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## Studies on biochemical and biomedical properties of *Conus betulinus* venom

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## PEER REVIEW

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**Comments**

This is a good study, in which the authors had evaluated the toxicity of *Conus* species and made a thorough analysis of the enzymes in the venom mixture and reported the presence of various pharmacological targets for drug development. Periodical analysis of these venomous creatures is necessary since they vary in their constitution as speciation occurs. Details on Page S109

## ABSTRACT

**Objective:** To study the biochemical and biomedical properties of predatory gastropod *Conus betulinus* venom.

**Methods:** Molecular weight of the crude venom extract was determined by using SDS–PAGE. Toxicity studies were carried out using haemolytic and brine shrimp lethality assays. Fibrin plate assay and substrate SDS–PAGE were used to determine the effect of sample on fibrin(ogen)olytic and proteolytic activity. The FTIR characterisation and chemical fingerprinting of amino acid were done with HPTLC. Enzymatic activities like phospholipase and hyaluronidase were measured spectrophotometrically and calculated in units. Anticancer screening was carried out by MTT assay.

**Results:** Studies on this deadly venom revealed six different molecular weight proteins of medical significance ranging between 20.0 kDa and 97.4 kDa. The protein content was estimated as 0.9 mg/mL. Haemolytic activity in erythrocytes was recorded and LC<sub>50</sub> (*Artemia*) at 31.5 µg/mL. Further the venom showed considerable enzymatic properties like gelatinolytic, caesinolytic, fibrinolytic and fibrinogenolytic activities. The hyaluronidase and phospholipase activities were recorded at meagre range. The venom exhibited significant activity against HeLa cell lines. Moreover the evolution of venom is the crucial nature of *Conus* peptides in their challenging ecosystem. Periodical study on these peptides will unveil more peptides of biomedical use.

**Conclusions:** Although quite a lot of works have dealt with paralytic effects of *Conus* venom, it still remains as an unexplored cocktail with promising molecules for drug development.

## KEYWORDS

Proteolytic, Fibrinogenolytic, Hemolytic assay, Phospholipase, Hyaluronidase, MTT assay

### 1. Introduction

Marine organisms are rich sources of structurally diverse bioactive compounds. Recently, a great deal of interest has been expressed regarding marine-derived bioactive peptides because of their numerous health beneficial effects[1]. Although bio prospecting of marine organisms had yielded many bioactive peptides, pharmacologists have great passion for venoms associated peptides and their

role in human metabolism. Since the medicinal value of venoms has been known from ancient times, venom toxins have been increasingly used as pharmacological tools and as prototypes for drug development. Venoms represent a huge and essentially unexplored reservoir of bioactive compounds that may cure disease conditions which do not respond to currently available therapeutics[2]. Milked venoms of *Conus* demonstrate direct lineage to US Food and Drug Administration approved and present in-trial drug

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leads. Yet the complexity of the milked venom has not been adequately investigated or characterized, in a sustainable manner[3]. Milked venom from marine cone snails represents a novel biological resource with a proven track record for drug discovery[4]. Most species within the genus *Conus* are considered to be specialists in their consumption of prey, typically feeding on molluscs, vermiform invertebrates or fish, and employ peptide toxins to immobilize prey[5]. Since they have developed many diverse types of biologically active peptides as part of an envenomation survival strategy for feeding and defence, it potentially corresponds to a rich pharmacological research tool. Surprisingly, there was no correlation between the peptides produced in the venom duct and those obtained after milking live cone snails, implying yet unknown mechanisms of selection and regulation[6]. The 700 or more species of cone snail attack prey by employing complex venom which can vary considerably both within species and from one species to another. Cone snail venom is remarkable for the high proportion of conotoxins with varied post-translational modifications and for the production of more diverse toxin scaffolds than any other known venomous animal[7]. Though the number of novel, active peptides within the *Conus* species is considered to be enormous, only few peptides have been characterised so far. Hence the present study was carried out with the objective of isolation, characterisation and screening *Conus betulinus* (*C. betulinus*) venom for several other biomedical applications.

## 2. Materials and methods

### 2.1. Collection of venom ducts

The *C. betulinus* specimens were aseptically transferred into a sterile room for dissection. The shell was broken with the help of hammer and the venom ducts were removed. The removed venom ducts were immediately processed for further study. All the process was carried out at 4 °C.

### 2.2. Isolation of crude extract

Crude toxin was isolated from the venom ducts by following McIntosh *et al.*[8]. The crude extract was collected from their venom duct followed by the homogenisation process with tris buffer (50 mmol/L tris hydrochloride, 120 mmol/L sodium chloride, 5 mmol/L potassium chloride, 1 mmol/L magnesium chloride and 2 mmol/L calcium chloride). The fresh venom ducts were homogenised with 2 mL of buffer in a manual tissue homogeniser and sonicated three times for 50 seconds/cycle (10 seconds ON, 20 seconds OFF). During sonication, the vessel was cooled in an ice

bath. The mixture was centrifuged at 17200 r/min for 10 min at 4 °C. The supernatant (considered as a crude extract) was lyophilized and stored at –20 °C for further analysis.

