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Biological Characterization of *Conus textile* venom for Medical Applications

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ABSTRACT
Cone snails are predatory marine gastropods that use venom as means of predation and defense. This venom is a complex mixture of conopeptides that selectivity binds to ion channels and receptors, giving them a wide range of potential pharmaceutical applications. In this study, crude venom from venom duct of the cone snail Conus textile was biologically characterized using BSLT, DNA-Binding activity, hemolytic activity, and mice bioassay. The crude venom extract contains 585 – 596 ppm protein. The proteins were proven to contain four protein bands, one band at 210 kDa, two bands between 90 kDa and 140kDa, and one band just below 20 kDa. The crude venom showed Brine Shrimp larvicidal activity, with a chronic LD_{50} value of 304.12 ppm, and has a strong DNA-binding capability. The crude venom also exhibited very strong hemolytic activity (87 %) at 500 ppm and a strong lethality on mice at 600 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 due to its very high hemolytic activity.
Keywords: *Conus textile, Conotoxin, DNA-binding, Venom.*

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INTRODUCTION

The member of genus *Conus* belongs to the phylum Mollusca, class Gastropoda, order Sorbeoconcha, and family Conidae, with 706 valid species currently recognized in the World Register of Marine Species [1]. Cone snails are predatory marine animals that kill their prey with powerful venom. The shells of the cone snails are spiral and conic, hence their name. Cone snails are found in warm seas and oceans throughout the world but are mostly in the Indo-West Pacific region. There are 300 species of *Conus* in the Philippines and more than 750 accepted species. Each species contains 100 to 200 different peptides. In the Philippines, only few studies have been done on cone snails [2].

Due to their limited mobility, cone snails have evolved to produce a vast library of structurally diverse bioactive peptides for prey capture and defense. The venom apparatus of the Cone snails produces potent venom called conotoxin peptides. These venoms are released to target receptors and ion channels in vertebrate and invertebrate neuromuscular system to help paralyzing their prey [3]. Since the venom can also be used as a defense against their predator, thus human fatalities occasionally occur [4].

More than 100 conotoxins purified from venoms have been classified into pharmacological families according to their molecular targets and their effect. They are also grouped based on these three arrangements: the similarities between the cDNA and its translated peptide sequence of the conotoxin precursors (gene superfamilies), the cysteine residue patterns found on the matured conotoxin (cysteine frameworks), and the specificities to pharmacological targets (pharmacological families) [6].

Conotoxins are classified into α-, δ-, μ-, κ-, and ω-conotoxins according to the targets of the conopeptides [7-11]. The receptors for α-, δ- and μ-, κ- and ω-conotoxins are nicotonic acetylcholine receptors, sodium channels, potassium channels, and calcium channels, respectively [12].

The specificity of the conotoxins is due to the disulfide bonds present and specific amino acids in the cysteine loops. Due to the target specificity and its actions, most of the conotoxin families have been

characterized and some of them have the potential to be developed into drugs, specifically in the development of novel analgesics. Furthermore, the specificity is one of the attributes that make them valuable diagnostic tools in the characterization of neural pathways and as therapeutic agents [13].
Conotoxins are used as tools of research including determining how specific receptors and ion channels work. Conotoxins have potential roles in the direct treatment of disease. The ω- conotoxins are used in neuroscience research to study calcium channel subtypes. Thus, due to their specificity to their respective target molecules, they have a huge potential in the medicinal field. In this study, we biologically characterize the crude venom extract form *Conus textile* to assess the potency and possible medical applications of the venom.

MATERIAL AND METHODS

SAMPLING AND SAMPLE PREPARATION:

The samples of *Conus textile* of lengths ranging from 60-85 mm were collected along the intertidal range of P-7, Bagumbayan, Kauswagan, Lanao del Norte, Philippines (8.197771°N, 124.09373°E). The specimens were kept alive in sand with seawater until they are dissected. The preparation of crude venom extract was made according to the method of Saravanan *et al*., 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.

PROTEIN ESTIMATION:

The concentration of the crude venom extract was determined using Bradford Assay. Standard solutions of bovine serum albumin with concentrations 0, 2, 6, 10, 14, 18, 20 µg/5 mL were prepared using the Bradford procedure. Then, a standard calibration curve was created. After that, the crude venom extract was serially diluted with the buffer solution until the concentration is in the range of the created calibration curve. Lastly, the concentration of the crude venom extract was calculated then recorded.

SDS-PAGE:

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which utilized 12% polyacrylamide gel was carried out following Laemmli method. 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.

EVALUATION OF BIOLOGICAL ACTIVITIES

BRINE SHRIMP LETHALITY ASSAY:

The method of Adoum 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.

DNA-BINDING ACTIVITY:

The method of Maier *et al*. 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 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.

