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Pharmacological studies on the venom of the marine snail *Conus lentiginosus* Reeve, 1844

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ABSTRACT

The bioactivities of the venom from *Conus lentiginosus* were studied from Mumbai coast, are characterized biomedically and pharmaceutically. Partial purification of the crude extract was carried out using DEAE cellulose anion exchange chromatography. The protein content of crude venom was found to be maximum $432.5 \pm 1.58 \mu\text{g/mL}$ and minimum $80.2 \pm 1.08 \mu\text{g/mL}$. The mice bioassay for lethality was performed on male albino mice weighing 20 ± 2 found to be lethal at 0.50, 0.75 and 1.0 ml. The crude venom exhibited hemolytic activity on chicken erythrocytes, which was estimated as 8 HU. Analgesic activity test was carried out on albino mice by tail flick method. The crude venom exhibited neurostimulatory response on mice brain AChE activity. Inhibitory effect on AChE activity ranging between 23% and 397% was caused by venom of *C. lentiginosus*. Molecular weight of purified toxins were determined by SDS-PAGE on 12.0% gel system using standard protein markers and yield 3 band 40 kDa, 71 kDa and 120 kDa. The present study reveals the pharmacological potential of the crude venom of *Conus lentiginosus* could be utilized for a better assessment of the clinical manifestations produced by the venomous marine animals.

Keywords: *Conus lentiginosus*; hemolytic activity; SDS-PAGE; venom; AChE; bioassay

1. Introduction

The member of genus *Conus* belongs to the phylum Mollusca, the class Gastropoda, the order Sorbeoconcha, family Conidae and genus *Conus* [1]. The predatory gastropods cone snails (genus *Conus*) are comprising approximately 700 species found in tropical marine habits around the world [2], with each *Conus* species producing a distinctive repertoire of 100-200 venom peptides [3]. More than 100 conotoxins purified from venoms have been classified into pharmacological families according to their molecular targets [4-6]. More than 300 venomous species are known of which forty are dangerous and believed to cause poisoning in human [7-9]. The most dangerous species of *Conus geographus* are deadly to humans [1]. Piscivores are more dangerous to human than other cone snails [10, 11]. Some species of this genus, such as *Conus geographus*, *C. textile* and *C. marmoreus* are known to paralyze and kill a man. Venom from *C. geographus* has delivered by disposable hypodermic like needle has indeed killed many unsuspecting human victims [12]. These peptides are potent and highly selective blockers or modulators of ion channel function involved in such disorders. Cone snails are predatory marine animals that kill their prey with powerful venom. Conotoxins are pharmacologically and chemically diverse group of toxins found in the venom. The lethality of *conus* snails was first noted by a Dutch naturalist [13] in the scientific literature 300 years ago. Extensive studies during last decade, on the purification and characterization of toxins from the piscivorous species, *C. geographus* and *C. magus*, revealed the presence of three pharmacologically distinct class of toxins, α -conotoxins acts on acetyl choline receptors; μ -conotoxins acts on skeletal muscle Na^+ channels and ω -conotoxins effects presynaptic neuronal Ca^{++} channels [8, 14-17]. These targets to mammalian receptors a remarkable number of venom components has been successfully developed as new research tools and therapeutic drugs [18, 19]. Several species of this genus including *Conus amadis*, *C. figulinus*, *C. lorosii*, *C. bitulinus*, *C. inscriptus* and *C. parvatus* also inhabit Indian coast. Therefore, the present study is aimed at obtaining a valuable piece of information on bioactive compounds from *Conus* for biomedical applications. The mechanism by which the *Conus lentiginosus* toxins cause death of mice was investigated and acquiring knowledge about its toxic factors its venom.

2. Materials and Methods

2.1. Collection of sample

The samples of *Conus lentiginosus* were collected along the of Khardanda beach, Khar, Mumbai. The specimens were kept alive in salt water till their scarified. A total amount of 2.5 g of crude venom was extracted from about 40-50 specimens. Venom was extracted from freshly sacrificed animals as described by [20]. The soft body of the animal was removed by cracking open the shell. The venom duct and venom bulb of each animal was dissected out. The venom duct was ground and mixed with distilled water. Then it was centrifuged at 10,000 rpm for 15 minutes. The supernatant was collected for lyophilisation and stored immediately at 4 °C. The lyophilized powder was resuspended in distilled water and it was considered as crude venom. The extracts were stored at -20 °C for further analysis.

