CHAPTER 3

Partial biochemical characterization of crude *Naja kaouthia* venom and determination of its cytotoxic activity against cancer cell lines

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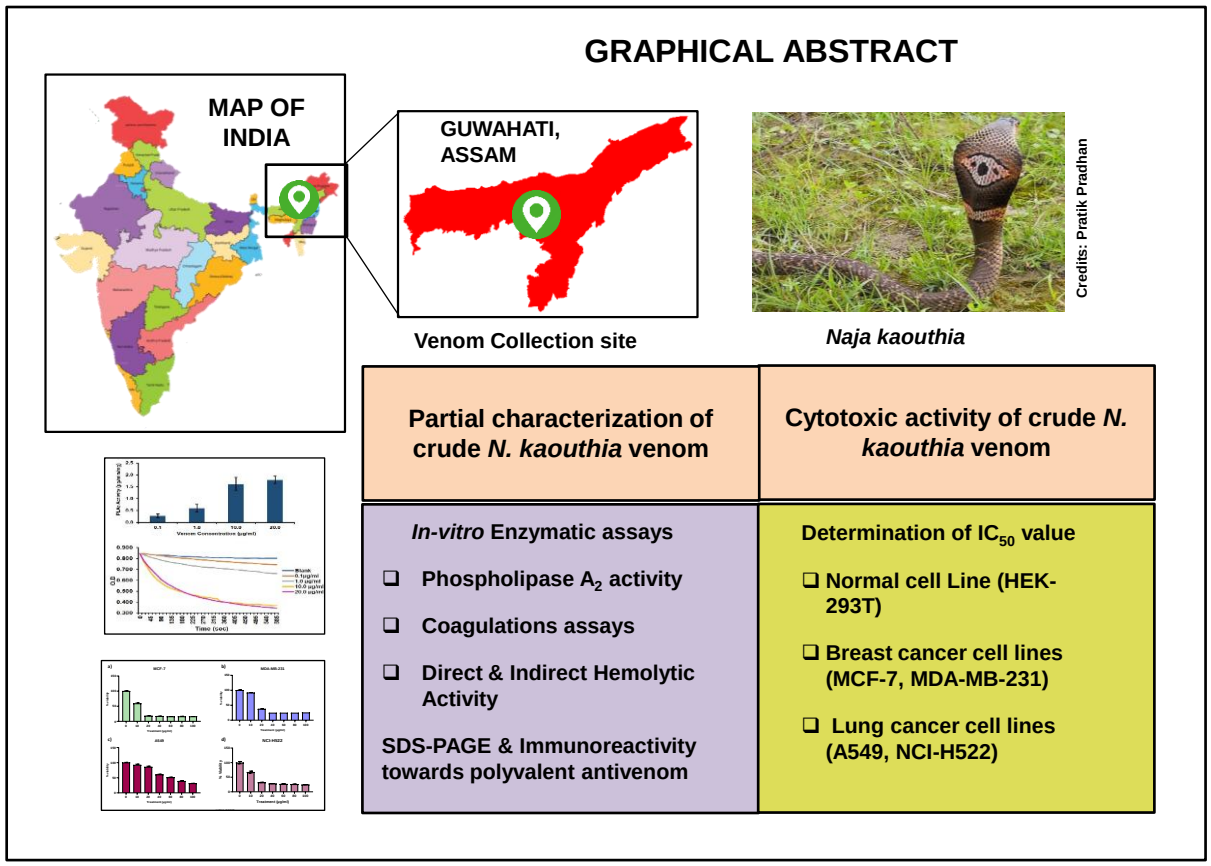

3.1 Introduction

India, known for its rich biodiversity, accommodates numerous venomous snakes, such as cobras. The "Big Four" snakes which include the Indian cobra (*Naja naja*), Russell's viper (*Daboia russelli*), common krait (*Bungarus caeruleus*), and the saw scaled viper (*Echis carinatus*) are the four venomous snake species that are of medical importance and are responsible for causing the greatest number of fatal bites in the Indian subcontinent [260]. However, many other venomous snake species from different geographical regions of India are also responsible for mild envenomation or fatality, which includes the Monocled cobra (*N. kaouthia*). The venom of *N. kaouthia* is neurotoxic and results in blistering and necrosis at the local bite site in envenomated

victims. Common symptoms of envenomation include ptosis, dysphagia, broken neck syndrome, hypertension, and respiratory paralysis which sometimes lead to death in the absence of timely and appropriate treatment [236,237].

Therefore, in this chapter we tried to understand the biochemical profile of *N. kaouthia* venom of North-East India, especially the cytotoxic potential towards breast and lung cancer cell lines.

3.2 Materials

3.2.1 Snake venom

Crude *N. kaouthia* venom was collected from North-East India (Guwahati, Assam) after obtaining necessary permission for milking the snake from the Chief Conservator of Forest (Wildlife) and Chief Wildlife Warden of Assam *via* letter no. WL/FG.27/tissue collection/09 dated 19th Aug 2011. The collected venom was lyophilized and stored at -20 ºC till use.

