BIOLOGICAL CHARACTERIZATION OF *Conus striatus* VENOM FOR MEDICAL APPLICATIONS

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ABSTRACT: *Gastropod molluscs of the genus Conus (cone snails) occur throughout the world's tropical coastal waters where they capture their prey such as fish, molluscs or worms using a complex battery of neurotoxins. In this study, crude venom from venom duct of the fish-hunting cone snail Conus striatus was biologically characterized using BSLT, DNA-Binding activity, hemolytic activity, and mice bioassay. The crude venom extract contains 205 – 212ppm protein. The proteins were proven to contain two bands above 210 kDa and between 210 kDa and 140 kDa. The crude venom showed Brine Shrimp larvicidal activity, with a chronic LC_{50} value of 446.76 ppm, and has a strong DNA-binding capability. The crude venom also exhibited high hemolytic activity, at half of the chronic LD_{50}, and a remarkable lethality on mice at, as low as, 100 ppm. This study strongly suggests that the conotoxin could potentially be utilized in medical applications such as DNA intercalating agent to induce arrest in DNA replication and transcription processes, as well as aid in cell membrane destruction, for cancer treatment.*

1. INTRODUCTION

Cone snails are venomous marine gastropod molluscs, which are characterized by a sophisticated venom apparatus responsible for the delivery of complex mixtures of toxin peptides [1]. These bioactive peptides are generally termed as "conotoxins" or "conopeptides". Molecules present in *Conus* venom can perform a variety of functions, acting as neurotoxins, carrier proteins, or hydrolytic enzymes such as glycosyl hydrolases [2], proteases, hyaluronidases and lipases, and in toxin processing [3]. These peptides either inhibit neuromuscular transmission, causing flaccid paralysis, or increase excitability at the target ion channel [4]. Specific nerve cell channel sites can also be blocked by conotoxins, which was believed to be due to the presence of disulfide-bonding network and specific amino acids in inter cysteine loops, making it a significant tool in the characterization of neural pathways, as well as therapeutic agents in medicinal applications [5]. Given this profile, it is not surprising that a number of conopeptides can reverse signs of pain in animal models, with several developed into potential novel analgesics [6] and a competitive blocker of selected neuronal-type nicotinic ACh receptors [7].

Conus striatus (*C. striatus*) is one of the largest fish-hunting *Conus* snails, a highly successful species found over the entire Indo-Pacific region from the Hawaiian Islands to the east African coast [8]. Studies have revealed that the venom of *C. striatus* contain more than one active material [9], which include the two paralytic toxins from *C. striatus* venom, α-conotoxin SI [10] and α-conotoxin SIA [11]. Both of these peptides have two disulfide linkages, which clearly belong to the nicotinic acetylcholine receptor targeted family of peptides [12]. In this study, we biologically characterize the crude venom extract form *C. striatus* to assess the potency and possible medical applications of the venom.

2. MATERIALS AND METHODS

A. Sampling and Sample Preparation

Live specimens of the marine snail *C. striatus* were collected near the coastal areas of Brgy. Bagumbayan, P-7, Kauswagan, Lanao del Norte, Philippines (8°11'32.0"N 124°05'27.5"E). The preparation of crude venom extract was made according to the method of Saravanan *et al*. [13], with modifications. The shell of *C. striatus* was cracked-open using a hammer followed by dissection of the soft body to obtain the venom duct. The contents of the duct was squeezed and added with 1.5 mL of the homogenizing buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$). The mixture was homogenized using a vortex then sonicated three times for 50 sec/cycle (10 sec on, 20 sec off) followed by centrifugation at 4,000 rpm for 10 minutes. The supernatant was transferred to a screw-capped container.

B. Protein Estimation

The protein content of crude venom extract was estimated using Bradford method [14].

C. SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) which utilized 12% polyacrylamide gel was carried out following Laemmli method [15]. A 10µL of molecular weight marker and crude protein were loaded in the gel. Upon completion of electrophoresis, the gel was washed with distilled water, stained with Coomassie Brilliant Blue R-250 for 2h at room temperature, and de-stained with methanol: Acetic acid:dH_2O (7:7:86 v/v) for 48 hours. Protein bands were visualized and analyzed using ImageJ software.

D. Evaluation of Biological Activities

D.1 Brine Shrimp Lethality Assay

The method of Adoum [16] was carried out to evaluate the toxicity of the venom extract. Nauplii of the brine shrimp *Artemia salina* were hatched by adding eggs in a hatching chamber containing filtered fresh seawater kept under an inflorescent bulb for 48 hours. A 20 mg of the freeze-dried extract was dissolved in 2 mL of the homogenizing buffer. From this, 5 mL of 500-, 250-, 125-, and 62.5 ppm concentrations were prepared by diluting it with 4.5 mL of the filtered fresh seawater. The vials were kept illuminated upon counting of survivor in the 24-h exposure of the nauplii to the sample. LC_{50} values were determined from the death count.

