Recovery of protein (mg) in each peak.*

**B:** *Hemorrhagic activity of *E. carinatus* venom and its inhibition by DEAE-Sephadex Peak 4 of *N. naja* venom (DEAE P4); (i) Dorsal surface of the skin showing hemorrhagic spot (ii) Area of hemorrhagic spots measured using graph sheet (iii) Photomicrographs of mice skin transverse sections: Mice were co-injected intradermally with constant 4 µg of *E. carinatus* venom along with various doses of DEAE P4 (0.2-0.6 µg) and further assay was performed as described in the methods section. (a) Saline, (b) *E. carinatus* venom alone, (c, d and e) *E. carinatus* venom with 0.2 µg, 0.4 µg, 0.6 µg of DEAE P4 and (f) DEAE P4 (0.6 µg) alone. The dark arrow head point the damaged portion and the light arrow head point the reduction in damage due to inhibition. ***p<0.01 and **p < 0.05 compared to *E. carinatus* venom-induced hemorrhage.
Figure 2.05: Determination of homogeneity and molecular mass of DEAE Peak 4 by SDS-PAGE and RP-HPLC:

(i) SDS-PAGE of *N. naja* venom and its chromatographic fractions: Electrophoresis was carried out on a 10% gel under non reducing conditions; lanes 1-4 were loaded with 50 μg each of crude *N. naja* venom, Sephadex G-75 P1, CM-sephadex P1 and DEAE-sephadex P4 (Purified anti-hemorrhagic protein; NNAh). Lane M represents the molecular weight markers from 98 to 16 kDa.

(ii) RP-HPLC of NNAh: Sunfire™ C18 (5 μm, 0.46 × 25 cm) column, pre-equilibrated with 0.1% TFA in water was used for elution. Protein was eluted using a 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 0.5 ml/min and monitored at 280 nm.
Figure 2.06: Inhibition of myotoxic activity of *E. carinatus* venom by NNAh:
A (i) Histopathology of skeletal muscle (ii) serum creatine kinase (CK) activity: Mice were co-injected intramuscularly with constant 5 µg of *E. carinatus* venom along with various doses of NNAh (0.2-0.6 µg) and further assay was performed as described in the methods section. The tissue sections were photographed at 10 X magnification. (a) Saline injected control section shows intact muscular striations and myocytes, (b) *E. carinatus* venom injected section shows damaged muscular striations and myocytes. (c, d & e) *E. carinatus* venom with 0.2 µg, 0.4 µg and 0.6 µg NNAh respectively and (f) NNAh (0.6 µg) alone. The dark arrow head point the damaged portion and the light arrow head point the reduction in damage due to inhibition. **p < 0.05 compared to *E. carinatus* venom-induced myotoxicity. Histogram represents the serum CK activity of the animals in the same order.
B: Gelatinolytic and collagenolytic activity of *E. carinatus* venom and its inhibition by NNAh; (i) Gelatinolytic activity and inhibition: 0.2% of gelatin was incorporated into 10% resolving gel. *E. carinatus* venom (5 µg) pre-incubated with various concentrations of NNAh were subjected to electrophoresis. Clear zones in the gel indicate the substrate hydrolysis due to enzyme activity. Lanes, 1: 5 µg *E. carinatus* venom, 2, 3 and 4: NNAh (0.5, 1 and 2 µg) pre-incubated for 5 min with constant 5 µg *E. carinatus* venom respectively, 5: 2 µg of NNAh alone. Intensity of gelatinolytic activity in each lane was measured using software image J (NIH, Bethesda, USA) to quantitate the inhibition. (ii) Collagenolytic activity and inhibition: 50 µg of collagen was incubated with 5 µg of *E. carinatus* venom with various concentrations of NNAh in 50 mmol/L Tris HCl buffer pH 7.6 for 2 h and cleavage pattern was analyzed using 7.5% SDS-PAGE under reducing condition. Lanes, 1: Collagen alone, 2: collagen + 5 µg *E. carinatus* venom, 3, 4 and 5: collagen + *E. carinatus* venom + 0.5, 1 and 2 µg of NNAh respectively, 6: collagen + 2 µg NNAh.
Figure 2.07: Partial sequence of NNAh aligned with homologous sequences from snake venom using Clustal Omega: (i) Alignment of the peptides obtained using peptide mass fingerprinting against amino acid sequences of protease inhibitors from other species showing highest homology. Accession numbers of the protein sequences are provided in table 02. * Indicates identical residues in all sequences; (:) highly conserved and (.) moderately conserved. (ii) Chromatogram of in gel tryptic digested peptides of NNAh analysed using MALDI-TOF mass spectrometry.
Figure 2.08: Caseinolytic and esterolytic activity of *E. carinatus* venom and its inhibition by NNAh;

**A; Caseinolytic activity and inhibition:** Reaction mixture (1 mL) contained 0.4 ml of casein (2%) in 0.2 mmol/L Tris-HCl buffer pH 8.5 was incubated for 150 min at 37 °C with 10 µg of *E. carinatus* venom and various concentrations of NNAh (0.5 to 2 µg).

**B; (i) Hydrolysis of BTEE by chymotrypsin (chymo) and its inhibition by NNAh:** Reaction mixture (0.5 mL) contained 40 mmol/L Tris-HCl buffer (pH 7.8), 0.58 mmol/L BTEE with 5 µg of chymo and various concentrations of NNAh (0.5 to 2 µg) and monitored for 5 min at 256 nm. *** p<0.01 compared to Chymo activity. (ii) Hydrolysis of BTEE by *E. carinatus* venom (ECV) and its inhibition by NNAh.

**C; (i) Hydrolysis of BAEE by trypsin (tryp) and its inhibition by NNAh:** Reaction mixture (0.5 mL) contained 40 mmol/L Tris-HCl buffer (pH 7.8), 0.23 mmol/L BAEE with 5 µg of tryp and various concentrations of NNAh (0.5 to 2 µg) and monitored for 5 min at 253 nm. (ii) Hydrolysis of BAEE by ECV and its inhibition by NNAh.

**D; (i) Hydrolysis of BAPNA by tryp and its inhibition by NNAh:** Reaction mixture (0.5 mL) contained 40 mmol/L Tris-HCl buffer (pH 7.8), 0.7 mmol/L BAPNA with 5 µg of tryp and various concentrations of NNAh (0.5 to 2 µg) and monitored for 5 min at 405 nm. (ii) Hydrolysis of BAPNA by ECV and its inhibition by NNAh.
Tables

Table 2.01: Summary of purification of NNAh from *N. naja* venom

<table>
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<th>Procedure</th>
<th>Loaded Protein (mg)</th>
<th>Total protein in peak of interest (mg)</th>
<th>Anti-hemorrhagic activity</th>
<th>Protein recovery (%)</th>
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Table 2.02: PLA$_2$ Activity of *E. carinatus* venom (ECV) in presence of NNAh

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<td>ECV + NNAh (2 µg)</td>
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(*) Dose of ECV used for radio labelled substrate hydrolysis was 10 µg whereas to study paw edema ratio it was 4 µg.
Table 2.03: MALDI-TOF MS identified peptides of NNAh with area and intensity

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