HEMOLYTIC ACTIVITY:

The preparation of erythrocytes suspension was made according to the method of Kumar *et al*., 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*. A volume of 0.5 mL

of the cell suspension was mixed with 0.5 ml of the venom extracts (62.5, 125, 250, and 500 ppm 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:

$$\% \text{ Hemolysis} = \frac{A_{sample} - A_{PBS}}{A_{Triton} - A_{PBS}}$$

where A_{sample} is the absorbance of the sample, A_{PBS} is the absorbance of the minimal control, and A_{Triton} is the absorbance of the maximal control.

MICE BIOASSAY:

The mice bioassay was carried out according to the method of Gouiffes *et al*. 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 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.

RESULT

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 585 – 596 ppm.

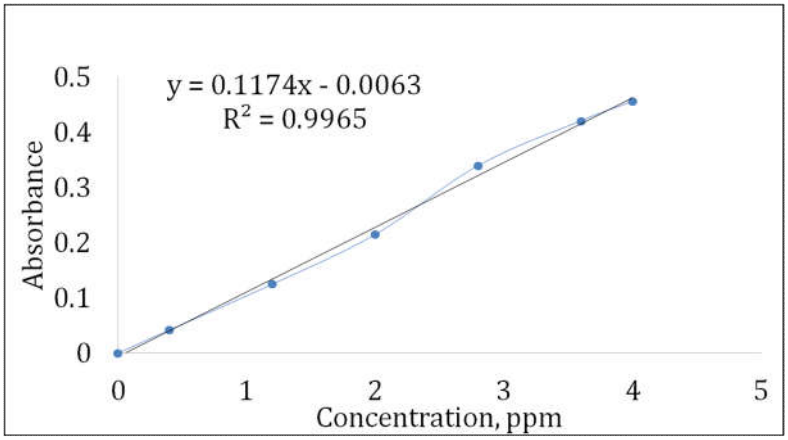

Figure 1: Standard calibration curve for Bradford protein estimation.

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

Sample	Dilution factor	Absorbance	Concentration, ppm
1	460	0.175	596
2	200	0.171	585

SDS-PAGE:

SDS-PAGE on 12.0% gel, pure protein extracts from crude venom of *C. striatus* yielded four protein bands, one band at 210 kDa, two bands between 90 kDa and 140kDa, and one band just below 20 kDa. The molecular mass distribution of the conopeptides in *C. textile* is shown in Figure 2 (pointed by blue 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 protiens is shown in Table 2.

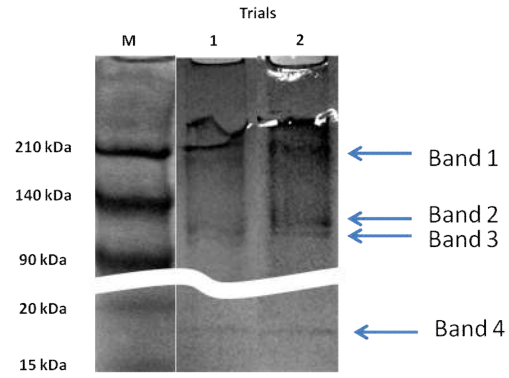

Figure 2: SDS-PAGE profile of crude venom extract of *C. textile*. M is the protein marker.

Table 2: Estimated percentage composition of protein bands in the SDS–PAGE profile of the crude venom extract from *C. textile.*

Bands	Ave. Area	Composition (%)
1	6199.562	33.85108
2	3830.903	20.91764
3	1254.569	6.850243
4	7029.186	38.38103
Total	18314.22	

EVALUATION OF BIOLOGICAL ACTIVITIES

BRINE SHRIMP LETHALITY ASSAY:

In this study, lethality of the marine snail *Conus textile* 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.

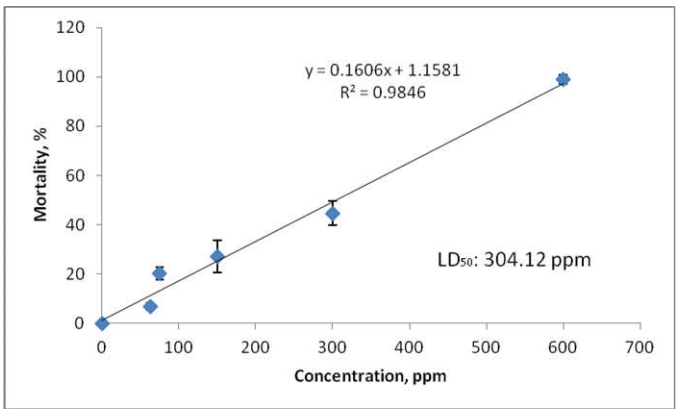

Figure 3: Cytotoxicity test results of *C. textile* crude venom extract against Brine Shrimp Nauplii after 24-h exposure. Error bars represent ± SD for n = 3.