D.2 DNA-Binding Activity

The method of Maier *et al*. [17] was followed to determine the DNA-Binding activity by obtaining random DNA. Salmon sperm DNA (ssDNA; 2mg/mL in distilled-deionized water) was homogenized by ultrasonication for 6 minutes,

heating at 95°C then cooled immediately in an ice bath. The DNA-binding activity of the crude venom sample was examined through 1D-Thin Layer Chromatography. The ssDNA was spotted above one spot prior to chromatography in TLC plates with 3.0 cm. x 8.5 cm dimension. The solvent

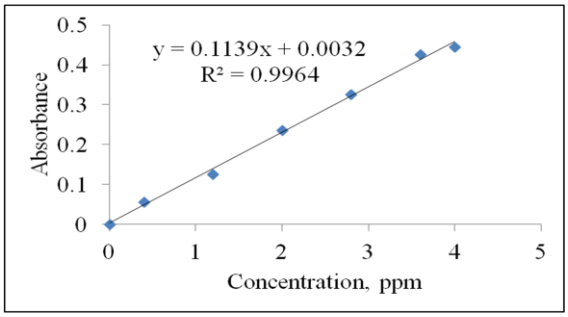

Figure 1: Standard calibration curve for Bradford protein estimation.

Table 1: Protein Concentration of the Crude Venom Extracts *(Conus striatus)*.

Sample	Dil. factor	Abs.	Concentration, ppm
1	460	0.054	205.15
2	200	0.124	211.93

system used was Hexane:Methanol with 3:7 ratio. Analysis was conducted in triplicates, where the Rf_1 (spot without ssDNA) and the Rf_2 (spot with ssDNA) were measured. The corresponding Rf_2/Rf_1 ratio value was also calculated.

D.3 Hemolytic Activity

The preparation of erythrocytes suspension was made according to the method of Kumar *et al*. [18], with modifications. A 5 mL of blood was collected from a healthy individual in a tube containing EDTA. The blood was centrifuged at 1500 rpm for three minutes in a laboratory centrifuge. Plasma (supernatant) was discarded and the pellet was washed three times with sterile phosphate buffer saline solution (pH 7.2±0.2) by centrifugation at 3000 rpm for 10 min. The cells were suspended in normal saline to 1.0%. *In vitro* hemolytic activity was performed by the micro hemolytic method of Yang *et al.* [19]. A volume of 0.5 mL of the cell suspension was mixed with 0.5 ml of the venom extract (200, 100, 50, 25 µg/ml concentrations in Tris-HCl buffer) in an eppendorf tube. The mixtures were prepared in triplicate followed by incubation for an hour at room temperature. Then, it was centrifuged at 2000 rpm for 5 min. Appropriate controls were included such as phosphate buffer ((-) control) and 1% Triton X-100 ((+) control). The released hemoglobin was quantified by reading the absorbance at 450 nm. Percent hemolysis was computed based on the formula:

$$\%Hemolysis = \frac{[\text{A(sample)} - \text{A(PBS)}]}{[\text{A(Triton)} - \text{A(PBS)}]} x100$$

D.4 Mice Bioassay

The mice bioassay was carried out according to the method of Gouiffes *et al*. [20]. From the calculated protein concentration, the crude venom sample was diluted to 150 ppm and 100 ppm sample solutions using phosphate buffer saline solution (pH 7.2 ± 0.2). Triplicate sets were prepared for each concentration, were the

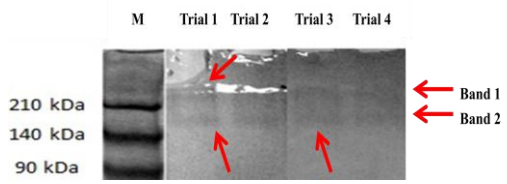

Figure 2: SDS-PAGE profile of crude venom extract of *C. striatus*. The image was converted to an 8-bit (black/white) image during ImageJ analysis.

solution was injected intraperitoneally to the mice. Symptoms of toxicity or behavioural changes for the injected mice were observed and recorded less than 24 hours.

3. RESULTS

A. Protein Estimation

The protein content of the crude venom extracts was calculated using the equation generated from the standard calibration curve, shown in Figure 1, upon reading the absorbance value at 595 nm for each concentration of BSA standard solution. The generated curve was used to calculate the protein concentration on the sample shown in Table 1. The amount of protein was found to be around 205 – 212 ppm.