2.2. Experimental Animal

Male albino mice of Kausauli strain weighing 20 ± 2 g were procured from the animal house of M/S Haffkine Bio-pharma, Mumbai, and were maintained in the laboratory, following the codal formalities of the Institute's Ethical Committee.

2.3. Partial Purification

Partial purification of the crude extract was carried out through DEAE cellulose anion exchange chromatography, following the method of Shiomi *et al.* [21].

2.4. Protein Estimation

The protein estimation was done as described by Lowry *et al.* [22], using bovine serum albumin (BSA) as a standard.

2.5. Mice Bioassay

The mice bioassay was carried out according to method Gouiffes *et al.* [23]. The crude venom dissolved @ 5 mg/mL in PBS was injected intraperitoneally (i.p.) to the mice in doses of 0.25, 0.50, 0.75 and 1.0 mL of each fraction was injected i.p. to the mice. Triplicate sets were maintained for each dose. The injected mice were kept under observation in mice rearing cages. The time of injection and the time of death were recorded, besides recording the behavioural changes before death.

2.6. Hemolytic Study

The haemolytic activity of crude venom on chicken was tested by micro haemolytic method [24]. The blood was centrifuged at 5,000 rpm for 5 minutes, the supernatant was discarded and the pellet suspended in normal saline (pH 7.4). The procedure was repeated thrice and 1% erythrocyte suspension was prepared by adding 99 mL normal saline to 1 mL of packed RBC.

The micro haemolytic test was performed in 96 well 'U' bottom microtitre plates. A row was selected for chicken erythrocyte suspensions. Serial two fold dilutions of the crude venom (100 µL; 1 mg crude in 1 mL PBS) were made in PBS (pH 7.2) starting from 1:2 ratio. An equal volume of 1% erythrocyte was added to all the wells. The plate was shaken to mix the RBC and venom extract. The plates were incubated at room temperature for two hours before reading the results. Appropriate control was included in the tests. Erythrocyte suspension to which distilled water was added (100 µL respectively) served as blanks for negative control. Button

formation at the bottom of the wells was taken as negative. The reciprocal of the highest dilution of the venom extracted showing the hemolysis was defined as one haemolytic unit.

2.7. Neuromodulatory Activity

2.7.1. AChE Activity

The method of Ellman *et al.* [25] was followed to see the AChE activity by preparing enzyme source obtained from male albino mouse.

Three mL phosphate buffer (pH 8.0) was taken in each tube to which 0.1 ml of enzyme source (2% w/v homogenate) was added and stirred. Then 100 µL of 0.01 M DTNB (5, 5-dithiobis-2-nitrobenzoic acid) was added and the initial color was measured spectrophotometrically at wave length of 412 nm. The test solution of toxin (100 µL) in different concentrations such as 100, 200, 400, 800 and 1000 µg were added. Control experiment was also run simultaneously with 100 µl of triple distilled water without toxins.

To start the reaction, 20 µL of acetyl thiocholine iodide (ATCI) (0.075 M) was added to each tube as substrate and then the reaction was allowed to continue for 15 minutes at room temperature. The colour developed was measured as final reading spectrophotometrically at 412 nm. All experiments were conducted in duplicate.

2.7.2. Immuno-modulatory Activity

Immuno-modulatory activity was analysed through *in vitro* phagocytosis of *Candida albicans* by polymorphonuclear cells (PMN) (slide method) following the procedure given by Kulkarni and Karande [26].

2.8. Evaluation of Analgesic Activity

2.8.1. Tail Flick Method

Analgesic activity was measured according to the D'amour and Smith [27] using a tail flick analgesia meter (Harvard, USA 50-9495, 230 V and 50 Hz) with a variable 150 W, 25 V quartz lamp as the heat source. During the testing period, the mice were restrained in a plastic tube, to which they had been previously adapted for 10 minutes twice a day for three days. The tail flick latency was recorded as the time onset of stimulation to the withdrawal of the tail from a light beam. The beam of light was focused on some spot, at about 6 cm from the tip of the tail of each animal. The intensity of the radiant heat was identical in all the experiments. The reaction time of the animal was then displayed and noted down.

