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Biochemical and biological characterization of the venoms of *Naja kaouthia* and *Naja mandalayensis* from Myanmar and neutralization effects of BPI cobra antivenom

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ABSTRACT

Snakebite is a neglected public health issue, with many scientific and medical issues to be solved. Cobras are among the most common venomous snakes in Myanmar and are responsible for a considerable number of severe snakebite envenoming. There are three species of cobra (*Naja kaouthia*, *Naja mandalayensis* and *Ophiophagus hannah*) in Myanmar. The study aims to characterize the *N. kaouthia* and *N. mandalayensis* venoms and to investigate the efficacy of anti-cobra antivenom (BPI) against the two venoms. Protein components and fibrinogenolytic activity were determined by SDS-PAGE. Enzymatic activities for PLA_2, protease and acetylcholinesterase were determined by spectrophotometric method. Anticoagulant activity was determined by recalcification time of citrated human plasma. Myotoxicity, necrotizing activity, median lethal dose (LD_{50}) and median effective dose (ED_{50}) were determined by WHO recommended methods. The SDS-PAGE displayed the proteins and enzymes containing in two venoms were different. *N. kaouthia* venom exhibited more in PLA_2, acetylcholinesterase, anticoagulant, fibrinogenolytic and necrotizing activities than *N. mandalayensis* venom. *N.* mandalayensis venom had more protease activity and myotoxicity than *N. kaouthia* venom. The median lethal dose (LD_{50}) of *N. kaouthia* and *N. mandalayensis* venom was 4.33 μg/mouse and 5.04 μg/mouse respectively. Both venoms induced fibrinogen Aα chain degradation in 30 min (*N. kaouthia*) and in 6 h (*N. mandalayensis*). The same median effective dose (ED_{50}) (19.56 μg/mouse) showed that anti-NK antivenom can neutralize against lethal effect of *N. mandalayensis* venom. It can also neutralize the protease activity, anticoagulant activity and fibrinogenolytic activity of both venoms. Immunodiffusion and immunoblotting studies showed that the antivenom recognized its homologous venom (*N. kaouthia*) and cross-reacted against the heterologous venom (*N. mandalayensis*). The anti-NK antivenom is suitable to use for *N. mandalayensis* bite if monospecific antivenom is not available.

1. Introduction

Envenoming and deaths resulting from snakebites are particularly important public health problem in rural tropical areas of Africa, Latin America and Papua New Guinea (World Health Organization, 2017). In Asia, cobra (*Naja* sp.) bites constitute the major cause of high mortality and morbidity associated with snake envenomation (Warrell, 2010). Snake antivenom is the only therapeutic products for the treatment of snakebite envenoming. The lack of available effective snake antivenom to treat envenoming is important medical problem in various regions of the world and it has become a critical health issue at global level (World Health Organization, 2017).

In Myanmar, only one type of anti-cobra antivenom is currently produced from Myanmar Pharmaceutical Factory (MPF), formally known as Burma Pharmaceutical Industry (BPI) and still used as brand name. There is a lack of specific antivenom for treatment of *Naja mandalayensis* bites as BPI produce monospecific anti-cobra antivenom (anti-

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Abbreviation

WHO	World Health Organization
MPF	Myanmar Pharmaceutical Factory
BPI	Burma Pharmaceutical Industry
ICR	Institute of Cancer Research
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
PLA_2	Phospholipase A_2
OD	Optical density
AChE	Acetylcholinesterase
MAD	Minimum anticoagulant dose
CK	Creatine kinase
MND	Minimum necrotizing dose
ED_{50}	Median effective dose
LD_{50}	Median lethal dose
PVDF	Polyvinylidene difluoride
BSA	Bovine serum albumin
SVMPs	Snake venom metalloproteinases

NK antivenom) against *Naja kaouthia* venom. Unfortunately, the previous study reported that anti-NK antivenom used in the region of Myanmar displayed lower efficacy against *N. mandalayensis* (Sai--Sein-Lin-Oo et al., 2020).

Further a previous study reported that the anti-NK antivenom is generally ten times low in neutralization of the lethal effect of *N. mandalayensis* venom. The study suggested that in the absence of monospecific antivenom for *N. mandalayensis*, currently available anti-NK antivenom could be used for treating *N. mandalayensis* bite, however higher amount of anti-NK antivenom will be needed to neutralize the lethal effect of the venom (Aye-Aye-Myint et al., 2004). When the requirement of very large amount of antivenom due to low potency of antivenom, it might be contributed to high treatment cost and greatly increases the risk of hypersensitivity. Therefore, the administration of a very large amount of antivenom is not desirable.

For this reason, the study was done to characterize *N. kaouthia* and *N. mandalayensis* venoms and to determine the efficacy, potency and cross-immunological reactivity of anti-NK antivenom against the two venoms. Findings from this study may provide clear facts about the possible use of the anti-NK antivenom in the spitting cobra bite from which unfortunately has no species-specific antivenom available as treatment.

2. Materials and methods

2.1. Cobra snake venoms and antivenom

The cobras were collected as wild specimens from two different geographical areas, *N. kaouthia* from Kyauk Se, Mandalay Region and *N. mandalayensis* from Taung Dwin Gyi, Magway Region. Ten specimens for each species were collected. Species identification was done by checking their distinguished physical characters visually. The two species differ from each other especially fangs, hook mark, throat pattern and ventral banding and coloration. *N. kaouthia* is recognized by distinct hood markings with the presence of a pale, oval or circular marking with a dark center and ventrolateral throat spots (Leviton et al., 2003). Neither hood marking nor throat spots is found in *N. mandalayensis*. However, fangs are highly modified for "spitting" venom in *N. mandalayensis* species (Slowinski and Wüster, 2000). The venom was collected from each snake and lyophilized in the freeze dryer. The lyophilized venoms were weighted and stored at −70 °C. Anti-NK antivenom (BPI) batch no. DG 19033 was used.