### 2.3. Protein estimation

Protein concentrations were determined by the method of Lowry *et al.*[9] using bovine serum albumin as a standard.

### 2.4. Molecular weight determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (10% gel) was carried out according to the method of Laemmli[10]. The molecular weight of the crude venom was estimated by comparing mobility with protein markers such as phosphorylase B (97400 Da), bovine serum albumin (66000 Da), ovalbumin (43000 Da), carbonic anhydrase (29000 Da), soyabean trypsin inhibitor (20000 Da), lysozyme (14300 Da) by using Gel Documentation System (Lark Fine Technologies, Version 4.0, India).

### 2.5. Haemolytic activity

Haemolytic assay was performed following the micro titre plate method of Paniprasad and Venkateshvaran[11]. Ethylene diamine tetraacetic acid solution (2.7 g/100 mL) was used as an anticoagulant. Red blood were obtained and washed three times with phosphate buffer solution. From these, 1% erythrocyte suspension was prepared by adding 99 mL normal saline to 1 mL of packed red blood cell. Samples (100 µL) of saline containing 1% red blood cell were mixed with 100 µL of different doses of *Conus* venom. Each sample was placed (duplicates) in microplates. As control (100% haemolysis) and phosphate buffer solution (0% haemolysis) were used. The plate was gently shaken and allowed to stand for 3 h at room temperature and the results were recorded. The uniform red colour suspension in the wells considered as positive haemolysis and a button formation in the bottom of the wells was considered as lack of haemolysis. Reciprocal of the highest dilution of the crude toxin showing the haemolytic pattern was taken as one haemolytic unit (HU).

### 2.6. Brine shrimp toxicity

Brine shrimp eggs were hatched out in dish filled with sea water. The freshly hatched phototrophic larvae (nauplii) were collected with a pipette and transferred (10 shrimps) to vials filled with sea water (5 mL). The samples were added at different concentrations into the vials. As control, a group of vials was filled with sea water. The vials were kept

illuminated during 24 h of contact with the substances and survivals were counted with the aid of a magnifying lens. This assay was carried out three times with five replicates for each fluid concentration tested. To calculate the  $LC_{50}$  (mean lethal concentration), the results were plotted as log of % mortality vs log concentration.

### 2.7. Proteolytic activity

Casein and gelatin were used as substrates and the method was adapted from Heussen and Dowdle<sup>[12]</sup>. Briefly 2 mg/mL (w/v) substrates were incorporated in the 10% resolving gel with a 5% stacking gel. The samples (10 mg) were loaded in non-reducing sample buffer. After electrophoresis, the SDS was removed by washing the gel twice for 20 min in 2.5% Triton X-100 before incubation in 20 mmol/L tris, 0.4 mmol/L calcium chloride pH 7.4 at 37 °C for 16 h and stained with 0.125% coomassie blue. The clear zone in the gel indicates the regions of enzyme activity.

### 2.8. Fibrinolytic activity

The fibrinolytic activity of the sample was analyzed in a fibrin plate by the method of Veiga *et al*<sup>[13]</sup>. Briefly, fibrin plate prepared by a solution containing 25 mg of fibrinogen was laid over agarose gel plates immediately after the addition of 10 mg of thrombin. The plates were incubated at 37 °C for 2 h. Then 20 µL of crude venom was laid over the fibrin plate. The same volume of water and plasmin (1 mU) were used as negative and positive controls, respectively. Plates were incubated at 37 °C for 24 h, dried out, and stained with Coomassie Blue stain. The presence of clear spots in the plate indicates fibrinolytic activity.

### 2.9. Fibrinogenolytic activity

The degradation of fibrinogen by sample was analyzed by using SDS-PAGE by the method of Shacter *et al*<sup>[14]</sup>. For the fibrinogenolytic activity, 50 µL of a bovine fibrinogen solution (2 mg/mL in 10mmol/L tris, pH 7.4) was incubated with 50 µL sample at 37 °C. At the indicated time (1 and 24 h), 20 µL of denaturing solution (10 mol/L urea, 4% SDS, 4% β-mercaptoethanol) was added to the reaction mixture. An aliquot of 15 µL of each reaction was used for analysis by SDS-PAGE. In this case, 5% stacking gel and 12% running gel were used.

### 2.10. Phospholipase activity

The method of Marinetti<sup>[15]</sup> was used for the evaluation of phospholipase activity with suitable modifications. Egg

yolk was suspended in tris-HCl buffer, pH 8.0, to an initial absorbance of 1.0 at 740 nm. Crude sample was added to 3 mL of this suspension. Phospholipase activity was assessed as the rate of a linear decrease in optical density over an incubation period of 5–15 min. One unit of phospholipase activity corresponds to a decrease of 0.001 of absorbance per minute. The activity was expressed as U/mg of toxin of six independent experiments.