Mice without administration of any toxin or known pain killer were used as controls while those injected intraperitoneally with Paracetamol (Crocin® @ 0.25 mL/ 20±2 g mice) served as reference standards. Experimental mice in triplicates received 0.25 mL of toxin i.p and subjected to a light intensity of 4 different current strengths viz. 3.0, 3.5, 4.0 amps. Analgesic activity was expressed as a ratio between the difference in reaction time of envenomated mice and control, since analgesic potential would be proportional to the difference in tail flick latency between the toxin and control. The mice were tested after 30 minutes of injection.

2.8.2. SDS-PAGE Analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) which utilized 5% stacking gel and 10% resolving polyacrylamide gels was carried out to estimate the molecular weight of the haemolytic toxin according to the

method of Laemmli [28]. The protein was analyzed by SDS-PAGE. Five molecular weight markers (9, 29, 40, 72 and 150 kDa) were used. Ten microlitre of the marker was loaded in the right well as marker and the crude proteins were loaded subsequently wells. Upon completion of electrophoresis, the gel was washed gently with distilled water to remove excess SDS, stained in Coomassie Brilliant Blue R-250 (Coomassie brilliant blue R-250, 1.25 g methanol, 227 mL; glacial acetic acid, 46 mL; distilled water to complete a volume of 500 mL) for two hours at room temperature and de-stained (methanol, 7 mL; glacial acetic acid, 7 mL; and distilled water to reach 100 mL) for 48 hours. Protein bands were visualized as dark blue bands on a light blue background. Samples were denatured by boiling in buffer containing SDS and β – mercaptoethanol prior to loading onto the gel. Following electrophoresis at 30 mA for four hours, gels were stained by Coomassie blue staining.

3. Results

3.1. Protein Estimation

The protein content in the crude extract of *C. lentiginosus* was found to be 432.5 $\mu\text{g/mL}$. Amount of protein in the purified fractions was found to vary between a minimum of 80.2 $\mu\text{g/mL}$ (F10) and a maximum of 281.2 $\mu\text{g/mL}$ (F1) (Table 1).

Table 1: Protein content of crude and partially purified fractions of *C. lentiginosus*

Sl. No	Samples	Protein ($\mu\text{g/ml}$)
1	Crude	432.5 \pm 1.58
2	F1	281.2 \pm 0.48
3	F2	243.7 \pm 0.55
4	F3	222.6 \pm 0.89
5	F4	209.5 \pm 0.53
6	F5	191.3 \pm 1.26
7	F6	183.8 \pm 0.76
8	F7	162.3 \pm 0.33
9	F8	152.3 \pm 0.68
10	F9	122.6 \pm 0.85
11	F10	80.2 \pm 1.08

3.2. Mice Bioassay for Lethality

The minimum lethal dose of venom extract was found to be 0.50 mL containing 220.0 $\mu\text{g/mL}$ of protein for 20 \pm 2 g mice, wherein death occurred in 52 minute, 0.75 mL containing 325.0 $\mu\text{g/mL}$ death occurred in 62 sec upon i.p. injection. When injected with the highest dose of 1.0 mL, containing 432.5 $\mu\text{g/mL}$ of protein death occurred in 43 sec. Toxicity of crude extracts of the conidiids injected intraperitoneally with different doses in male albino mice are shown the symptoms of toxicity observed (Table 2).

Table 2: Toxicity of crude extracts of the *C. lentiginosus* injected intraperitoneally in male albino mice (20 \pm 2 g with different doses).

Injected volume (ml)	Amount of protein ($\mu\text{g/mL}$)	Death time	Symptoms of toxicity	Remarks
		(Second)		
0.25	115.0	-	-----	Non-lethal
0.50	220.0	52	Palpitation, excess urination, gasping for breath, exophthalmia, eye becomes opaque before death.	Lethal
0.75	325.0	62	Palpitation, excess urination, gasping for breath, exophthalmia, eye becomes opaque before death.	Lethal
1.0	432.5	43	Palpitation, excess urination, gasping for breath, exophthalmia, eye becomes opaque before death.	Lethal

3.3. Hemolytic Assay

Hemolytic assay conducted on chicken erythrocytes revealed that the crude venom of column fractionated venom of *C. lentiginosus* induced spontaneous hemolysis of chicken blood found to be 8 Haemolytic Units (HU) from the protein content in the crude extract of *C. lentiginosus* was 432.5 $\mu\text{g/mL}$.