2.2. Laboratory animals

ICR (Institute of Cancer Research) (*Mus musculus*) mice were obtained from Laboratory Animal Services, Department of Medical Research and the Department of Veterinary (BPI), respectively. The study was approved to conduct from the Ethical Review Committee, University of Pharmacy, Yangon. The number of the approved ethical certificate is January 2020. It was approved on 24-2-2023.

2.3. Characterization of the venom protein profile of *N. kaouthia* and *N. mandalayensis*

2.3.1. Determination of protein content

Total protein content of each venom was determined by the Lowry's method (Lowry et al., 1951).

2.3.2. Separation of venom proteins by SDS-PAGE

SDS-PAGE was carried out according to the Laemmli method (Laemmli, 1970). The venoms were prepared to a concentration of 2.5 mg/mL and each venom preparation (40 μL) was individually diluted with 10 μL of sample buffer containing 63 mM Tris-Cl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue and 5% β-mercaptoethanol. Both venom sample solutions were kept at room temperature for 30 min for non-reducing condition. For reducing condition, 1% β-mercaptoethanol was added to the venom samples, and heated at 95 °C for 2 min. Each venom sample (10 μL) and molecular weight markers (Precision Plus Protein Standard, BioRad, USA) were loaded to 12% polyacrylamide gel. The Dual cool electrophoresis system (DCX-700, CBS Scientific) was used to run the electrophoresis using Tris-glycine buffer (pH 8.3), initially 180V, 90 mA for 40 min, followed by 200 V, 60 mA for 2 h. The gels were stained by Coomassie blue and the images were taken with Bio-Rad Molecular image Gel Doc.

2.4. Enzymatic assays and biological activities

2.4.1. Phospholipase A_2 (PLA_2) activity

Phospholipase A_2 activity was determined by a method of Tan and Tan (1988). One mL of phosphatidylcholine (egg-yolk lecithin) (8.1 mM) was emulsified with one mL sodium deoxycholate (8.1 mM). Then one mL of $CaCl_2$ (18 mM) was added to the above mixture. Then the substrate mixture (3 mL) was pre-incubated in a 37 °C for 5 min. After 5 min, 0.5 mL venom solution was added to the substrate mixture, and the optical density was immediately measured at 925 nm. The OD was measured again in next 15 min. The difference in OD was measured as PLA_2 activity.

2.4.2. Protease activity

Protease activity of each venom was determined by using 2% casein in 50 mM potassium phosphate buffer, pH 7.5, as a substrate. Briefly, 50 μL of venom was incubated with 500 μL of casein for 15 min at 37 °C. The reaction was stopped by adding 500 μL 5% trichloroacetic acid and the mixture was centrifuged at 10,000 rpm for 5 min. The digested casein in the supernatant was determined at 660 nm using 20% Folin-Ciocalteu's reagent (Anson, 1938).

2.4.3. Acetylcholinesterase (AChE) activity

Acetylcholinesterase activity was measured by the Ellman method using the acetylcholinesterase activity assay kit (MAK 119, Sigma-Aldrich) (Ellman et al., 1961). Firstly, 200 μL water (as assay blank) and 200 μL calibrator (MAK 119B) were transferred into each well of a 96 well plate. A 10 μL sample was added into each well. Aliquots of 190 μL freshly prepared working reagent were transferred to sample wells. The plate was incubated at room temperature. The initial absorbance at 412 nm $(A_{412})_{initial}$ was taken, after 2 min. Then the final measurement $(A_{412})_{final}$ was taken at 10 min. The AChE activity is calculated as follow.

$$\text{AChE activity } (units/L) = \frac{(A_{412})_{final} - (A_{412})_{initial} \times n \times 200}{(A_{412})_{calibrator} - (A_{412})_{blank}}$$

200 = Equivalent activity (units/L) of the calibrator when assayed is read at.

2 min and 10 min

n = Dilution factor.

$(A_{412})_{calibrator}$ = Absorbance of the calibrator at 10 min.

$(A_{412})_{blank}$ = Absorbance of the blank at 10 min.

2.4.4. Anticoagulant activity

Anticoagulant activity was determined by recalcification time of citrated human plasma (Frommeyer and Epstein, 1949). It was measured by adding 100 μL of various concentrations of venom solutions to 200 μL citrated human plasma in a glass tube and the solution was mixed thoroughly and kept at 37 °C for 5 min. The clot formation was initiated by adding 100 μL 25 mM $CaCl_2$ solution and observed for coagulation at 60 s thereafter. The clotting time was recorded in seconds till to complete coagulation of the plasma. The normal coagulation time with plasma without venom was also recorded and normal saline was used as negative control. The minimum anticoagulant dose (MAD) is defined as the minimum dose of venom that prolonged by two times of the recalcification time of the control (Sánchez et al., 2018).