### 2.11. Hyaluronidase assay

Hyaluronidase enzyme activity was determined by the Dorfman A<sup>[16]</sup>. The assay mixture contains bovine serum albumin (1 mg/mL) pH 6.9 in 50% HCl and 0.3 mL of enzyme diluents were added to it and mixed at 37 °C. At 1 min intervals, the reaction was started by adding 0.5 mL of substrate (4 mg hyaluronic acid potassium salt in phosphate buffer pH 5.3) at 37 °C. The samples were incubated for 30 min, then the reaction was stopped by adding 5 mL acid albumin at a minute intervals. Repeatedly the samples were incubated for 10 min at 37 °C and measured the absorbance at A600 nm at minute intervals. One unit is defined as the amount of enzyme which causes the reduction in turbidity under specified conditions similar to that caused by one unit of an international standard.

### 2.12. Determination of cell viability by MTT assay

The cytotoxicity of samples on Vero and HeLa cells was determined by the MTT assay<sup>[17]</sup>. HeLa cell line and Vero cell line were procured from the National Centre for Cell Sciences, Pune, India. When the cells reach 80% confluence, they were used for cytotoxicity assay with venom of target species. The growth medium minimum essential medium (MEM) was removed without disturbing the cell sheet. The monolayer of cells washed with MEM to remove dead cells and excess fetal calf serum. The crude samples were diluted in MEM at a rate of 1 mg/mL and serial 10 fold dilutions were made. The dilution without the venom served as control. Each dilution of the crude sample ranged from 1:1 to 1:64 were added to respective wells of the 24 well titre plates. The plates were incubated at 37 °C in 5% carbon-di-oxide environment. The cells were observed under an inverted microscope for changes in their morphology. The toxin treated plates after 48 h of incubation, was taken for MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] cell viability test. Each well washed with MEM without fetal calf serum for 2–3 times and add 200 µL of MTT (5 mg/mL and incubated for 6–7 h in 5% carbon-di-oxide incubator. Then the medium was replaced by 1 mL of dimethylsulfoxide and incubated for 45

seconds on a micro plate shaker to dissolve the formazon crystals of MTT. The suspension was transferred into the cuvette of spectrophotometer and read at 595 nm using dimethylsulfoxide as blank. The graph is plotted by taking concentration of crude sample on x-axis and relative cell viability on y-axis.

$$\text{Cell viability (\%)} = (\text{mean OD/control OD}) \times 100$$

Where OD is outside diameter.

### 2.13. Fourier transform infrared spectroscopy (FTIR) analysis

The solid sample of *C. betulinus* toxin was analysed by using IR spectroscopy. One part of the crude sample was mixed with ninety nine parts of dried KBr and then compressed to prepare a salt disc (3 mm diameter). These discs were analysed under the IR-spectrophotometer. The absorption was read between 450 and 4000  $\text{cm}^{-1}$ .

### 2.14. Analysis of free amino acid through high performance thin layer chromatography (HPTLC)

About 2 mL crude toxin of *C. betulinus* was taken and centrifuged at 10000 r/min for 1 min at 4 °C. The pellet was discarded and supernatant was further taken for HPTLC analysis. TLC aluminium plate silica gel 60 F254 (Merck), 20 × 10 was used for the analysis. About 2  $\mu\text{L}$  of sample and standard (21 amino acids) were loaded using Hamilton syringe at the band width of 4 mm. Solvent mixture containing *n*-Butanol:acetic acid:water (8:2:2) was used as the developing agents. The plate was then air dried for 3 min. To view the bands, derivatization reagent containing 0.1% ninhydrin in acetone was added and heated for 10 min at 100 °C. The bands in the plate were scanned using CAMAG TLC scanner 3 and Wincats software version 1.4.4 (Switzerland).

## 3. Results

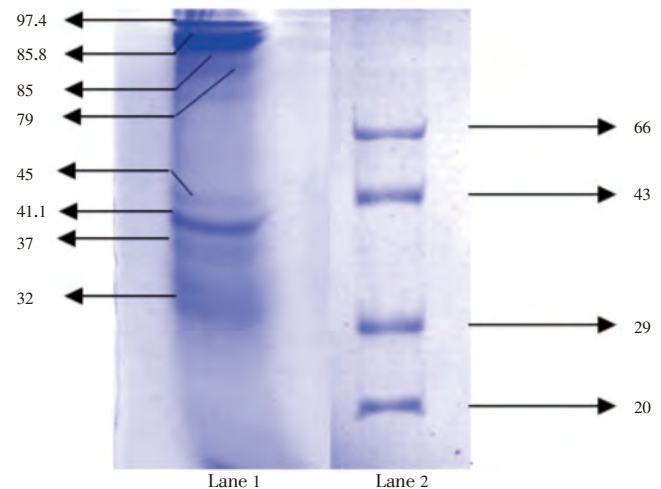
### 3.1. Protein estimation

The protein content of the sample was estimated as 0.9 mg/mL of lyophilised crude extract.