3.2.2 Chemical and reagents

Uniplastin and Liquicelin-E were purchased from Tulip Diagnostics Pvt. Ltd., India. Bovine serum fibrinogen, Sodium chloride, Sodium bicarbonate, Sodium lauryl sulphate (SDS) and Trisodium citrate of analytical grade, glycerol, acrylamide, bisacrylamide, glycine, tris base, bromophenol blue, glacial acetic acid, methanol, Ammonium Per Sulfate (APS), N,N,N´N´-Tetramethylethylenediamine (TEMED), ethanol, 2-mercaptoethanol (BME) and Coomassie brilliant blue R-250 were obtained from Merck (Sigma Aldrich, USA). Cell lines MCF-7, MDA-MB-231, A549, NCI-H522 and HEK-293T were procured from National Centre for Cell Science (NCCS), Pune, India. Fetal Bovine Serum (FBS) was purchased from Gibco, USA. Dulbecco's Modified Eagle Medium (DMEM), Trypsin-EDTA, Penicillin-Streptomycin and Mitomycin were purchased from HiMedia Laboratories, India. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma Aldrich, USA. Precision Plus protein^{TM} standard protein marker was purchased from Bio-Rad, USA.

3.2.3 Cell culture

Cell lines MCF-7, MDA-MB-231, A549, NCI-H522 and HEK-293T were maintained routinely in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C. Culture conditions provided for the experiment were atmosphere air 95% and 5% CO_2. Cells were passaged in accordance with American Type Culture Collection (ATCC) guidelines, with trypsin-EDTA (0.05% trypsin and 0.02% EDTA) and cryopreserved in its respective complete medium with 5% DMSO.

3.3 Methods

3.3.1 SDS-PAGE (Sodium dodecyl sulfate- polyacrylamide gel electrophoresis)

Crude venom of *Naja kaouthia* (Assam) was subjected to SDS-PAGE as per the methodology outlined by Laemmli et al. [261]. A 12.5% resolving gel consisting 2.5% glycerol was prepared using acrylamide/bisacrylamide. For reducing conditions, crude venom (20 µg) was incubated at 100ºC for 3 min with loading dye (5%) which is composed of β-mercaptoethanol, followed by loading into the wells of stacking gel. The electrophoresis was performed at a constant voltage of 60V in the stacking gel, and 120V in the resolving gel. After the gel was run, it was detached from the plates and 100 ml of Coomassie Brilliant Blue (0.25%) containing methanol: acetic acid: water (4:1:5 ratio) was utilized to stain the gel for 4-5 hours. Destaining was performed using 100 ml of destaining solution containing methanol: acetic acid: water (4:1:5 ratio) until the protein bands were visible. The gel/ptotein bands were visualized in a Chemidoc (BioRad, USA) and the molecular weight was estimated by comparing with the standard protein marker (10-250 kDa).

3.3.2 Phospholipase A_2 activity

The PLA_2 activity of the crude *N. kaouthia* venom was estimated using the turbidometric method outlined by Joubert and Taljaard, with modifications by Doley and Mukherjee [262,263]. Crude *N. kaouthia* venom at different concentrations of 0.1 µg/ml, 1.0 µg/ml, 10.0 µg/ml and 20.0 µg/ml initially added in a total volume of 60 µl with 20 mM Tris, pH 7.4 was added to 140 µl egg yolk substrate (one egg yolk mixed with 0.9% NaCl (250 ml)), with optical density (O.D) pre-adjusted to 1.0 at 740 nm. The decrease in absorbance was documented continuously at 15 secs interval for 10 mins at 740 nm

utilizing a spectrophotometer (MultiSkan GO, Thermo Fisher Scientific, USA). Tris-Cl (20 mM) was taken as the blank and bee venom (0.05 µg) was considered as the positive control. The PLA_2 activity (µg/min/mg) is defined as a unit reduction in absorbance by 0.01 O.D in 10 mins at 740 nm.

3.3.3 Anti-coagulant activity

The anticoagulant activity of crude *N. kaouthia* venom was determined by calculating Recalcification Time (RT), Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) using platelet poor plasma (PPP) following the methods previously described by Sharma *et al.* [264]. Crude venom of *D. russelii* was taken as positive control in each of these clotting experiments.

Preparation of platelet poor plasma (PPP):

Goat blood (45 ml) was freshly collected from a local butcher shop in a tube containing 3.8% sodium citrate (5 ml). The collected blood was centrifuged at 5000 rpm for 20 min at 4 ºC immediately after collection and the pale yellowish supernatant is pipetted out and collected as PPP and stored at -20 ºC until further use.

Recalcification time (RT):

RT was calculated using PPP as per the protocol followed by Deka et al. [245]. Crude venom of *N. kaouthia* (0.1, 1.0, 10.0 and 20.0 µg/ml) were pre-incubated with PPP (50 µl) 37 ºC for 2 min. Initiation of plasma clot was obtained by adding 50 mM $CaCl_2$ (25µl) and the absorbance shift was recorded at 10 sec intervals for 15 min at 405 nm using a MultiSkan GO Spectrophotometer (Thermo Scientific, USA). The clotting time of PPP without venom sample was taken as the Normal clotting time (NCT).

