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Original article

Mass spectrometric identification and *denovo* sequencing of novel conotoxins from vermivorous cone snail (*Conus inscriptus*), and preliminary screening of its venom for biological activities *in vitro* and *in vivo*

Ruchi P. Jain [a], Benjamin Franklin Jayaseelan [b], Carlton Ranjith Wilson Alphonse [a], Ahmed Hossam Mahmoud [c], Osama B. Mohammed [c], Bandar Mohsen Ahmed Almunqedhi [d], Rajesh Rajaian Pushpabai [a,*]

[a] *Molecular & Nanomedicine Research Unit, Centre for Nanoscience and Nanotechnology, Sathyabama Institute of Science and Technology, Chennai 600119, India*
[b] *Department of Marine Conservation, Bombay Natural History Society, Hornbill House, SBS Road, Mumbai-400001, Maharashtra, India*
[c] *Department Zoology, College of Science, King Saud University, P.O Box 2455, Riyadh 11451, Saudi Arabia*
[d] *Department of Botany and Microbiology, College of Science, King Saud University, P.O Box 2455, Riyadh 11451, Saudi Arabia*

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ABSTRACT

Venom of *Conus inscriptus*, a vermivorous cone snail found abundantly in the southern coastal waters was studied to yield conotoxins through proteomic analysis. A total of 37 conotoxins (4 with single disulfide bonds, 20 with two disulfide bonds and 11 three disulfide-bonded peptides) were identified using mass spectrometric analysis. Among them, amino acid sequences of 11 novel conopeptides with one, two and three disulfides belonging to different classes were derived through manual *de novo* sequencing. Based on the established primary sequence, they were pharmacologically classified into α conotoxins, μ conotoxins and contryphans. Except In1696 all other conopeptides have undergone C-terminal amidation. The natural venom exhibited 50% lethality at 304.82 μg/mL against zebrafish embryo and 130.31 μg/mL against brine shrimp nauplii. The anticonvulsant study of natural venom effectively reduced the locomotor activity against pentylenetetrazole (PTZ) treated zebrafish. This concludes that the venom peptides from *Conus inscriptus* exhibit potential anticonvulsant function, which leads to the discovery of lead molecules against seizures.

1. Introduction

Marine invertebrates comprise an enormous number of bioactive molecules, particularly medium-sized peptides play a crucial role in provoking biological functions with negligible side effects. Among them, the gastropod mollusc cone snail (genus Conus) adds up with a vast array of bioactive peptides. The mixture of venom components produced at the venom duct of cone shells is primarily used in the feeding process, which ultimately sedates and paralyses the prey before they engulf them (Norton and Olivera, 2006). Conotoxins are venom peptides typically made of 6–50 amino acid residues with 1–5 disulfide-bonds (Sonti et al., 2013). Based on the extended diversity of structure, disulphide connectivity, and cysteine framework of conotoxins, it is presumed that conotoxins possess various mechanisms of action, most of which have not yet been determined (Robinson and Norton, 2014).

Among the vast array of peptide libraries discovered until now, few conotoxins were analysed for the existence of biological activity by Olivera, his group, and few other groups. Researchers found that conotoxins act on neurotransmitter transporters, voltage/ligand-gated ion channels and G-protein coupled receptors, which block or modulate the central nervous system more precisely (Kaas et al., 2012; Terlau and Olivera, 2004). Because of the significant and specific biological activity generated by different classes of conotoxins, it is important to find novel structures with a novel

* Corresponding author.
E-mail address: jeshran@sathyabama.ac.in (R. Rajaian Pushpabai).
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Fig. 1. *Conus inscriptus*.

mode of action. Even though conotoxin gene superfamilies share similar signal peptides, their mature peptides differ significantly in their amino acid sequence, 3-D structure and functional targets (Kaas et al., 2012).

Epilepsy is a chronic neurological disorder, recurrent, unprovoked seizures, which affects approximately 65 million people worldwide, where about >80% of these patients are from average or low-income countries (Leffler et al., 2017). Currently, existing antiepileptic drugs (AEDs) are efficient, but 30% of patients fail to respond to these drugs. Those patients suffer from drug-resistance, surgery is one of the ways. Still, 10% of patients do not respond to any available medical drugs or practice. These pitfall in the treatment of epilepsy has led researchers to work on discovering of new anticonvulsant compounds with a novel mode of action (Dave and Lahiry, 2012).

Zebrafish is a successful model for studying quite a few human diseases (Goessling and North, 2014). It has a complex nervous system capable of sophisticated behaviours and susceptible to seizures. The pentylenetetrazole (PTZ) treated zebrafish seizure model displayed increased locomotor activity and characteristic seizure-like actions, which makes it a perfect model for screening molecules with anticonvulsant property (Afrikanova et al., 2013). Voltage-gated sodium channels are important in the transmission of action potentials and they also play a chief role in epilepsy. Many mu conotoxins isolated from the cone snails, such as KIIIA, MIIIA, PIIIA are known inhibitors of these Voltage-gated sodium channels (Jacob and McDougal, 2010). Epilepsy based seizures are also controlled by antagonists of voltage-sensitive calcium channels and have been proved in epilepsy animal models. ω-conotoxin MVIIC acts on P and/or Q subtype voltage-sensitive calcium channel and prevents tonic and clonic seizures in mice model (McDonough et al., 2002).

Conus inscriptus is a vermivorous cone snail abundantly found in Indian coastal waters. Studies pertaining to the function and venomics of *C. inscriptus* are scanty. So we chose this species for this study to identify different classes of venom peptides and to evaluate its biological function (Rajesh, 2015).