B. SDS-PAGE

SDS-PAGE on 12.0% gel, pure protein extracts from crude venom of *C. striatus* yielded two bands, above 210 kDa and between 210 to 140 kDa. The molecular mass distribution of the conopeptides in *C. striatus* is shown in Figure 2 (pointed by red arrows). After the staining process, the electrophoretic gel was analyzed using ImageJ to quantify the percent composition of the separated proteins. The percentage composition on the proteins is shown in Table 2.

C. Evaluation of Biological Activities

C.1 Brine Shrimp Lethality Assay

In this study, lethality of the marine snail *Conus striatus* crude venom extract to brine shrimps were determined on *A. salina* after 24-h exposure to the samples. The results of this particular exposure, including the chronic LD_{50} determination, were shown in Figure 3.

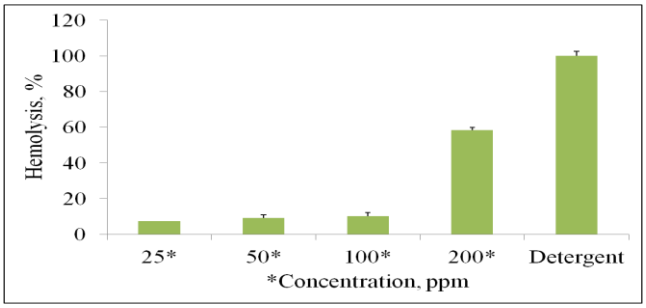

Figure 3: Cytotoxicity test results of *C. striatus* crude venom extract against Brine Shrimp Nauplii after 24-h exposure. Error bars represent ± SD for n = 3

Table 2: Percentage composition of protein bands in the SDS – PAGE of the crude venom extract of *C. striatus.*

Bands	Area	Composition (%)
1	11458.7	7.217341
2	147307	92.78266
Total	158766	

C.2 DNA-Binding Activity

The crude venom sample of *C. striatus* was analysed for its DNA-binding properties in a solvent system consisting of n-Hexane: Methanol (3:7), and was performed in a 1D-TLC method on silica gel plates. Table 4 showed the Rf values obtained with and without ssDNA The spot without ssDNA served as the reference.

C.3 Hemolytic Activity

The human blood erythrocytes were vulnerable to lysis provoked by *C. striatus* venom extract. The hemolytic assay conducted on human erythrocytes revealed that hemolysis occurs at half the concentration of the calculated chronic LD_{50} of the sample, as shown in Figure 4.

Table 3: Retention factor values of the DNA-Binding Activity of *Conus striatus.*

Trial	Rf_1	Rf_2	Rf_2/Rf_1	Average Rf_2/Rf_1
1	0.947	0.147	0.155	
2	0.913	0.147	0.161	0.175
3	0.953	0.2	0.21	

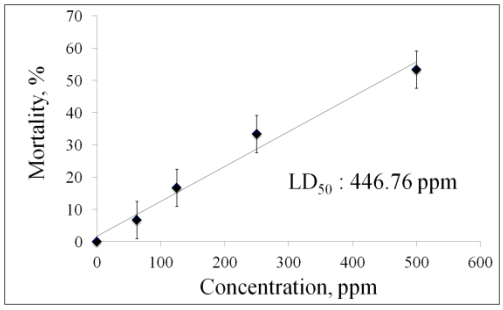

Figure 4: Percent hemolytic activity of *C. striatus* with respect to the positive control (Triton/Detergent). Hemolytic activity is more pronounced at 200 ppm. Error bars represent SD for n= 3.

C.4 Mice Bioassay

The minimum lethal concentration injected intraperitoneally is 100 ppm, which showed lethality or toxicity to mice, followed by 150 ppm, then 211.930 ppm which is the concentration of the crude venom extract. Each mice was injected with 300 µL (0.3 mL) of the varied concentration, which has shown certain behavioural changes after administration. Observation time lasted for 24 hours. Table 6 summarizes the recorded behavioural changes of mice before death.

4. DISCUSSION

The crude venom extract of *C. striatus* was found to contain around 205 – 211 ppm of proteins, as estimated by the Bradford assay, shown in Table 1, calculated from the standard calibration curve in Figure 1. The crude venom contains two major protein components, as confirmed by SDS-PAGE in Figure 2, with molecular weights above 210 kDa (Band 1) and between 210-140 kDa (Band 2), respectively. Upon ImageJ analysis, Band 1 comprised 7.22 % of the total protein while Band 2 is 92.78 % of the total protein concentration as summarized in Table 2. These results were in agreement with the ones reported by Kobayashi *et. al.*, in 1982 [9]. These observed protein bands reportedly belong to the nicotinic acetylcholine receptor [12], the α-conotoxin SI [10] and α-conotoxin SIA [11].