### 3.2. Molecular weight determination

*C. betulinus* crude venom was subjected to 12% SDS-PAGE analysis and documented for band visualisation. Figure 1 shows the electrophoretic pattern of *Conus* venom. After SDS-PAGE under reducing conditions many components with more or less similar molecular masses, mainly above 20 kDa were observed. Distinct bands were observed at 97.4, 85.8

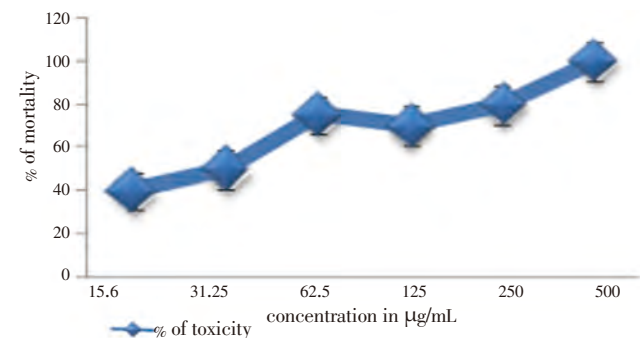
and 41.1 kDa range for crude venom of *C. betulinus*. Apart from this, diffused band between 37 and 32 kDa and some faded bands were also observed at 85, 79, 45 kDa ranges.



**Figure 1.** Electrophoretic profile of the *C. betulinus* toxin was analysed using 12% SDS-PAGE under non reducing conditions. Gel was stained with Coomassie Blue. Numbers on the right correspond to the position of molecular mass markers.

### 3.3. Brine shrimp toxicity

Brine shrimp lethality test were performed, usually with 6 different dose levels and six replicates per dose of *C. betulinus* venom. It showed the 50% mortality against brine shrimps ( $\text{LC}_{50}$ ) at 31.5  $\mu\text{g/mL}$  (Figure 2).



**Figure 2.** Effect of *C. betulinus* toxin on *Artemia*.

Newly hatched *Artemia* were incubated for 24 h with increasing doses of *C. betulinus* toxin. Mortality rate of *Artemia* increased with the increasing concentration of toxin. Each value represents the mean  $\pm$  SE of six experiments.

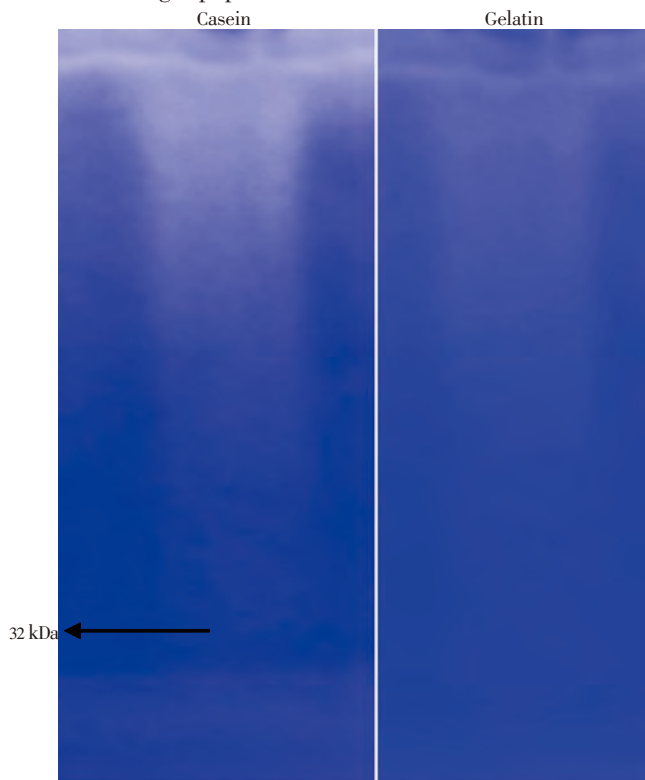
### 3.4. Haemolytic activity

It exhibited haemolytic activity on washed chicken blood erythrocytes, transpired in dose dependant manner. The haemolytic effects were observed up to 128 HU. (One HU is defined as the quantity of protein catalyzing lysis of 50% of red blood cells present).

### 3.5. Proteolytic activity

Casein and gelatin were incorporated as substrates in 12.5% acrylamide gels to assay enzymatic activities of crude

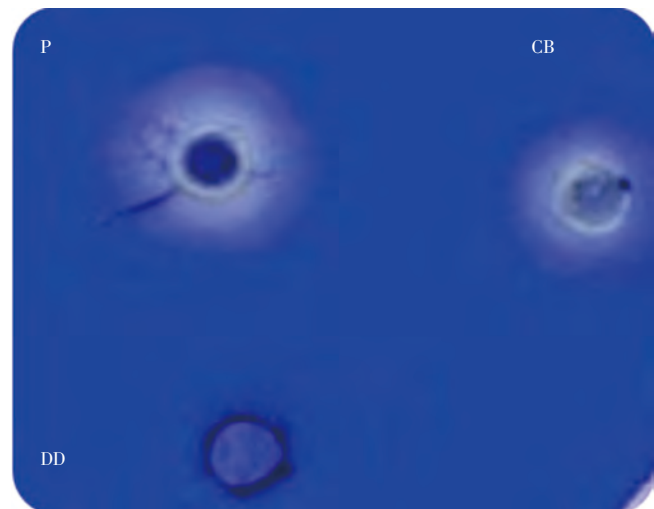
toxin. The profile of enzymatic degradation by *C. betulinus* toxin indicated the presence of proteases with broad substrate specificity. The clear zones in the gel indicated the regions of enzyme activity. The proteolytic activity was detected as colourless bands in blue gel (Figure 3). Though the proteolytic (casein and gelatin) activity crop up throughout the lane, it was more perceptible above 30 kDa molecular weight peptides.