3.4. Neuromodulatory Activity

3.4.1. In vitro effect on AChE activity

Crude venom of *C. lentiginosus* showed a neurostimulatory response on the mice brain. The level of modulation was inhibitory effect on AChE activity ranging between a minimum of 23% and at the maximum concentration 397% caused by venom. The activity increased with increased in concentration (Table 3).

Table 3: In vitro effect of the venom of *C. lentiginosus* on the mouse brain AChE activity

Sample (μg)	μ moles of Acetylcholine hydrolyzed/mg protein/hr	Activity (%)
Control	0.08990	--
100	0.02010	23
200	0.08024	85
400	0.09758	108
800	0.26332	285
1000	0.35538	397

3.5. Immuno-modulatory Activity

Stimulating activity up to 19.23% was shown by lower concentration (100 μg) of *C. lentiginosus* venom whereas suppressive activity, between 12.35% and 20.85% was exhibited by higher concentrations (Table 4).

Table 4: Phagocytosis of *Candida albicans* by Polymorphonuclear cells (PMN) when treated with venom

Concentration ($\mu\text{g/mL}$)	Phagocytic Index	Level of modulation (%)
100	2.558	19.23
200	2.129	-12.35
400	2.211	-14.45
800	1.932	-19.82
1000	1.886	-20.85

3.6. Analgesic Activity

3.6.1. Tail Flick Method

The crude proteins of the *C. lentiginosus* were tested, exhibited pronounced analgesic activity. The analgesic ratio decreased with increase in time. Analgesic activity in terms of tail flick response observed response time before injection 28.8 sec and after injection 145.5, 185.0 and 190.5 after 30, 60 and 90 min respectively. The crude protein of *C. lentiginosus* showed an analgesic ratio (AR) of 7.185, 2.558 and 1.726 after

30, 60 and 90 min respectively, when compared with the standard (Paracetamol), *C. lentiginosus* showed more

prominent effect (Table 5).

Table 5: Analgesic activity in terms of tail flick response and analgesic ration (%) in mice injected with the crude venom

Particulars	Response time (sec) before injection	Response time (sec) after injection		
		30min	60min	90min
Control	27.8	27.9	27.0	28.5
Paracetamol	28.5	47.2	90.2	135.3
<i>C. lentiginosus</i>	28.8	145.5	185.0	190.5
		Analgesic Ratio (AR %)		
<i>C. lentiginosus</i>	-----	7.185	2.558	1.726

3.7. SDS-PAGE

SDS-PAGE on 12.0% gel, crude protein of *C. lentiginosus* yielded four bands ranging from 40, 71 to 120 kDa indicating that these samples possess some protein bands in common. The molecular weight of protein was found to be approximately 15 kDa (Fig 1).

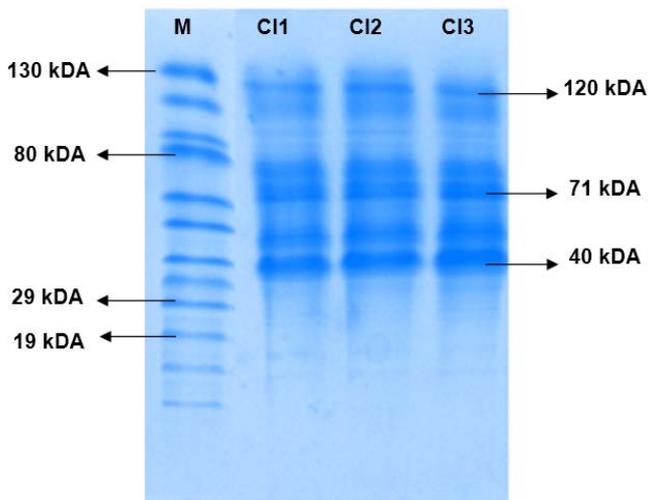


Fig 1: SDS-PAGE analysis of crude protein extracts of the *C. lentiginosus*. Lane M: protein molecular

4. Discussion

4.1. Protein Estimation

The present investigation found the respective crude protein toxin contents to be 432.5 µg/mL fraction one 281.2 µg/mL and fraction ten 80.2 µg/mL. The observed symptoms of toxicity compared well with those reported for other cone snails also viz. *C. striatus*, *C. amadis* and *C. mutabilis* [29-33]. Nayak [34] estimated crude venom protein of *C. inscriptus* was 330 µg/mL fraction one 210 µg/mL fraction ten 50 µg/mL.