Prothrombin time (PT):

Uniplastin was used to calculate the PT according to the manufacturer's manual. Different doses of crude venom (0.1, 1.0, 10.0 and 20.0 µg/ml) were pre-incubated with goat PPP (50 µl) for 2 min at 37ºC. Initiation of plasma clot was obtained by adding Uniplastin (50 µl) and the absorbance shift was recorded at 405 nm at the interval of 2 sec for 2 min using spectrophotometer. The clotting time of PPP without venom sample was taken as the NCT.

Activated partial thromboplastin time (APTT):

Liquicelin-E was used to calculate the APTT according to the manufacturer's manual. Crude venom (0.1, 1.0, 10.0 and 20.0 µg/ml)) were pre-incubated with PPP (50 µl) and Liquecelin-E (50 µl) for 3 mins at 37 ºC. Initiation of plasma clot was obtained by adding 25 mM $CaCl_2$ (50 µl) and the absorbance shift was recorded at 2 sec intervals for 120 sec at 405 nm using a spectrophotometer. The clotting time of PPP without venom sample was taken as the NCT.

3.3.4 Hemolytic activity

The direct and indirect hemolytic activity of crude *N. kaouthia* venom was carried out following the method previously described by Das *et al*. [265].

Preparation of Red Blood Cells (RBC) suspension:

Goat blood (45 ml) was freshly obtained from a local butcher shop in a tube containing 3.8% sodium citrate (5 ml). The collected blood was centrifuged at 5000 rpm for 20 min at 4 ºC immediately after collection. The pellet containing RBCs was washed with 0.9% NaCl at 5000 rpm for 15 mins and then re-suspended in 0.9% NaCl. This step was repeated for 3-4 times.

Direct hemolytic activity

A 10% RBC suspension (150 µl) was incubated with different concentrations of crude *N. kaouthia* venom (0.1, 1.0, 10.0 and 20.0 µg/ml) and the volume of the reaction mixture was adjusted to 2 ml. This mixture was then incubated for 1 hour at 37 ºC and then centrifuged at 10,000 rpm for 10 mins. The supernatant was collected and absorbance was measured in a multiplate reader at 540 nm. RBC mixed with distilled water was taken as the positive control and the RBC suspension mixed with 0.9% NaCl was taken as negative control. Hemolysis caused by the positive control was considered 100%.

Indirect hemolytic activity

Egg yolk solution (20 µl) (chicken egg yolk suspended in solution containing 250 ml of 0.9% NaCl) was added in 10% RBC solution. Different concentrations of crude venom (0.1, 1.0, 10.0 and 20.0 µg/ml) were added to the solution and the reaction volume was

adjusted to 2 ml. RBC mixed with distilled water and egg yolk solution was considered as the positive control, whereas, RBC suspension mixed with 0.9% NaCl and 20 µl of egg yolk solution was taken as the negative control. These mixtures were then incubated for 1 hour at 37 ºC followed by centrifugation at 10,000 rpm for 10 mins. The supernatant was then collected and the absorbance was measured in a multiplate reader at 540 nm.

3.3.5 MTT Assay

In vitro cytotoxicity of crude *N. kaouthia* venom was tested against normal (HEK-293T), breast cancer (MCF-7 and MDA-MB-231) and lung cancer (A549 and NCI-H522) cell lines. The viability of the cells was determined by MTT assay [266]. Aliquots of 100 µl of optimum cells (10,000 cells per well) were seeded into 96-well microtiter plates. Following overnight incubation, cells were treated with different concentrations of crude venom (10-100 µg/ml) for 24 hours. Cells were then incubated with 10% MTT in the dark for 3-4 hours at 37 ºC post venom treatment. NADPH dependent cellular oxidoreductase enzymes in viable cells reduces MTT to purple formazan which was dissolved with MTT dissolving solution containing SDS, 0.2 M HCl and Isopropanol. Finally, data was collected by obtaining the absorbance at 595 nm using a spectrophotometer (Thermo Scientific, USA). Cell viability percentage was calculated as follows:

$$\%\ \textbf{Cell viability} = \frac{(\textbf{Absorbance of experimental sample}) - (\textbf{Absorbance of blank sample})}{(\textbf{Absorbance of untreated sample}) - (\textbf{Absorbance of blank sample})} \times 100\%$$

3.3.6 IC_{50} determination of crude *Naja kaouthia* venom

The IC_{50} value for each of the cell lines were calculated using the online tool AAT Bioquest Graph™ IC_{50} Calculator (https://www.aatbio.com/tools/ic50-calculator).

3.4 Results

3.4.1 Gel electrophoresis: SDS-PAGE

Crude venom of *N. kaouthia* (Assam) was subjected to SDS-PAGE to separate and visualize the proteins that are present based on their molecular weight (Figure 3.1a). Electrophoresis was conducted in reduced condition and molecular weight of various protein bands were determined by comparing with a pre-stained protein marker (10-250 kDa).