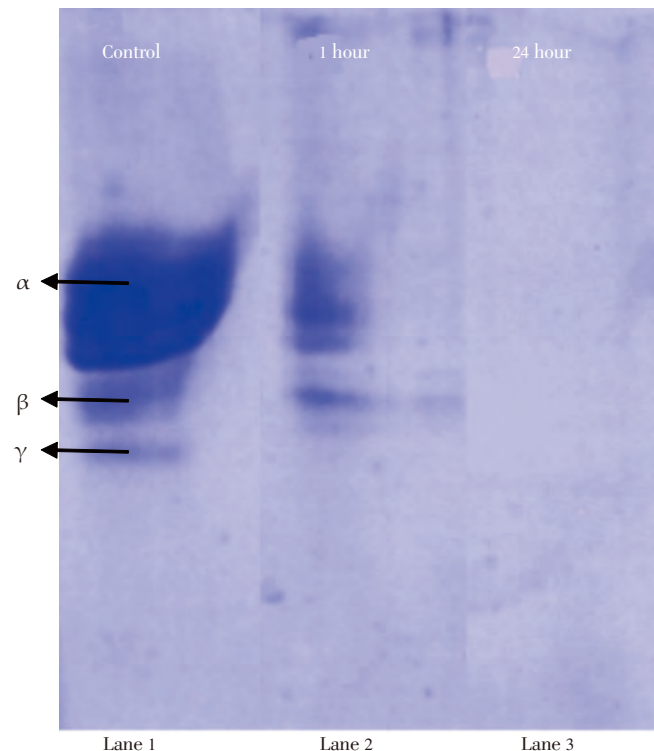
**Figure 3.** Caesinolytic and gelatinolytic activities of *C. betulinus* toxin were determined using the technique of substrate SDS-PAGE 12.5%. Clear regions in the gel indicate regions of enzymatic activity.

### 3.6. Fibrinolytic and fibrinogenolytic activity

The fibrinolytic zone caused by *C. betulinus* venom (1 mg/mL) when applied to a fibrin plate was 6 mm after the incubation period. The formation of lysis zone around the sample in the plate confirms the fibrinolytic activity of *C. betulinus* toxin (Figure 4). Specific cleavage of fibrin precursor, fibrinogen was also determined by SDS-PAGE at incubational intervals of 1 h and 24 h. *C. betulinus* venom after 1 h incubation, partially degraded the  $\alpha$ -chain,  $\beta$ -chain,  $\gamma$ -chain of fibrinogen molecule (Figure 5). When the incubation time was prolonged to 24 h, crude venom digested all three chains of fibrinogen ( $\alpha$ -chain,  $\beta$ -chain, and  $\gamma$ -chain) completely, showing a prominent fibrinogenolytic activity. The amount of hyaluronidase enzyme which causes a reduction in turbidity under specified conditions similar to that caused by one unit of an international standard was about 40 NF units for *C. betulinus*. Minimal phospholipase activity was observed in *C. betulinus* toxin. About 2540 U/mg of phospholipase activity was recorded for the venom when tested with egg yolk.



**Figure 4.** The fibrinolytic activity of *C. betulinus* (CB) toxin. Plasmin (P) was used as positive control and double distilled water (DD) was used as negative control.



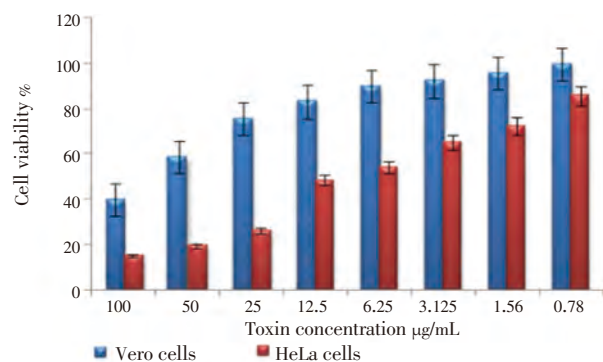
**Figure 5.** Fibrinogenolytic activity of *C. betulinus* toxin was determined using fibrinogen as substrate in 12% SDS-PAGE technique.

Lane 1 shows the three bands responsible for  $\alpha$ ,  $\beta$ ,  $\gamma$  subunits of fibrinogen. Lane 2 shows the partially digested  $\alpha$ ,  $\beta$ ,  $\gamma$  subunits by *C. betulinus* toxin after 1 h of incubation time. Lane 3 shows the complete digestion  $\alpha$ ,  $\beta$ ,  $\gamma$  subunits by *C. betulinus* toxin after 24 h of incubation time.

### 3.7. MTT assay

The viability of the cell was found to decrease with the increasing concentration of crude toxin (Figure 6). The crude toxin showed medium toxicity ( $LC_{50}$ ) against cancer cell lines at 12.5  $\mu\text{g/mL}$ . The minimum toxicity (14.04%) was observed at 0.78  $\mu\text{g/mL}$  and higher toxicity (84.74%) was observed at 100  $\mu\text{g/mL}$  respectively. Whereas sample showed only lower toxicity (60.05%) even at higher concentrations of 100  $\mu\text{g/mL}$  when screened against normal cell lines. Hence the isolated

toxin could be used as a significant anticancer agent.