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

Trial	Rf_1	Rf_2	Rf_2/Rf_1	Average Rf_2/Rf_1
1	0.9	0.4071	0.4524	
2	0.8786	0.3786	0.4309	0.49
3	0.8571	0.5	0.5833	

DNA-BINDING ACTIVITY:

The crude venom sample of *C. textile* 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 3 showed the Rf values obtained with and without ssDNA. The spot without ssDNA (Rf_1) served as the reference.

HEMOLYTIC ACTIVITY:

The human blood erythrocytes were vulnerable to lysis provoked by *C. textile* venom extract. The hemolytic assay conducted on human erythrocytes revealed that high hemolysis occurs at 500 ppm concentration, which is nearly half of the calculated chronic LD_{50} of the sample, as shown in Figure 4.

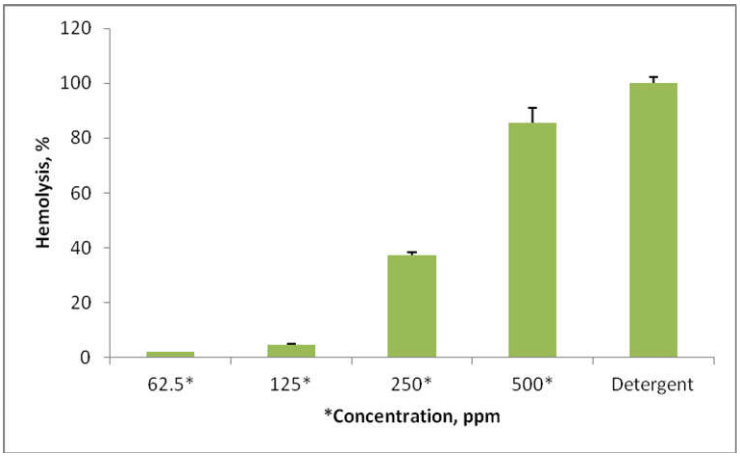

Figure 4: Percent hemolytic activity of *C. textile* with respect to the positive control (Triton/Detergent). Hemolytic activity is more pronounced at 500 ppm and is comparable to the positive control triton. Error bars represent SD for n= 3.

MICE BIOASSAY:

The minimum lethal concentration injected intraperitoneally, which showed lethality or toxicity to mice, is 300 ppm at 50 % lethality then followed by a 100 % lethality at 600 ppm, which is the original concentration of the crude venom extract. Each mouse 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 4 summarizes the recorded behavioural changes of mice before death.

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	150	Immediate "calming" effect, intense color of urine	0/2	20.60	N/A
	2				22.98	N/A
2	1	300	Immediate "calming" effect, loss of grasp	1/2	11.29	N/A
	2				10.40	12.05
3	1	600	Immediate "calming" effect, "calming effect", hair standing, loss of grasp, convulsion, eye becomes opaque	2/2	11.41	13:17
	2				11.65	13:50

DISCUSSION

The crude venom extract of *C. textile* was found to contain around 585 – 596 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 four major protein components, as confirmed by SDS-PAGE in Figure 2, with molecular weights around 210 kDa (Band 1), two bands between 90-140 kDa (Bands 2 & 3), and another one just below 20 kDa (Band 4). Upon ImageJ analysis, Band 4 (38.38 %) was found to have the highest concentration of the four components followed by Band 1 (33.85 %), then Bands 2 and 3 with 20.92 % and 6.85 %, respectively. The total protein and their respective percentage compositions were summarized in Table 2. These result suggested that the venom in the venom duct of the *C. textile* only contains four basic protein components.

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 304.12 ppm. The chronic LD_{50} value

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 is considered non-toxic [9,25]..

The toxicity result was confirmed by the DNA-binding activity of the crude venom extract as it 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 average calculated value of the ratio of Rf_2/ Rf_1 (0.49) fits that of moderate to strong DNA intercalating 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 hemolytic. 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 hemolytic 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. Therefore, this cellular system could be very useful to study oxidative stress and the protective effect of *C. textile* venom extracts. Results showed that the crud extract has very high hemolytic activity towards human erythrocytes, as shown in Figure 4, of about 85.7 % compared to the positive control (Triton) at 500 ppm. This indicated that, indeed, conotoxins causes *in vitro* hemolysis in human erythrocytes membrane resulting in the release of hemoglobin. Hemolytic activity is indicative of cytolytic activity and most cytotoxins have considerable potential as anticancer and antiviral agents. Lattore [21] postulated that the lethality of *C. textile* venom arose not from its neurotoxic properties but from the hemotoxic activities but Kobayashi *et al.* [22] presented evidence to the contrary.

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* at 600 ppm of the crude venom, while 50 % mortality was observed at 300 ppm after intraperitoneal administration of the venom. The mice were dead as early as 12 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 could 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 indicted 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.

CONCLUSION

C. textile 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 erythrocytes. Moreover, the study strongly suggested that the conotoxin could be investigated for 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 emphasized the need for isolation and molecular characterization of the most active component of the *C. textile* venom for establishing its potential medical application.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in publishing this manuscript.

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