2.4.5. Fibrinogenolytic activity

Fibrinogenolytic activity was assayed by the method of Ouyang and Teng, using bovine fibrinogen (2 mg/mL) (Ouyang and Teng, 1976). An equal volume (150 μL) of fibrinogen solution and venom solution (750 μg/mL) were mixed and incubated for different time intervals at 37 °C. Fibrinogen solution was incubated with 0.05 M Tris buffer only for control. At different time intervals (0, ½, 1, 4, 5, 6 and 8 h for *N. kaouthia* and 0, ½, 6, 12, 18 and 24 h for *N. mandalayensis*), 40 μL of aliquots were withdrawn from the digested mixture and heated at 95 °C for 2 min with 10 μL of 1M Tris-HCl buffer, pH 6.8 containing glycerol, β-mercaptoethanol, SDS and bromophenol blue. The denatured mixture were then loaded on a 12% SDS-PAGE gel. Staining was done with 0.2% Coomassie brilliant blue R250 and the gel was destained till the protein bands were visible.

2.4.6. In vivo myotoxicity

Venom test doses (4 μg crude venom) were prepared in a constant volume of 50 μL and injected into the right gastrocnemius muscle of five mice (20±2g) in test group. Control mice were injected 50 μL saline solution. In the present study, myotoxic activity of venom was found only after 6 h of injection of venom. According to WHO guidelines, creatine kinase (CK) was measured after 3 h of venom administration but CK activity of plasma was not observed at that time in this study. Therefore, blood sample was collected after 6 h injection according to Hardy et al. (2014) method. After 6 h, the blood sample was drawn using cardiac puncture and centrifuged at 3000 rpm for 10 min. The serum obtained by centrifugation was diluted with saline at a 1:10 ratio. Then, CK activity of serum was measured in 10 μL of serum according to the creatine kinase activity assay kit protocol (MAK 116, Sigma-Aldrich-Lot 116BJ10A23) (Hardy et al., 2014; World Health Organization, 2017).

2.4.7. Necrotizing activity

Group of three mice were injected intradermally, in the dorsal region, with varying amount (5, 6, 6.5, 7 μg) of cobra venom in a constant volume of 50 μL (using saline solution as diluent). Control mice were injected with the same volume of saline solution. After three days of injection, the mice were sacrificed by diethyl ether inhalation and measured the diameter of necrotic lesion on inner side of the skin. The minimum necrotizing dose (MND) was defined as the dose which induced an area of necrosis with 5 mm diameter three days after injection (Theakston and Reid, 1983).

2.5. Neutralization activity of antivenom against both venoms

2.5.1. In vitro enzymatic activities

For neutralization of protease activity, 50 μL of venom solution (500 μg/mL) was mixed with 5 μL, 10 μL and 20 μL of anti-NK antivenom (1 mg/mL) respectively. These mixtures and control venom sample without antivenom were incubated at 37 °C for 10 min and mixed with 150 μL casein (20 mg/mL) solutions in 50 mM potassium phosphate buffer solution. The protease activities was measured by Anson method (Anson, 1938). The anti-protease activity of anti-NK antivenom was calculated in percentage inhibition as follow.

$$\% \text{ inhibition} = 100 - [(A / B) \times 100]$$

A = protease activity of mixture of venom and antivenom solution.

B = protease activity of venom solution.

For neutralization of anticoagulant activity, five doses of the mixtures containing of venom (10 μg/mL) and antivenom (100–500 μg/mL) (in the ratios of 1:10, 1:20, 1:30, 1:40, 1:50) and control sample without antivenom were incubated at 37 °C for 30 min. Aliquots of 100 μL of each pre-incubated mixture were added to 200 μL citrated human plasma. For recalcification time, 100 μL 25 mM $CaCl_2$ solution was added and clotting time was recorded. Neutralization of anticoagulant activity of both cobra venoms expressed as percentage inhibition.

$$\text{Percent inhibition } (\%) = A / B \times 100.$$

A = recalcification time of mixture of venom and antivenom solution.

B = recalcification time of venom solution.

For neutralization of fibrinogenolytic activity, 75 μL of 750 μg/mL venom solution was mixed with 75 μL of 1 mg/mL antivenom solution. The sample mixture (150 μL), control sample without antivenom were incubated at 37 °C for 30 min. Then, the sample solutions were added to 150 μL fibrinogen solution (2 mg/mL) and anti-fibrinogenolytic activity was assayed according to the method of Ouyang and Teng (1976).

2.5.2. Lethality analysis

2.5.2.1. Determination of LD_{50} (median lethal dose). The lethal activities of both venom samples were determined according to the method of Meier and Theakston (1986). Venoms of various doses (0.2 mL) were injected into tail vein of six mice (20 ± 2 g) and the numbers of death mice were recorded after 24 h. The median lethal doses (LD_{50}) of the venoms were calculated by Spearman-Karber method (Spearman, 1908; Karber, 1931). One venom LD_{50} dose is defined as the amount of venom causing death in 50% of injected mice. The LD_{50} and 95% confidence limits were calculated by using MedCalc® Statistical Software version 22.009 (https://www.medcalc.org, 2023).

2.5.2.2. Determination of median effective dose (ED_{50}). ED_{50} of antivenom (in μL) was determined by the WHO guidelines (World Health Organization, 2017). The study was performed by pre-incubating the different doses of antivenom with 5 LD_{50} venom challenge does at 37 °C for 30 min. The venom and antivenom mixture (0.2 mL) were subsequently injected into tail vein of six mice (20 ± 2 g). The numbers of survivals were recorded after 24 h. The ED_{50} was calculated by Spearman-Karber method. ED_{50} was defined as the volume dose of antivenom (μL) at which 50% of mice survived. The ED_{50} and 95% confidence limits were calculated by using MedCalc® Statistical Software version 22.009 (https://www.medcalc.org, 2023).