**Figure 6.** Effect of *C. betulinus* toxin on normal (Vero) and cancer (HeLa) cell lines.

Increasing concentrations of *C. betulinus* toxin were incubated with Vero and HeLa cells for 72 h at 37 °C. The cell viability was estimated by a calorimetric test with MTT and expressed as a percentage of viable cells. Results are expressed as mean±SE of six independent experiments.

### 3.8. FTIR

The FTIR spectrum of crude toxin between the 400–4000 spectral ranges was measured. Totally 19 peaks were observed for sample. The list of functional groups assigned for the wave number was tabulated (Table 1).

**Table 1**

Functional groups assigned for *C. betulinus* toxin after analysing through FTIR spectroscopy.

Group Frequency <sup>a</sup>	Assignment <sup>b</sup>
3420	Heterocyclic amine NH stretch
2968	Methyl C–H asymmetric/symmetric stretch
2360	Out of range
2342	Out of range
2267	Aliphatic cyanide/ nitrile
2082	Isothiocyanate (–NCS)
1773	Open chain acid anhydride carbonyl compound
1636	Alkenyl C=C stretch
1473	Methylene C–H bend
1457	Methylene C–H bend
1405	Vinyl C–H in plane bend
1339	Methane C–H bend
1219	Aromatic C–H in plane bend
1149	Tertiary alcohol, C–O stretch app. Centre of range for the group frequency
1046	Primary amine, CN stretch
934	Aromatic phosphates (P–O–C stretch)
900	Aromatic C–H out of plane bend
668	Alkyne C–H bend
617	Sulfate ion inorganic group frequencies

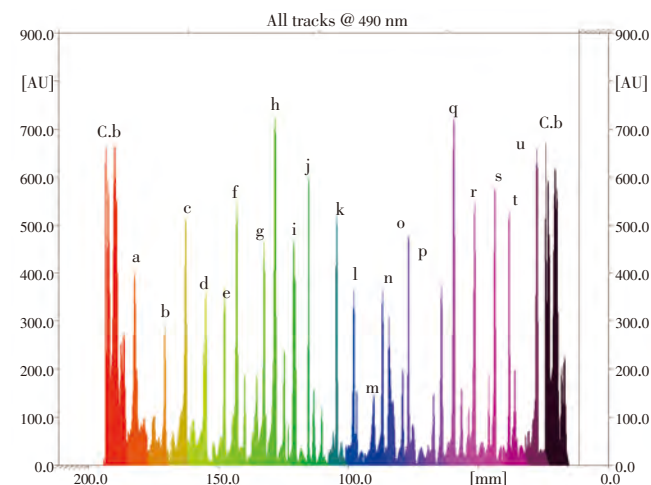
The IR bands indicating the functional groups were assigned for the wave numbers were tabulated.

a: wave number; b: IR bands indicating the functional groups.

### 3.9. HPTLC screening of amino acids

The HPTLC profiling revealed the free amino acids pattern in the crude toxin. The well resolved HPTLC profile of amino acids and crude toxin was showed in Figure 7. The spectrum of peaks corresponding to the amino acids of standard and crude toxin was analysed. The  $R_f$  values obtained for the samples were compared with standards and tabulated

(Table 2). From superimposition study, a band ( $R_f$  0.22) corresponding to arginine, histidine, lysine and a band ( $R_f$  0.29) corresponding to Cys–HCl was confirmed in the sample. Band responsible for the presence of aspartic acid, glycine, proline, asparagine, glutamine, serine, hydroxyl proline was confirmed from the  $R_f$  value 0.45. Similarly band with  $R_f$  value 0.62 responsible for amino acids like glutamic acid, threonine confirms their presence in *Conus* toxin. The presence of band at  $R_f$  point 0.75 confirms the presence of valine and methionine in the sample. Band with the  $R_f$  value of 0.92 corresponding to the amino acids tyrosine, tryptophan, phenyl–alanine, leucine indicates their presence in the sample.



**Figure 7.** HPTLC profile of sample and 21 standard amino acids (scanning at 490 nm using CAMAG TLC scanner 3).

First and last peak (C.b) corresponds to the sample *C. betulinus*. Alphabets (a to u) represents the peak for standard (21) amino acids.

**Table 2**

Free amino acids analysed based on their  $R_f$  values through HPTLC at 490 nm using CAMAG TLC scanner were tabulated.

Sample peaks ( $R_f$ )	Possibility of amino acids in sample by comparing with standard $R_f$ value
0.07	Unknown
0.22 <sup>a</sup>	Arginine, histidine, lysine
0.29	Cysteine
0.45 <sup>b</sup>	Glycine, aspartic acid, glutamine, proline, asparagine, serine, hydroxyproline
0.62 <sup>c</sup>	Glutamic acid, threonine
0.75 <sup>d</sup>	Valine, methionine
0.92 <sup>e</sup>	Tyrosine, tryptophan, phenyl–alanine, leucine

The amino acids present in the sample were profiled using HPTLC. Some of the amino acids have same migration point, hence they have same  $R_f$  values (a, b, c, d, e).