The crude venom was found to consist major percentage of proteins at molecular mass range of <15 kDa as evident from the intense thick band of protein smear observed at ~15 kDa (Figure 3.1). Further, a single protein band was observed between 20 to 25 kDa. Three protein bands were observed at a molecular weight range of ~150 kDa, ~75 kDa and ~50 kDa respectively (Figure 3.1). The SDS-PAGE profile of crude *N. kaouthia* venom suggests the presence of low molecular weight proteins (3FTx family, PLA_2 and its isoforms, or KSPI) and larger molecular weight proteins (LAAO or proteases).

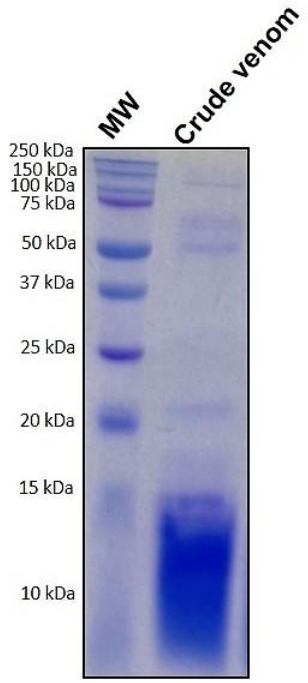

Figure 3.1: SDS-PAGE (12.5%) of *Naja kaouthia* crude venom. (MW: Precision Plus protein™ standard protein marker). 20 µg of crude venom sample was separated by 12.5 % SDS-PAGE under reducing condition and subsequently stained with Coomassie Brilliant Blue stain. Biorad Precision Plus Protein™ dual color standard was used as the protein marker.

3.4.2 Phospholipase A_2 activity

Crude venom of *N. kaouthia* exhibited a dose-dependent PLA_2 activity. The PLA_2 activity at different concentrations of 0.1, 1.0, 10.0 and 20.0 µg/ml were calculated to be 0.54, 1.01, 4.52 and 4.57 µg/min/mg respectively (Figure 3.2). The maximum effect was exhibited at 20.0 µg/ml.

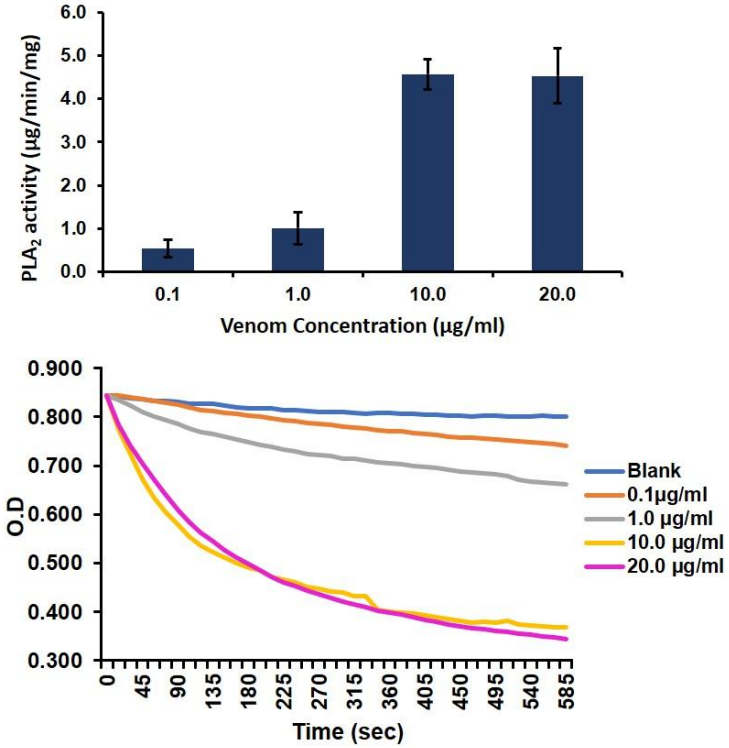

Figure 3.2: PLA_2 activity of crude *Naja kaouthia* venom from North-East India estimated using turbidometric method. Doses ranging from 0.1-20.0 µg/ml were checked for the PLA_2 activity. Absorbance was recorded at 15 sec intervals for 10 mins at 740 nm. Bee venom was taken as positive control.

3.4.3 Coagulation activity

The crude venom affected the *in vitro* coagulation time of PPP as observed in RT, APPT and PT. The results are described as follows:

Recalcification Time (RT):

N. kaouthia crude venom exhibited a concentration dependent increase in RT (*i.e.*, the time required for clotting) with respect to the NCT of PPP (Figure 3.3). At a

concentration of 0.1µg/ml, the RT was calculated to be 285 sec, which increased steadily up to 535 sec for 1.0 µg/ml and 900 sec for 10.0 µg/ml treatment, which remained constant upon further increase in dosage up to 20.0 µg/ml (900 sec). However, the NCT of PPP was calculated to be 240 sec and RT for positive control was 15 sec (Figure 3.3).