2.5.3. Potency of antivenom

The neutralization efficacy of the antivenom was calculated using the following formula. and expressed as potency (P). "P" is the amount of venom, expressed in mg/mL, means that is completely neutralized per unit volume of antivenom (Morais et al., 2010).

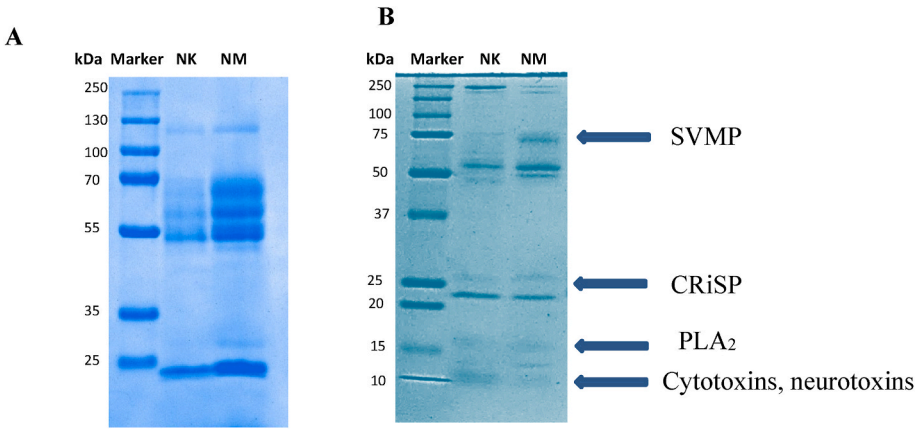

Fig. 1. SDS-PAGE analysis of *N. kaouthia* and *N. mandalayensis* venom. The venom were subjected to electrophoresis in a 12 % sodium dodecylsulfate polyacrylamide gel under reducing (A) and non-reducing (B) conditions. In each line, 18 μg (for reducing gel), 10 μg (for non-reducing gel) venom samples and a molecular weight marker (kDa) 4 μg was used for electrophoresis. NK=*N. kaouthia* venom, NM= *N. mandalayensis* venom. Protein families are identified on the far right and include SVMP (snake venom metalloproteinase), CRiSP (cysteine-rich secretory protein), PLA_2 (phospholipases A_2) and 3FTx (three-finger toxins). Identities are based on previously published work (Leong et al., 2015 & Tan et al., 2016).

$$\text{Potency } (\text{mg}/\text{mL}) = (\text{n}-1)\ \text{LD}_{50}/\text{ED}_{50}$$

Where, n = the number of LD_{50}
LD_{50} = median lethal dose.
ED_{50} = median effective dose.

2.6. Cross-immunological analysis

2.6.1. In vitro immunodiffusion

Immunological cross-reactivity studies were performed by Ouchterlony immunodiffusion technique (Bailey, 1996). Immunodiffusion was carried out using 1% Nobel agar plate. Both venoms were prepared to a protein concentration of 2.5 mg/mL with 0.9% normal saline solution. The outer wells were filled with 50 μL of *N. kaouthia*, *N. mandalayensis* venom solutions, 0.9% normal solution and 2.8 mg/mL Myanmar green pit viper venom solution respectively. The central well contained 50 μL of 13 mg/ml anti-NK antivenom solution. After 24-h incubation, the protein precipitin lines between venom and antivenom were checked visually.

2.6.2. Immunoblotting

Immunoblotting experiments were carried out after the completion of 14 μg venom protein separation in SDS-PAGE gel. Separated venom proteins were electro-transferred from gel onto PVDF membrane in a blotting unit at 100 V, 300 mA for 2 h. After completion of electro-transfer, the membrane was immersed in blocking solution (1% BSA in TTBS) for 30 min at room temperature and then washed thoroughly with washing buffer, TTBS (0.1% Tween 20 in Tris-NaCl, TBS) for 5 min, for three times. Blocked membrane was incubated overnight at 4 °C with primary antibodies (0.5 mg/mL cobra antivenom solution). Membrane was then washed again and incubated for 3 h with 1:1000 (v/v) secondary antibody anti-horse IgG solution. After washing the blot with TTBS for two times and incubated in enzyme substrate solution (0.5 mg/mL 4-chloro-1-napthol in TBS) for 45 min (Towbin et al., 1979).Venom-protein and antivenom-antibody binding were visualized by using a UV detector.

3. Results and discussion

Cobra envenomation is characterized by local tissue necrosis and neuromuscular paralysis that leads to respiratory failure. Clinical signs and symptoms developed after *N. kaouthia*'s and *N. mandalayensis*'s bites were similar. Local swelling, bleeding at the site of bite, blistering, necrosis and lymphadenopathy were found in patients bitten by either species. Systemic neurotoxic signs and symptoms such as ptosis, blurred vision, slurred speech, difficulty in swallowing, respiratory distress, drowsiness and respiratory paralysis were occurred in both types of envenoming (Mon-Mon-Myint-San, 1997; May-Mya-Win et al., 2001; Sai-Sein-Lin-Oo et al., 2020). However, patients suffered redness, lacrimation, periocular swelling, severe burning pain in the eyes following spitting of venom into the eyes by the spitting cobra (*N. mandalayensis*) (Tun-Pe et al., 2002; Sai-Sein-Lin-Oo et al., 2020).