## 4. Discussion

It is estimated that venoms of marine cone snails (genus *Conus*) contain more than 100000 different small peptides with a wide range of pharmacological and biological actions. Some of these peptides were developed into potential therapeutic agents and as molecular tools to understand biological functions of nervous and cardiovascular systems[18]. The present exertion was an underway to investigate the crude venom of *C. betulinus* venom. Here

the high protein content observed confirm that our sample has high toxicity since toxin comprises majorly proteins. It is estimated that between 500 to 700 *Conus* species exist, each possessing 50–200 conopeptides in their venom, which acts as a rich source of biochemically active enzymes, proteins, peptides and low molecular weight substances. Toxins isolated from the venom either inhibit or activate a vast number of targets such as ion channels, acetylcholine receptors, acetylcholinesterase, membranes, coagulant/anticoagulant pathways, and metalloproteases, with high selectivity and affinity<sup>[19]</sup>. Since venom is a concoction of biochemically active substances, these molecular mass components documented for *C. betulinus* may play shared role in making the venom lethal to predators.

The brine shrimp lethality test was used as a surrogate tool to evaluate their toxicities, and also to identify their potential for other biological activities<sup>[20]</sup>. Previously, Bragadeeshwaran *et al.*<sup>[21]</sup> have reported the LC<sub>50</sub> values of the crude extract of ascidians *Polyclinum madrasensis* and *Phallusia nigra* as 92 µg/mL and 130 µg/mL respectively. The crude methanol extract of ascidians (*Didemnum psammatoide*) indicated the highest activity with LC<sub>50</sub> value of 106.965 µg/mL and the ethanol extract of ascidian *Phallusia arabica* exhibited lower brine shrimp lethality with LC<sub>50</sub> value of 227.055 µg/mL<sup>[22]</sup>. Earlier in the brine shrimp test, extracts of *Zeyheria tuberculosa* showed toxic effects (LC<sub>50</sub> 29.55 to 398.05 µg/mL) and some extracts were non-toxic or showed weak lethality (LC<sub>50</sub> 705.02 to >1000 µg/mL)<sup>[23]</sup>. Comparatively our sample showed higher toxicity at lower concentration wherein LC<sub>50</sub> values of less than 250 µg/mL can be considered significantly for further investigation. Accordingly our sample showed toxicity at lower concentration, substantiating it for further study. The potent haemolytic activity of extracted crude toxin was further confirmed with cytolysis of various methanol and aqueous extracts prepared from sponges<sup>[24]</sup>. Earlier Sarumathi *et al.*<sup>[25]</sup> have recorded very less haemolytic units for gastropod *Cantharus* species. Comparatively our venom sample showed higher cytolysis against chicken erythrocytes. Similarly soletti *et al.*<sup>[26]</sup> reported the cytotoxicity of two sea anemone through pore-forming cytolysins, toxin Bc2, and equinatoxin (EqTx-II). Rahman *et al.*<sup>[27]</sup> have also recently demonstrated that crude venom of the vermivorous snail *Conus vexillum* (*C. vexillum*) caused an array of cytotoxic effects in mammalian systems which were attributed to the venoms ability to induce oxidative stress. Further, the most commonly used physical definitions of life are based on the principle that an intact impermeable cell membrane is essential for life. In assay based on this principle, a toxin induces membrane permeabilization resulting in erythrocytes cell wall lysis and liberation of haemoglobin. Therefore from the results procured (including brine shrimp toxicity and haemolysis), it can be concluded that erythrocytes lysis may be due to stoichiometric mechanism.

Enzymes are an important and common component of

venom of many animals including bees, spiders, scorpions and snakes with several functions probably involved in the toxic action<sup>[28]</sup>. The proteolytic activity of venom extract against casein and gelatin were assayed due to the probable involvement of proteases in the instability of biological activities. The proteolytic activity through zymography analysis showed that isolated toxin could degrade distinct protein such as casein and gelatin effectively. These results suggest that, such proteases could contribute to degradation of proteins and diffusion factors of extra cellular matrix or they are directly involved in the degradation of proteins. Hyaluronidases are considered as spreading factors, that facilitate the tissue diffusion of toxins by degrading hyaluronan<sup>[29]</sup> and enhances local systemic envenomation in the victim's tissues. From the previous reports, we could confirm that hyaluronidase present in our sample could play a substantial role in spreading of other lethal components of toxin in the victim. The presence of phospholipase enzyme in the venom concoction may help in digestion of phospholipid bilayer of the intact cells and paves the way for other lethal molecules into the host of predators. Therefore it can be concluded that hyaluronidase and phospholipase may play mutual role in spreading of lethal components of toxin. These enzymatic properties of *Conus* venom can be corroborated for their anticancer potential, since venom components entirely affect the live cells functional organization. Likewise Feng *et al.*<sup>[30]</sup>, reported that hyaluronidase venom of Chinese red scorpion *Buthus martensi* had hydrolyzed hyaluronan into relatively smaller oligosaccharides and modulated the expression of CD44 variant in the breast cancer cell line MDA-MB-231. Thus from the MTT assay, it could be concluded that *C. betulinus* venom showed potent anticancer activity against HeLa cells screened. Further Jimenez *et al.*<sup>[31]</sup> suggested that the use of peptides from marine sources has potential for the prevention and treatment of cancer, and that they might also be useful as molecular models in anticancer drug research.