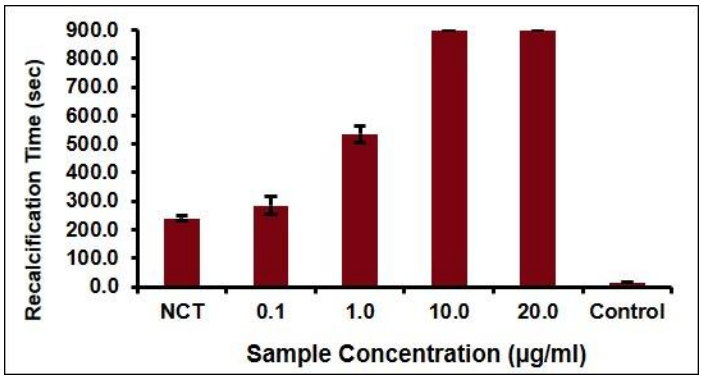

Figure 3.3: Recalcification Time of crude *Naja kaouthia* venom from North-East India. The normal clotting time (NCT) of PPP is taken as the control.

Activated Partial Thromboplastin Time (APTT):

The APTT was observed to increase steadily in a time and dose-dependent manner. At 0.1 µg/ml, it was calculated to be 28.0 sec, which further increased to 37.0 sec for 1.0 µg/ml. With a further increase in dosage, the APTT was calculated to be 79.60 sec and 106.50 sec for 10.0 µg/ml and 20.0 µg/ml respectively. The NCT of PPP was 24.70 sec whereas, APTT for the positive control was 10.10 sec (Figure 3.4).

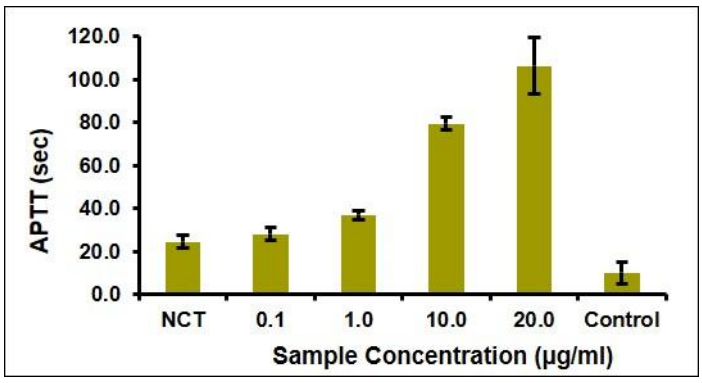

Figure 3.4: Activated Partial Thromboplastin Time of crude *Naja kaouthia* venom from North-East India. The normal clotting time (NCT) of PPP is taken as the control.

Prothrombin Time (PT):

The crude *N. kaouthia* venom exhibited a dose-dependent increase in PT. At concentrations 0.1, 1.0, 10 and 20µg/ml, the PT was calculated to be 8.7, 19.5, 35.7 and 47.6 sec respectively, whereas the NCT of PPP was 7.6 sec and PT for the positive control was 3.3 sec (Figure 3.5).

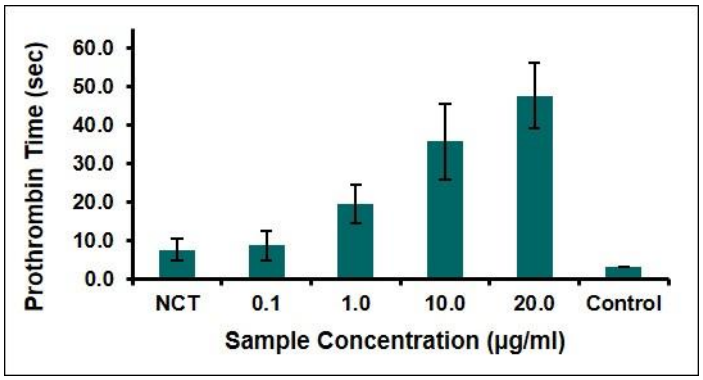

Figure 3.5: Prothrombin Time of crude *Naja kaouthia* venom from North-East India. The normal clotting time (NCT) of PPP is taken as the control.

3.4.4 Hemolytic activity

To test the cytotoxic effect of crude venom, hemolytic assay was performed using goat blood RBC. The crude venom of *N. kaouthia* when incubated in a dose-dependent manner with RBCs exhibited poor direct hemolytic activity of 5.4%, 5.8%, 7.2% and 7.6% at doses of 0.1, 1.0, 10.0 and 20.0 µg/ml respectively, as compared to the positive control (distilled water) which exhibited 100% hemolysis (Figure 3.6).