4.2. Mouse Bioassay

The death time varied between 43 sec and 62 sec in *C. lentiginosus* from the protein at dose of 1.0, 0.75, 0.50 mg/mL. Upon envenomation, the common symptoms that were exhibited by the test mice in the present study were, Palpitation, excess urination, gasping for breath, exophthalmia, eye becomes opaque before death. In the present investigation, instances of toxicity of various *Coniids* have been well established. Lewis and Garcia [18] have stated that a number of *Coniids* exhibited toxicity on insects and fishes. Bingham *et al.* [35] showed that *Coniid* venom composition varies among different species. Nallathambi [31] reported that only the fractions, III and V were able to elicit any symptoms in mice by intracerebral injection (i.c.) with most of the activity concentrated in I and V which produced

shaking, convulsions and sleep like activity. Purified conotoxin GIV from *C. geographus* by at first subjecting the venom to a Sephadex G-50 column chromatography followed by DEAE cellulose chromatography, *C. lentiginosus* and *C. mutabilis* venom extracts were partially purified by DEAE-cellulose chromatography, but out of all the fractions, only fraction 8 in case of *C. mutabilis* proved lethal, whereas all other fractions proved non-lethal, although they elicited symptoms of toxicity in various degrees [33].

4.3. Hemolytic Assay

The hemolytic unit (HU) recorded for *C. lentiginosus* (8) is less than those reported earlier for *C. lentiginosus* and equivalent to *C. mutabilis* by Sakthivel [33]. Hemolytic activity is indicative of cytolytic activity and most cytotoxins have considerable potential as anticancer and antiviral agents. Lattore [36] postulated that the lethality of *C. textile* venom arose not from its neurotoxic properties but from the hemotoxic activities but Kobayashi *et al.* [37] presented evidence to the contrary. Hashimoto [38] reported that the hemolytic factor was not adsorbed on DEAE cellulose from 0.1% NaCl solution. Potent hemolytic activity was discernible in the venom of *C. lentiginosus*. Results of the hemolytic property of these venom were studied confirm to those reported in earlier studies such as those of Nallathambi [31], Ramu [32] and Sakthivel [33]. Shanmuganandam [39] reported that the venom of the vermivorous cone snail *C. figulinus* does not contain any hemolytic peptide, besides paralytic peptides.

4.4. Neuromodulatory activity

A dose dependent neuromodulatory response on mouse brain AChE activity was also evinced by the venom of *Conus inscriptus*. Venom showed inhibitory effect on AChE activity ranging between 23% and 397% was caused by venom of *C. lentiginosus* at higher concentrations are in conformity with earlier findings of Elancheran [40], who reported elevation of AChE activity to be caused at higher doses of tetrodotoxin, while results from these species are in conformity with the findings of Wankhede [41] who reported crude ovarian extracts of the horseshoe crab to enhance mouse brain AChE activity at lower doses and suppressing the same at higher doses (800 and 1000 µg). Reports on effects of other fish poisons include those of ciguatoxin and jellyfish poison [42]. Kuriaki and Nagano [43] reported that the Acetyl Choline Esterase (AChE) has been the most sensitive enzyme to the puffer poison.

4.5. Immunomodulatory Activity

The present study revealed that the venom *C. lentiginosus* had an immune-stimulating effect at lower concentrations but at higher concentrations, they exhibited immunosuppressive effects. Immuno-stimulation was found to decrease with the

increasing concentration of the venom in all the cases. Al-Hassan *et al.* [44] had shown that the wound healing activity exhibited by the epidermal secretion of the Gulf catfish was associated with immuno-modulation as well as the prostaglandin pathway. Immuno-modulation by marine toxins is a poorly studied subject [45]. The present results thus open up new vistas for research on the effects of these *Conus* venom on wound healing, tissue regeneration, and related activities.