Two major toxin families of cobras (*Naja* sp.) venoms are three-finger toxins (3FTx) and PLA_2. The 3FTx family contains neurotoxins (NTXs) and cytotoxins (CTXs). NTXs are responsible for the rapid onset of neuromuscular paralysis and death in most elapid envenomation, via the blockade of postsynaptic nicotinic cholinergic receptors (Barber et al., 2013) and inhibition of acetylcholinesterase (Katali et al., 2020). CTXs involved in cell death and tissue damages, contributing to necrotic (Osipov et al., 2008) and myotoxic (Ownby et al., 1993) activities. PLA_2 are responsible for neurotoxicity, cytotoxicity, cardiotoxicity, coagulopathic, haemorrhage, tissue damage in the patients (Kini, 2003). Myotoxic PLA_2 causes degeneration of muscle cells and leaking large quantity of CK into the circulation of the patients (Saaiman and Buys, 2019). Snake venom metalloproteinases (SVMPs) are contributed to fibrinogenolytic activity in the venom of cobra (Das et al., 2013). SVMPs, PLA_2s and LAAOs have been associated to tissue inflammation and local necrosis of Elapidae snakes (Neto et al., 2021).

3.1. Characterization of N. kaouthia and N. mandalayensis venom protein profile

The main components of snake venom are proteins and peptides. Various research groups have explored protein content determination of snake venoms for identification and quantification for the purpose of finding potential candidates in drug development (Almeida et al., 2017). According to the WHO technical report series for biological standardization, specific tests such as the protein concentration per gram and SDS-PAGE scans of venom are included for venom batch consistency. *N. kaouthia* venom has lower protein content than *N. mandalayensis*.

SDS-PAGE profiles of *N. kaouthia* and *N. mandalayensis* (Fig. 1) revealed that the majority of venom proteins were within the molecular weight range of 10–25 kDa and 50–75 kDa. The venom proteins within 10–15 kDa might include cytotoxins, neurotoxins and PLA_2 that their molecular masses are comparing to *N. sumatrana* (Indonesia) and *N. kaouthia* (Thailand) cobra venom (Leong et al., 2015 & Tan et al., 2016). The protein bands between 23 and 30 kDa might comprise cysteine-rich secretory proteins (CRiSPs). High molecular weight of 250

Table 1
Biochemical characteristics of *N. kaouthia* and *N. mandalayensis* venom.

Test	*N. kaouthia* venom	*N. mandalayensis* venom
Phospholipase A_2 activity (Unit/mg protein)	63.17 ± 0.027	12.35 ± 0.004
Protease activity (Unit/mg protein)	141.18 ± 0.011	248.64 ± 0.01
Acetylcholinesterase activity (Unit/mg protein at 1 mg/mL)	62 ± 0.42	0.054 ± 0.006
Anticoagulant activity (MAD[a], μg/mL)	2.7 ± 0.5	3.8 ± 0.3
Fibrinogenolytic activity	Fibrinogen Aα chain degradation in 30 min	Fibrinogen Aα chain degradation in 6 h
In vivo Myotoxicity (Creatine kinase activity)(Units/L)	1534 ± 324	2489 ± 109
Necrotizing activity (MND[b], μg)	6.5 (4.55–7.67)	7 (6.37–7.41)

Results are in means ± SD (n = 3); MND are in mean (95% confidence limits).
[a] Minimum anticoagulant dose (MAD).
[b] Minimum necrotizing dose (MND).

kDa band did not appear to represent a monomeric form upon reducing both venoms. These high molecular weight proteins might represent snake venom metalloproteinases (SVMPs).

3.2. Enzymatic and biological activities

The information regarding enzymatic activity of the venoms is essential for characterization of venom. It has proved to be useful for medication and this information are valuable in biochemical research (World Health Organization, 2017).

PLA_2 activities of *N. kaouthia* and *N. mandalayensis* venoms were dose dependent. Enzymatic activity of PLA_2 in *N. kaouthia* venom displayed five times higher than that of *N. mandalayensis* venom (Table 1). Venom of both cobra species contained a common protein family, PLA_2, represented by 15 kDa in SDS-PAGE profile (Fig. 1). In *Naja* species venom, phospholipase A_2 are the second most abundant enzyme (Tan et al., 2015b). Their major pathophysiological activities were acute muscle necrosis, paralysis and local inflammatory reactions (Gutiérre and Ownby, 2003).

Regarding proteolytic activity, *N. kaouthia* venom exhibited low proteolytic activity than *N. mandalayensis* venom (Table 1). Proteolytic activity is contributed by proteases. Venom proteases are divided into two classes of enzyme; snake venom serine protease (SVSPs) and snake venom metalloproteinases (SVMPs) (Samel et al., 2002). The high proteolytic activity in *N. mandalayensis* venom might be due to its high abundant SVMPs (Neto et al., 2021).

Naja kaouthia venom has 1148 times more acetylcholinesterase (AChE) activity than *N. mandalayensis* venom (Table 1). The AChE activity variation may also reflect the variation of species (Yap et al., 2011). Frobert et al. (1997) described that the low AChE activity found in some *Naja* venom batches results in proteolytic degradation by venom protease. Low AChE content in *N. mandalayensis* venom may be due to high content of protease enzymes and reverse in *N. kaouthia* venom.