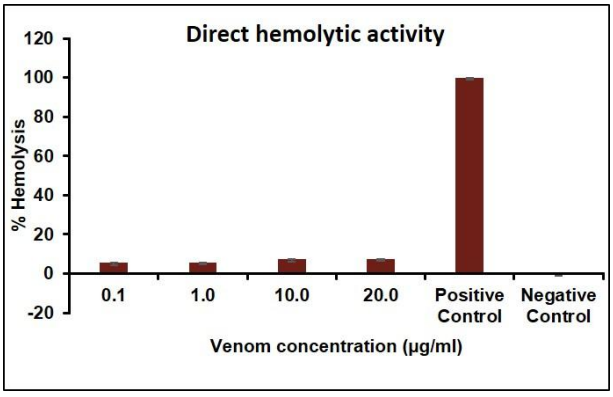

Figure 3.6: Direct hemolytic activity of crude *Naja kaouthia* venom from North-East India. Different concentrations of crude *Naja kaouthia* venom was incubated with 150 µl of 10% RBC suspension and volume adjusted to 2 ml. RBCs mixed with distilled water was taken as the positive control. RBC suspension with 0.9% NaCl was taken as negative control.

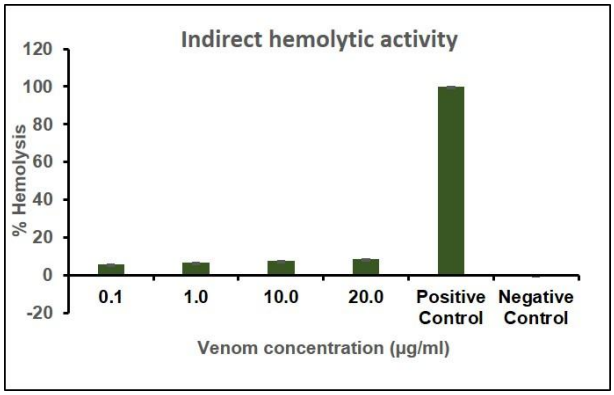

Figure 3.7: Indirect hemolytic activity of crude *Naja kaouthia* venom from North-East India. Different concentrations of crude *Naja kaouthia* venom was incubated with 150 µl of 10% RBC suspension and 20 µl of egg yolk solution and volume adjusted to 2 ml. RBCs mixed with egg yolk solution and distilled water was taken as the positive control. RBC suspension with 0.9% NaCl was taken as negative control.

Indirect hemolytic assay is an indirect method of assessing the phospholipase A_2 activity. The crude venom samples exhibited low indirect hemolytic activity of 5.7%, 6.6%, 7.5% and 8.6% at doses of 0.1 µg/ml, 1.0 µg/ml, 10.0 µg/ml and 20 µg/ml respectively, as compared to control which showed 100% hemolysis (Figure 3.7).

3.4.5 MTT Assay

The cytotoxic activity of the crude *N. kaouthia* venom was studied against breast cancer cell lines MCF-7 and MDA-MB-231, lung cancer cell lines A549 and NCI-H522 and normal human embryonic kidney cell line HEK-293T. The crude venom induced cytotoxicity in breast and lung cancer cell lines in a dose and time-dependent manner. In A549 cells, treatment with 40 µg/ml crude venom resulted in 50% cell death (Fig. 3.8). However, treatment with the same concentration of crude venom led to increased cell deaths in the following order MCF-7 ≥ MDA-MB-231 ≥ NCI-H522 ≥ HEK-293T cells.