The qualitative aspect of infrared spectroscopy is one of the most powerful attributes of this diverse and versatile analytical technique. The frequency of 3420 is responsible for heterocyclic amine NH stretch, strongly influence the chemistry and the relativity of the nitrogen and the N-H group. Frequency 2968 is responsible for methyl C-H asymmetric/symmetric stretch forms the basic structure. Frequency 2267 and 2082 are thiol substituted compounds which are highly polarisable producing stronger spectral activity. Group frequency 1773 is responsible for the open chain acid anhydride carbonyl group present in the toxin and 1636 frequency corresponds to the presence of alkenyl C=C stretch in the sample. Frequency 1473 and 1457 form the methylene C-H bend. Group frequencies including 1219, 934 and 900 responsible for presence of aromatic groups (C-H in plane bend, P-O-C stretch, C-H out of plane bend) were also observed. Similarly an alkyne group (C-H bend) was detected at 668 frequency and a simple inorganics groups at 617.

The free amino acids present in the crude venom was analysed using HPTLC. The presences free amino acids were analysed with the standards and some of unidentified spots were also recorded as well, because besides 22 standard amino acids, there are a vast number of non–standard amino acids present. Thus through this simple chromatographic fingerprinting presence of possible free amino acids were also resolved.

Innovation of toxins, especially from marine resources is racing ahead because of their extremely complex and unique properties. Over two decades of research conopeptide has led to several compounds targeting neuropathic pain. Startlingly none of these conotoxins are considered on the basis of discovery and validation of new therapeutic enzymes. Since vast majority of them are yet to be identified and characterised. The present study was attempted in search of identifying potential peptide from *Conus* toxin for biomedical value. Through the evaluation of toxin, we have identified the presence of significant therapeutic enzymes which can be used in the thrombolysis and anticancer treatment. Hence further exploration of these peptides will shed light on discovery of new leads for life threatened diseases of the world as evolving conotoxins on sure will provide novel peptides of biomedical impact.

### Conflict of interest statement

We declare that we have no conflict of interest.

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### Comments

#### Background

The study of the marine toxins has very good scope, since by the very nature of the complexity of these substances and their ability to destroy life by complicated and sometimes undetermined means. But pharmacologists always have love affair with venoms and toxins because of their specific mode of action towards their target. Neurotoxic venoms are common among tropical marine creatures, which have specialized apparatuses for delivery of the venoms. These include jellyfish and anemones, venomous cone snails, venomous fish, stingrays, sea snakes, and venomous octopuses. In particular, *Conus* venoms contain

a remarkable diversity of pharmacologically active small peptides which targets mainly ion channels and receptors in the neuromuscular system.

#### Research frontiers

Researchers recently demonstrated that crude venom of the vermivorous snail *C. vexillum* caused an array of cytotoxic effects in mammalian systems which were attributed to the venom’s ability to induce oxidative stress. Similar sort of study was conducted by Clork and Olivera, wherein A toxin from the venom of the marine snail, *Conus geographus* Linne, doesn’t showed any detectable toxicity to mice on *i.p.* injection, but identified as a potent convulsant following intracerebral injection.

#### Related reports

Moller *et al.* reported the proteolytic properties of venom of *Conus purpurascens* and *Conus ermineus* (piscivorous), and the dissected venom of *Conus purpurascens*, *Conus marmoreus* (molluscivorous) and *Conus virgo* (vermivorous) through zymograms which greatly correlates with our findings. In particular the statement on fibrinogenolytic activity and their effects on the hemostatic system of the prey were similar supports the current article reports.

Rahman *et al.* reported the cytotoxicity of specimens of *C. vexillum* collected from two different sites (Hurgada and Sharm El–Shaikh) in the Red Sea, Egypt. Their dosage level is different from the current one. This may state the degree of toxicity associated with the species.

#### Innovations & breakthroughs

In specific living *Conus* are likely to express more than 70000 different peptides. The venom peptide complement of any one species is distinct from that of any other, as venom peptide sequence diverges rapidly as speciation occurs. Hence evaluation of the venom of *C. betulinus* was first reported in terms of their medicinal value.

#### Applications

The results of the present study suggest that many biomedical enzymes like fibrinolytic, fibrinogenolytic and protease were available in the venom mixture. They have significant role in the clinical treatment of thrombosis. Further investigation of these leads will pave way for the drug development.

#### Peer review

This is a good study, in which the authors had evaluated the toxicity of *Conus* species and made a thorough analysis of the enzymes in the venom mixture and reported the presence of various pharmacological targets for drug development. Periodical analysis of these venomous creatures is necessary since they vary in their constitution



as speciation occurs.

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