Snake venom neurotoxins give rise to blockade of neuromuscular transmission eventually leading to death by asphyxiation in the clinical setting (Tsetlin and Hucho, 2004). Although both cobra envenomation sign and symptoms were difficult to differentiate, *N. kaouthia* envenomation might be more severe than *N. mandalayensis* due to the high level of AChE and PLA_2 enzyme of *N. kaouthia* found in current study.

Minimum anticoagulant dose (MAD) was expressed by correlating with the minimum dose of venom that extended by two times of the recalcification time of the control (Sánchez et al., 2018). *N. kaouthia* venom possesses more anticoagulant activity than *N. mandalayensis* (Table 1). The stronger anticoagulant activity might be caused by higher content of PLA_2 enzyme. PLA_2 are strong anticoagulants in snake venom (Kini, 2006).

Table 2
Neutralization of lethality of *N. kaouthia* and *N. mandalayensis* venom by BPI cobra antivenom.

Venom	LD_{50} (μg/mouse)	ED_{50} (μL/mouse)	Potency (mg/mL)
N. kaouthia	4.33 (3.88–4.90)	19.56 (3.65–38.23)	0.89
N. mandalayensis	5.04 (4.44–5.90)	19.56 (6.09–47.94)	1.04

Values in bracket for LD_{50} and ED_{50} indicate 95% confidence intervals.

Effect of both venoms on bovine fibrinogen revealed significant differences in fibrinogenolytic activity. Clear α-chain digestion was observed after 30 min and 6 h incubation of *N. kaouthia* and *N. mandalayensis* venom with fibrinogen solution, respectively (Table 1). This finding suggested that the toxins responsible for fibrinogenolytic activity might be more abundant in *N. kaouthia* than *N. mandalayensis* venom. Metalloproteinases purified from *N. kaouthia* hydrolyzed the Aα-subunit of the fibrinogen (Wijeyewickrema et al., 2007; Chanda et al., 2016).

Serum creatinine kinase (CK) level increased 1.6 times and 2.4 times 6 h after the injection of *N. kaouthia* and *N. mandalayensis* venom into mice. Compared to the two venoms, *N. mandalayensis* was more myotoxic than *N. kaouthia* (Table 1). The severity and development of myotoxicity is caused by different groups of snake myotoxins such as small myotoxins, cardiotoxins and PLA_2 (Charoenpitakchai et al., 2018). The more myotoxicity of *N. mandalayensis* venom might be due to its solely highly significant content of cardiotoxins compared to *N. kaouthia* venom (Neto et al., 2021).

N. kaouthia venom is more necrotizing than *N. mandalayensis* (Table 1). It may be due to the fact that *N. kaouthia* venom has more PLA_2 content than *N. mandalayensis* venom. Cobra PLA_2 may play a role in pathogenesis of necrosis by promoting cytotoxin induced cytotoxicity (Gasanov et al., 1997). The differences in some venom activities could also be relevant differences in the enzymatic activity of different components from the same protein family in addition to differences in toxin abundance between the venoms of two different species.

The median lethal dose (LD_{50}) for *N. kaouthia* and *N. mandalayensis* were 4.33 μg/mouse and 5.04 μg/mouse respectively (Table 2). LD_{50} of *N. kaouthia* for Malaysia was 0.9 μg/mouse, Thailand was 0.18 μg/mouse and Vietnam was 0.9 μg/mouse (Tan et al., 2015a). Comparing LD_{50} of venoms from different countries, *N. kaouthia* (Thailand) was the most lethal than any other cobras. Khow et al. (1997) found that the lethality of *N. siamensis* (spitting cobra) venom is 21.4 μg/mouse and Aye-Aye-Myint et al., (2004) showed that *N. mandalayensis* (spitting cobra) venom was 56.1 μg/mouse. The lethality of spitting cobra from Tharzi (Mandalay Region) was (5 times) less than that of *N. kaouthia* (monocled cobra) venom (Aye-Aye-Myint et al., 2004). In the current findings, *N. kaouthia* venom was (about 1 time) more lethal than *N. mandalayensis* venom (Taung Dwin Gyi, Magway Region), but not much in five times as in the previous study. This is due to the geographical variation of the spitting cobra species.

The proteomic study of *N. mandalayensis* (Myanmar) elucidated its major composition is three-fingers toxins (3FTXs) (especially cardiotoxins), metalloproteinases followed by less abundant PLA_2 (Neto et al., 2021). That study also described that neurotoxins are highly more abundant in *N. kaouthia* than *N. mandalayensis*.

There is lack of data for complete proteomic study of Myanmar *N. kaouthia* species up to date. The venom of *N. kaouthia* (Thailand) is primarily composed of 3FTXs and PLA_2 isoforms (Kulkeaw et al., 2007; Namiranian and Hider, 1992; Lausten et al., 2015; Tan et al., 2015a). The 3FTXs of Thai *N. kaouthia* venom also composed especially more neurotoxins than cardiotoxins (Tan et al., 2015a).

In cobra envenomation, local tissue inflammation and necrosis are prevalent with or without systemic neurotoxicity. The monocled cobra *N. kaouthia*, *N. siamensis* and *N. naja* cause severe neurotoxic

Table 3
In vitro neutralization activities of BPI cobra antivenom.