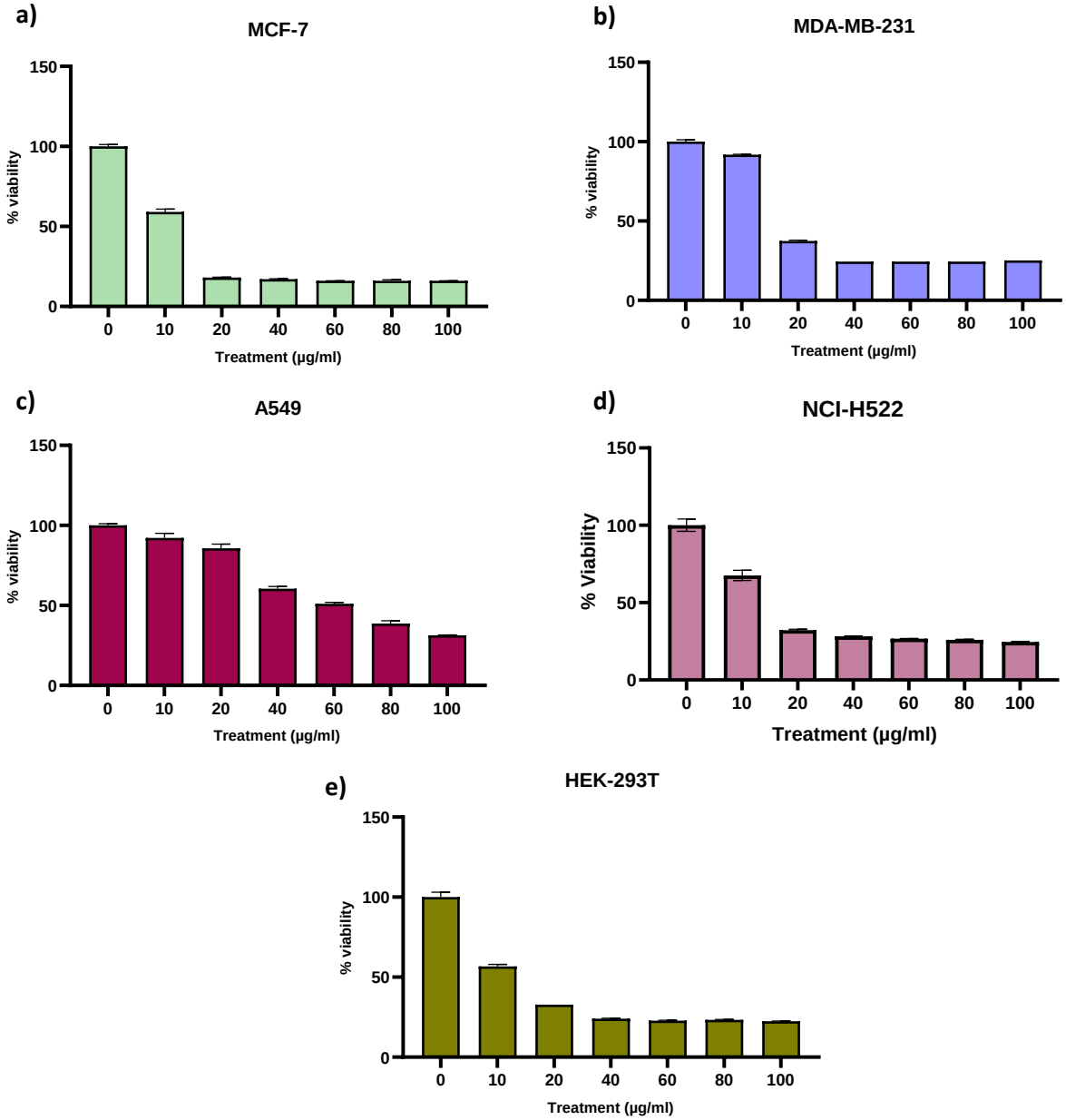

Figure 3.8: Cytotoxic effects of crude *Naja kaouthia* venom on cancer and normal cell lines. a) MCF-7, b) MDA-MB-231, c) A549, d) NCI-H522 and e) HEK-293T cells treated with crude venom for 24 hours. Dose dependent effect on cell viability was quantified by MTT Assay. The cells without crude venom treatment were considered as control (100%). Half maximal inhibitory concentration (IC_{50}) of cell lines treated with crude venom was calculated for each of the cell lines.

3.4.6 IC_{50} value of crude *Naja kaouthia* venom against cancer and normal cells

IC_{50} value for crude *N. kaouthia* venom against MCF-7, HEK-293T MDA-MB-231, A549, NCI-H522 and HEK-293T was calculated to be 10.08 µg/ml, 14.87 µg/ml, 30.12

µg/ml, 15.30 µg/ml and 13.00 µg/ml respectively (Table 3.1). Therefore, it can be observed that crude venom was most effective against MCF-7 > HEK-293T > MDA-MB-231 > NCI-H522 > A549. Thus, IC_{50} value suggested that crude venom is most effective towards breast cancer MCF-7 cell line.

Table 3.1: Calculated IC_{50} values of *Naja kaouthia* crude venom and purified Cytotoxin 10 against all experimental cell lines.

Tissue origin	Cell line	Crude venom (µg/ml)
Breast (cancer)	MCF-7	10.08
	MDA-MB-231	14.87
Lung (cancer)	A549	30.12
	NCI-H522	15.30
Kidney (normal)	HEK-293T	13.00

3.5 Discussion

N. kaouthia has been reported for numerous snakebite incidents in Eastern and North-Eastern India. For instance, Kakati et al. have reported 22 cases of *N. kaouthia* bites from a primary health centre in Sivasagar district of Assam between 2018-2022 [267]. However, the actual number of bites is difficult to accurately estimate owing to a number of factors, such as, the unavailability of epidemiology, clinical and management data, and absence of snake venom detection kits to accurately identify and segregate *N. naja* and *N. kaouthia* bites [268]. Although rare, but recurrent neurotoxicity has also been reported in a patient envenomated by *N. kaouthia* in one of the studies from Assam [269]. Previously, the venom of *N. kaouthia* from West Bengal, India has been studied by various authors [241,270-272] and few studies were also reported from North-East India (Assam) [234,265]. Understanding the biochemical properties of venom from medically important snakes from a particular region or geographic location is important, because snakes exhibit variations in their venom composition which not only affect the clinical manifestations but also require special medical consideration for treatment [265]. In this

chapter, the *in vitro* biochemical properties of *N. kaouthia* venom from North-East India (Assam) have been studied.

One of the most common venom protein families present in snake venom is the PLA_2 superfamily, which are esterolytic enzymes that break down glycerophospholipids and release lysophospholipids and free fatty acids. They form a major component of the venom in both elapids (Group I PLA_2s) and viperids (Group II PLA_2s) [273]. PLA_2s have been previously isolated from *N. kaouthia* venom from West Bengal, India as well as from North-East India [234,263,274]. A dose dependent PLA_2 activity was exhibited by *N. kaouthia* (Assam) venom sample with maximum effect at 20 µg/ml dose. PLA_2s are associated mainly with presynaptic neurotoxicity and the presence of PLA_2 activity can be correlated to the neurotoxic symptoms reported from envenomated patients like dizziness, blurred vision, ptosis, dysarthria, dysphagia and muscle and respiratory paralysis [267,269,273,275].