The crude extract was also found to be very toxic after 24-hour exposure of *A. salina* in the brine shrimp lethality assay, as shown in Figure 3, with a calculated chronic LD_{50} of 446.76 ppm. The chronic LD_{50} value of 446.76 ppm is characterized as toxic according to Meyer's toxicity index wherein an LC_{50} < 1000 µg/ml is considered toxic, while an LC_{50} > 1000 µg/ml isconsidered non-toxic [21]. This particular chronic LD_{50} value is also characterized as in medial toxicity based on Clarkson's toxicity criterion [22].

The DNA-binding activity of the crude venom extract was found to be very strong as shown in Table 3. The spots without ssDNA (Rf_1) appear to have travelled farther than the spots with ssDNA (Rf_2). The Rf_2/Rf_1 ratio of < 0.85 implies moderate to strong binding and > 0.85 implies weak to no binding activity.

The calculated value of the ratio of Rf_2/ Rf_1 was found to be 0.175, which implies strong DNA-binding activity. It seems likely that the toxicity of the crude venom could be correlated to its strong DNA-binding ability, which could potentially arrests DNA replication and transcription, once it interacts with DNA inside the living system.

Most reported toxins are usually haemolytic. Such toxins like sea anemone toxins and melittin from bee venom form a channel in the lipid membrane by assembly of several molecules. These toxins are also known to increase their α-helical structure content upon binding to the lipid membrane.

In this study, erythrocytes, the most abundant cells in the human body, were used to determine the haemolytic activity of the crude venom. The membranes of erythrocytes are rich in polyunsaturated fatty acids, hemoglobin and high cellular concentration in oxygen, which render them extremely susceptible to oxidative damage. Oxidative damage of erythrocyte membrane may be implicated in hemolysis.

Table 4: Observations recorded after 24 hours of intraperitoneal injection of venom with different concentrations in each group of mice.

Group no.	Mouse no.	Concentration (ppm)	Observations	No. of Deaths	Weight (g)	Time of death (hr:min)
1	1	212	Immediate "calming" effect, increased respiratory rate, gasping for air and seizures before death	3/3	14.5	2:47
	2				14.9	~2:00
	3				16.2	2:49
2	1	150	Immediate "calming" effect, increased respiratory rate, defecation, urination, seizure before death	2/3	12.8	~3:00
	2				13.8	-
	3				13.7	1:30
3	1	100	Immediate "calming" effect, increased respiratory rate, defecation, urination, seizure before death	3/3	12.6	~3:00
	2				13.2	~3:00
	3				13.2	~3:00

Therefore, this cellular system could be very useful to study oxidative stress and the protective effect of *C. striatus* venom extracts [23]. Results showed that the crude extract has high hemolytic activity towards human erythrocytes, as shown in Figure 4, of about 60 % compared to the positive control (Triton) at 200 ppm, which is below half of the calculated chronic LD_{50}. This indicated that, indeed, conotoxins causes *in vitro* hemolysis in human erythrocytes membrane resulting in the release of hemoglobin [13].

In *in vivo* envenomation using mice, the common symptoms exhibited by all mice were, immediate "calming" effect – where they would sit still in one spot and their respiratory rate (breathing) is increased. Major convulsions were also observed before death, as well as defecation and urination. The summary of the observations were shown in Table 4. The crude venom exhibited a very strong toxicity *in vivo*, from as low as 100 ppm of the crude venom. All mice, at 100 ppm, were dead 3 hours after intraperitoneal administration of the venom, while at higher concentration, 212 ppm undiluted venom, the mice were dead as early as 2 hours after administration.This elicited strong *in vivo* toxicity of the venom on mice could be correlated to its high hemolytic activity and strong DNA-binding ability. The crude venom likely caused hemolysis of the mouse erythrocytes, once inside the body, thereby decreasing oxygen in the blood and on the different organs of the mouse, as indicated by the observed increase respiratory rate, which could be a compensatory mechanism for the decreased oxygen distribution on different organs in the body. The strong DNA-binding ability of the crude venom could likely interfere in the biological processes, by potentially arresting DNA replication and transcription processes, contributing to increased mortality.

5. CONCLUSION

Conus striatus crude venom is highly toxic both in Brine Shrimp Lethality and Mouse assays. The toxicity of the venom could likely be correlated to its strong DNA-binding ability and its high hemolytic activity on human erythrocytes. Moreover, the study strongly suggests that the conotoxin could be utilized as a probing tool to investigate its pharmacological potential. The strong DNA-binding ability of the venom could potentially be used as DNA intercalating agent, like that of the chemotherapeutic agent cisplatin, to induce arrest in DNA replication and transcription processes for cancer treatment. The high hemolytic activity of the crude venom could highly aid in cell membrane destruction in cancer cells. These characteristics emphasize the need for isolation and molecular characterization of the active component of the *C. striatus* venom.

6. ACKNOWLEDGEMENT

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