Test	% inhibition by BPI cobra antivenom	
	N. kaouthia	N. mandalayensis
Anti-protease activity (1.25 mg/mL)[a]	40 %	15 %
Anti-anticoagulant activity (0.03 mg/mL)[a]	50 %	100 %
Anti-fibrinogenolytic activity (0.03 mg/mL)[a]	Fibrinogen Aα chain present till to 12 h	Fibrinogen Aα chain present till to 18 h

All experiments were triplicated. We used mean value in calculation of % inhibition.
[a] Neutralization of venom activities in mg of venom per mL of antivenom.

envenomation in addition to local tissue toxicity. On the other hand, the spitting cobra *N. atra*, *N. sputatrix* and *N. sumatrana* gave severe local tissue toxicity but rarely severe neurotoxicity (Tan et al., 2019). This is coincided with the systemic neurotoxic symptoms/signs attributed to 67% of those bitten by *N. kaouthia* and 33% of patients bitten by *N. mandalayensis* in Myanmar (Sai-Sein-Lin-Oo et al., 2020).

In Myanmar, more than half (51%) of cobra bite (*N. kaouthia*) needed referral to intensive care unit and 30% of these needed ventilation support (San-Mya and Tun-Pe, 2004). High acetylcholinesterase activity of *N. kaouthia* in addition to more abundant neurotoxins and high PLA_2 activity reflect the common local tissue toxicity with severe neurotoxicity occurs in the patients bitten by *N. kaouthia*.

3.3. *Neutralization of venom activities by anti-NK antivenom*

In vitro neutralization activity of anti-NK antivenom against the venoms of *N. kaouthia* and *N. mandalayensis* were done on anti-protease activity, anti-anticoagulant and anti-fibrinogenolytic activity (Table 3).

At venom: antivenom ratio of 5:4, the anti-NK antivenom could neutralize the protease activity of *N. kaouthia* and *N. mandalayensis* venoms in 40% and 15% inhibition, respectively. Less neutralization effect of anti-NK antivenom on *N. mandalayensis* than *N. kaouthia* might be due to the higher protease enzyme content in *N. mandalayensis* than *N. kaouthia*. The antivenom rose against *N. kaouthia* venom could not neutralize the extra proteases included in *N. mandalayensis* venom.

The ability of anti-NK antivenom to neutralize anticoagulant effect of the venoms related with the amount of PLA_2 enzyme containing in venom. The anti-NK antivenom was able to exhibit 100% neutralization of anticoagulant activity at a venom/antivenom ratio (1 : 30) (5 μg/300 μL) and (1 : 15) (5 μg/150 μL) for *N. kaouthia* and *N. mandalayensis* respectively. The venom of *N. kaouthia* contained more PLA_2 enzyme than *N. mandalayensis* venom and less amount of anti-NK antivenom was needed to neutralize the anticoagulant activity of *N. mandalayensis*. The current finding highlighted anti-NK antivenom can neutralize the anticoagulant PLA_2 in both venoms.

Moreover, fibrinogenolytic activities of both cobra venoms described the significant differences. Their degradation of alpha chain of fibrinogen was inhibited by anti-NK antivenom in different time manner (Table 3). It was found that the anti-NK antivenom retained the capability of cleaving the alpha chain fibrinogen by *N. kaouthia* venom till 12 h and by *N. mandalayensis* venom till 18 h. Our result indicated that the anti-NK antivenom can neutralize fibrinogenolytic activity of both venoms.

Median effective dose (ED_{50}) for *N. kaouthia* and *N. mandalayensis* venom was determined according to World Health Organization, 2017 guidelines. The same median effective dose of 19.56 μL/mouse was observed for both venoms. According to the study of Sai-Sein-Lin-Oo et al. (2020), the anti-NK antivenom has lower preclinical efficacy against *N. mandalayensis* because of the disparities between the lethality (LD_{50}) of the two cobra venoms (Aye-Aye-Myint et al., 2004). However, current finding of same ED_{50} dose of antivenom suggested that the anti-NK antivenom is effective against the lethal activity of Mandalay spitting cobra (*N. mandalayensis*) venom.

Fig. 2. Immunodiffusion pattern of *N. kaouthia* and *N. mandalayensis* venoms against Myanmar cobra antivenom (BPI); (1) *N. kaouthia venom*, (2) *N. mandalayensis venom*, (3) Normal saline, (4) Myanmar green pit viper, Centre well = Anti-snake venom.

Two studies (Khow et al., 1997; Tan et al., 2015a) showed that ED_{50} values obtained with the heterologous test venom were higher than those obtained with the homologous test venom. However, the current study showed the same ED_{50} values for both homologous and heterologous venoms. This might be due to the LD_{50} of two cobra venoms were not statistically significant difference.

Potency of anti-NK antivenom is 0.89 mg/ml against *N. kaouthia* venom and 1.04 mg/ml against *N. mandalayensis* venom (Table 2). The anti-NK antivenom was more efficacious in neutralizing the lethality of *N. mandalayensis* than *N. kaouthia*, but the difference was not statistically significant ($p > 0.05$, Tukey's HSD test). The potency value provides the rough estimation of the number of an antivenom vial (dose) that may be required for the treatment. Although *N. kaouthia* is more lethal than *N. mandalayensis*, according to potency values, the anti-NK antivenom dose needed to neutralize lethality might be less for *N. mandalayensis* venom. This finding is contrast to the study of Aye-Aye-Myint et al., (2004), in which higher amount of anti-NK antivenom will be needed for treating *N. mandalayensis* bite.

The potency of an antivenom against different venom is also contributed by the factors during antivenom production such as preparation of venom immunogen, immunization of horses, cross-contamination with heterologous venom. It is also need to verify that the horses used for production of monovalent venom (anti-NK antivenom) should not be previously immunized with any other types of venoms.