The electrophoresis profile of crude *N. kaouthia* venom suggested that it contains proteins of low molecular weight which implies that the venom might contain various protein families, such as, 3FTx, PLA_2 and its isoforms or KSPIs. Also, three distinct protein bands observed at a molecular weight range of ~150 kDa, ~75 kDa and ~50 kDa suggested the presence of larger molecular weight proteins like LAAOs or proteases.

The venom samples exhibited mild direct (7.6% at 20 µg/ml) and indirect (8.6% at 20 µg/ml) hemolytic activity. The indirect hemolytic activity is observed due to the lytic nature of free fatty acids (e.g. arachnidonic acid), which are released due to the PLA_2 activity of crude venom. This causes the breakdown of membrane phospholipids of RBCs, leading to hemolysis [276]. The direct hemolytic activity observed in the venoms of *Naja* sp. is due to the presence of direct lytic factors (DLFs) in their venom which cause direct hemolysis of RBCs [277,278].

The crude venom also exhibited anti-coagulant activity as observed from the delayed coagulation time of PPP (RT, PT and APTT) in dose-dependent manner. This implies that the venom exerts its anticoagulant activity involving both the intrinsic (RT and APTT) and the extrinsic (PT) blood coagulation pathways. Previously, the anti-coagulant nature of *N. kaouthia* venom from Assam has been reported [234,265] and this nature may be

attributed to the presence of PLA_2, 3FTx and KSPIs [279-281]. Similar anticoagulant nature of elapid venom has also been reported from other species of the *Naja* genus such as *N. naja, N. atra, N. siamensis* and *N. nigricolis* from different geographical locations [282-285]. Symptoms of coagulopathy, although not common, has been reported from a *N. kaouthia* envenomated victim from USA [286].

In this study, the crude venom of *N. kaouthia* exhibited concentration-dependent cytotoxicity towards breast and lung cancer cells. The IC_{50} values suggested that crude venom is most cytotoxic towards breast cancer MCF-7 cell line as compared to other cell lines that were tested. The anti-proliferative and cytotoxic activity of various elapid venoms and their proteins have been studied and reported previously. In one of the studies by Debnath et al., the anticancer effects of *N. kaouthia* crude venom from West Bengal have been reported against Ehrlich-ascites cells (EAC), human lung lymphoblasts (U937) and leukemia cells (K562) [212,241]. The cytotoxicity of *N. kaouthia* venom of North-East India origin was previously reported for normal kidney cell line HEK-293, macrophage cell line RAW 264.7, MCF-7 cells and skeletal muscle cells (L6) [234,238,245]. The crude venom of the Caspian cobra (*N. oxiana*) has also exhibited anticancer activity against breast cancer (MCF-7), prostate carcinoma (DU145) and hepatocellular carcinoma (HepG2) *via* apoptosis and the IC_{50} values were reported to be 28.85 µg/ml, 26.59 µg/ml and 21.17 µg/ml respectively [287]. Crude venom from the Moroccan cobra (*N. haje*) exhibited anticancer activity on hepatocellular carcinoma (Huh7.5) cells by decreasing the size of multi-cellular tumor spheroids [288]. Moreover, anticancer activity of crude venom has also been reported from other elapid and viperid snakes. For instance, the crude venom of *Ophiophagus hannah*, *N. kaouthia, N. annulifera* and *E. carinatus* exhibited *in vitro* anticancer cancer activity against pancreatic tumor (PaTu) cells with IC_{50} values being 1.39 ng/ml, 1.42 ng/ml, 1.89 ng/ml and 2.09 ng/ml respectively by inducing cytotoxicity *via* apoptosis and antimetastasis. Further, crude venom from *O. hannah* also inhibited angiogenesis in the tumor cells [259]. Similarly, *Vipera lebentina turanica* venom induced apoptosis in ovarian cancer cells by downregulating NF-κB and STAT3 signaling pathways and suppression of p50 and p65 translocation into the nucleus. Moreover, pro-apoptotic proteins (Bax and caspase-3) were expressed in high levels, although the expression of anti-apoptotic protein like Bcl-2 was inhibited [200].

The cytotoxic nature of *N. kaouthia* venom may be correlated to the presence of protein families like PLA_2 and 3FTx which includes cytotoxins/cardiotoxins [194,280]. The presence of cytotoxic activity in *N. kaouthia* crude venom indicates towards the possibility of the presence of specific venom toxins with cytotoxic activity, which may have anti-cancer potential or may be utilized to develop anti-cancer drugs. Therefore, further studies on the venom proteins present in the *N. kaouthia* venom of North-East India (Assam) were carried out in subsequent chapters in order to isolate and characterize cytotoxic protein/s.