4.6. Analgesic activity

The present result indicates the potent analgesic activity of the venom studied about as much as 3 times more than that of paracetamol. Gouiffes *et al.* [23] reported that no local anesthetic activity or analgesic effect was observed after administration of Bistramide 'A'-toxin. Intracisternal injection of the substance a dose of 1.5 mg/kg of the body weight did not cause mortality in mice but immobility with loss of muscle tone was rapidly apparent (5 min after injection). Shanmuganandam [39] showed the effectiveness of *Conus figulinus* venom on guinea pig skin as infiltration anesthetic agent while Marwick [46] reported *Conus magus* venom had an analgesic effect 1,000 times stronger than morphine. In the present study the analgesic activity was measured only with the crude venom and therefore, the analgesic activity is likely to increase many fold if the purified fraction or fractions are tested for this activity. The present study exhibited pronounced analgesic activity. The analgesic ratio decreased with increase in time. Analgesic activity in terms of tail flick response observed response time before injection 28.8 sec and after injection 145.5, 185.0 and 190.5 after 30, 60 and 90 min respectively. The crude protein of *C. lentiginosus* showed an analgesic ratio (AR) of 7.185, 2.558 and 1.726 after 30, 60 and 90 min respectively, when compared with the standard (Paracetamol), *C. lentiginosus* showed more prominent effect.

4.7. SDS-Polyacrylamide Gel Electrophoresis of protein

In present study SDS-PAGE on 12.0% gel of crude protein of *C. lentiginosus*, yielded 3 prominent bands could be observed at 40-71 kDa ranging from 40 to 120 kDa. This is in agreement with the results of Shiomi *et al.* [47] that the partially purified echotoxins extracted from *Monoplex echo*, has molecular mass 7 kDa by gel filtration on Sephadex G-75 column. Saravanan *et al.* [48] isolated 14 kDa protein from the *Conus figulinus*. Periyasamy *et al.* [49] different molecular weight marker proteins were used for *C. inscriptus* 97, 63, 61, 42 kDa and *C. betulinus* 93, 61, 42, 40 kDa band was detected in the gel that represented protein of 97-2 kDa. Alam [50] reported that molecular weight calculated for *Conus catus* 13.50 kDa by gel filtration marker on Sephadex G-50 column. Nearly equal to molecular weight of conotoxin GIV (13.0 kDa) from *C. geographus* calculated using SDS-PAGE [29]. Two factors, one lethal to fish and other to mice have been purified from *Conus striatus* having molecular weight between 10.0 kDa to 14.0 kDa and 10.0 kDa respectively [17, 51]. From the present investigation on the venoms of *C. lentiginosus*, it can be concluded that the venoms of these *C. lentiginosus* are having higher potential to be evaluated as compounds with clinical significance, as these venoms are thermostable upto 70 °C, viable even after long storage and for a period of 18 months, beyond which there was decrease in the potency. The present results are in accordance with those of Kobayashi *et al.*

[37] who reported that the venom of *C. geographus*, *C. textile* and *C. imperialis* were stable up to 100 °C when heated for 15 min. The venom of *C. amadis* was stable upto 60 °C [32], and that of *C. betulinus* upto 63 °C [31]. At higher temperatures the venom lost its activity. Preliminary investigations on *C. mutabilis* and *C. lentiginosus* revealed a potent analgesic activity to be exhibited by their venoms [33]. Similar instances of stability have been encountered by the other researchers.

5. Conclusion

The study demonstrates the effects of crude extract on column chromatography, SDS PAGE, characterization of the protein responsible for the bioactivity. Further purification and structural elucidation of compounds are required to confirm the designation of venoms in the proposed groups. This will greatly help utilize these compounds for the prosperity and well-being of human kind. Thus, the results of the present study indicate a very strong hemolytic activity of *C. lentiginosus*. The study strongly suggests that these conotoxins could be utilized as a probing tool to investigate the pharmacological potential. These characteristics emphasize the need for isolation and molecular characterization of new active toxin in *C. lentiginosus* in near future.

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