3.4. *Immunoreactivity of cobra antivenom*

The anti-NK antivenom (Batch no.19033) was cross-reacted with both *N. kaouthia* and *N. mandalayensis* venoms but varied in the extent of cross-reactivity by immunodiffusion and immunoblot profile. After 24 h of immunodiffusion (Fig. 2), three protein precipitate lines were seen between *N. kaouthia* venom and antivenom while two protein lines also were seen in *N. mandalayensis* venom and antivenom. No precipitin band was formed towards the well containing normal saline solution and Myanmar green pit viper venom solution. It showed that anti-NK

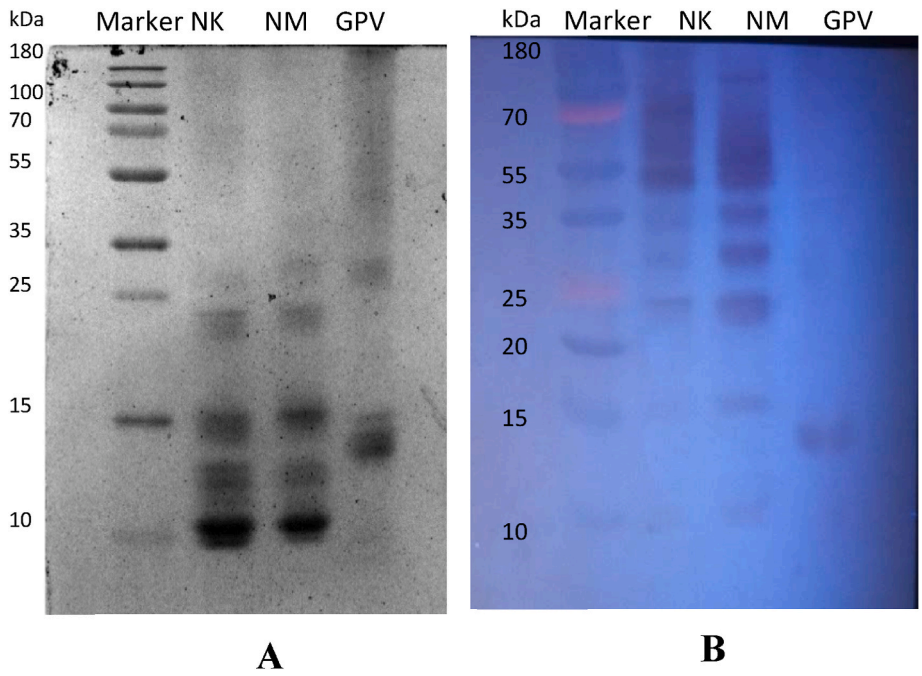

Fig. 3. Immunoblot detection of *N. kaouthia* and *N. mandalayensis* venom using cobra antivenom (BPI), NK=*N. kaouthia* venom, NM= *N. mandalayensis* venom, GPV = Green pit viper venom. (A) SDS-PAGE, (B) Blot developed against the cobra antivenom.

antivenom can be cross-neutralized with *N. mandalayensis* venom. This finding was in agreement with the previous study done by Aye-Aye--Myint et al., (2004).

3.5. Immunoblotting

The analysis of cross-reactivity of anti-NK antivenom with two venoms by immunoblotting (Fig. 3) revealed that antibodies from the antivenom recognized to antigens of two venom samples especially high molecular weight venom proteins. The low molecular weight proteins were poorly recognized by anti-NK antivenom which might be due to antibodies against such proteins are either smaller amounts or absent. Notably, it did not recognize green pit viper venom well. As the intensity and range of venom protein binding by anti-NK antivenom was comparable to the other venoms, *N. mandalayensis* venom protein binding seems to be similar pattern as that of *N. kaouthia* venom. More venom proteins with high molecular weight (25–180 kDa) from *N. mandalayensis* were recognized by anti-NK antivenom than those from *N. kaouthia* venom.

4. Conclusion

This study demonstrated the variations of protein composition, enzymatic and toxicological activities between *N. kaouthia* and *N. mandalayensis* venoms. The anti-NK antivenom can neutralize some of tested enzymatic, biochemical and biological activities of both venoms under *in vitro* and *in vivo* conditions. The anti-NK antivenom has no significant different potency for *N. kaouthia* and *N. mandalayensis* venom neutralization. If there is no monospecific antivenom for *N. mandalayensis* is available, our results indicated that the anti-NK antivenom can be used for both *N. kaouthia* and *N. mandalayensis* envenomations in Myanmar.

Ethical statement

The authors hereby stated that all procedures involving animals were conducted in ethical manner. Ethical approvals were obtained from relevant Institutional Animal Care and Use Committee and Ethical Review Committee (ERC) of the University of Pharmacy, Yangon.

CRediT authorship contribution statement

Mya Nila Win: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Khin Than Yee:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kyae Mhon Htwe:** Writing – review & editing, Supervision, Resources. **Ei Ei Thin:** Writing – review & editing, Supervision, Project administration. **Su Mon Win:** Investigation, Formal analysis, Data curation. **Aung Myat Kyaw:** Investigation. **Myo Myo Aye:** Investigation. **Kyaw Kyaw Khaing:** Investigation. **Wai Myat Thwe:** Investigation. **Khin Khin Htwe:** Writing – review & editing, Supervision. **Aung Zaw:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mya Nila Win reports financial support was provided by Ministry of Health, Myanmar.

Data availability

Data will be made available on request.

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