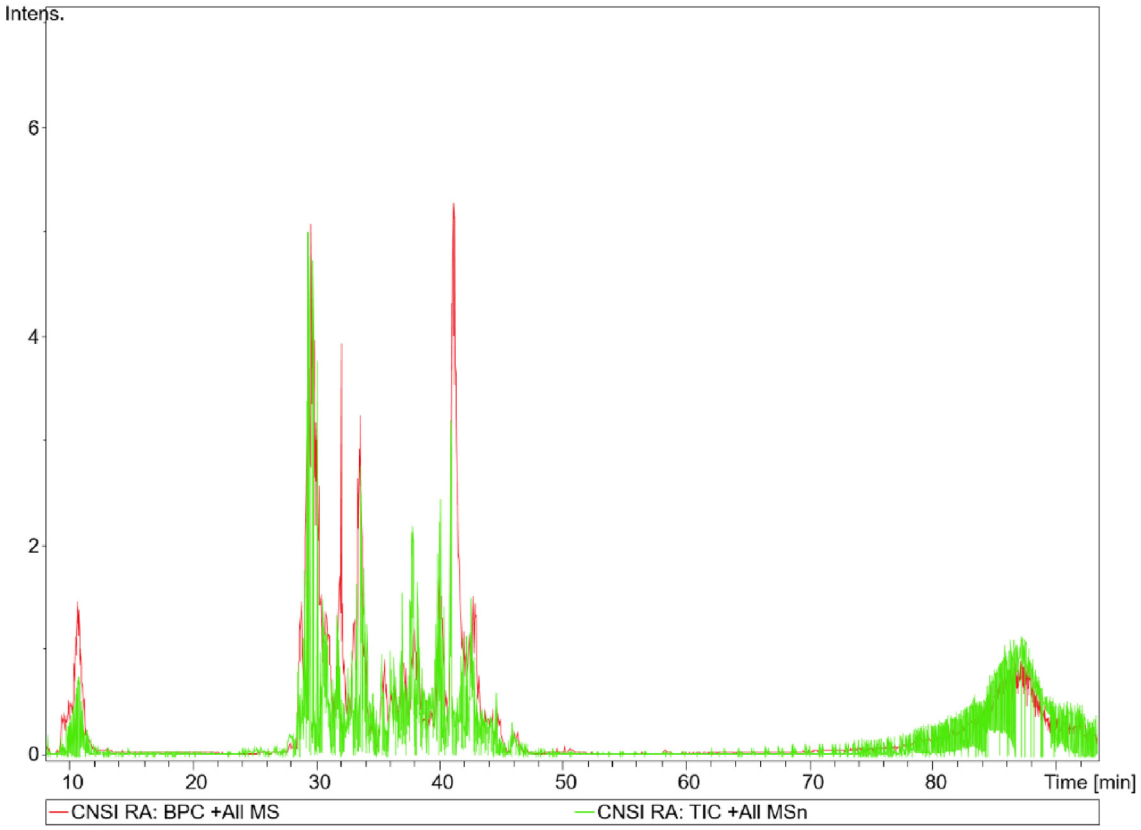

Fig. 2. Mass spectrometric analysis of Venom complex of *Conus inscriptus* presenting the total ions throughout the analysis.

Table 1
List of peptides with their reduced and alkylated mass along with the sequence.

S.No.	Name	Mass	Residues	Sequence	Reference
	Single Disulfidecontryphans				
1	In1172	1172.4	9	**RCPWDPWCN-NH2**	This Work
2	In896	895.3	7	**CVLYOWC-NH2**	This Work
3	In880	879.3	7	**CVLYPWC- NH2**	Sonti et al. (2013)
	Two Disulfide A-Superfamily conotoxins				
4	Lo1a	1929.69	18	**EGCCSNPACRTNHPEVCD**	Lebbe et al. (2014)
5	In1907	1907.7	17	**EGCCSNPOCRHNHOEVC-NH_2**	This Work
6	In1891	1891.7	17	**EGCCSNPOCRHNHPEVC-NH_2**	This Work
7	In1874	1874.7	17	**ZGCCSNPOCRHNHPEVC-NH_2**	This Work
8	In1857	1857.6	17	**ZGCCSNPPCRHNHPEVC-NH_2**	This Work
9	In1878	1878.7	17	**EGCCSNPOCRHTHPEVC-NH_2**	This Work
10	In1761	1761.7	17	**GCCSHPOCNVNNPHICG-NH_2**	This Work
11	OmIA	1719.6	17	**GCCSHPACNVNNPHICG-NH_2**	Talley et al. (2006)
	Three Disulfide M-Superfamily conotoxins				
12	In1696	1696.4	15	**CCEWPCSHGCIPCCY**	This Work
13	In1746	1746.5	15	**CCEWPCHHGCIPCCY- NH_2**	This Work
14	In1762	1761.6	15	**CCEWOCHHGCIPCCY- NH_2**	This Work
15	Pr3a	1691.5	15	**CCNWPCSF GCIPCCY**	(Jimenez and Olivera, 2010)

2. Materials and methods

2.1. Collection identification and extraction of natural peptides

The *Conus inscriptus* samples are collected from the fish landing sites (trash fish) located in Rayapuram fishing harbour (13°07′45.2″N 80°17′52.0″E), Tamil Nadu, India and identified following standard keys. *C. inscriptus* specimens were dissected to separate the venom duct and subsequently extracted with 50:50%, Acetonitrile: water mix and the crude extract was stored in a refrigerator at −20 °C for further studies (Rajesh, 2015; Rajesh et al., 2019).

2.2. LC-MS of the natural venom extract from Conus inscriptus

The crude venom extract was solubilised in Acetonitrile: Water mix and filtered using a 0.2 μM filter. ESI-MS (Esquire 3000-plus mass spectrometer- Bruker Daltonics, Germany) was performed

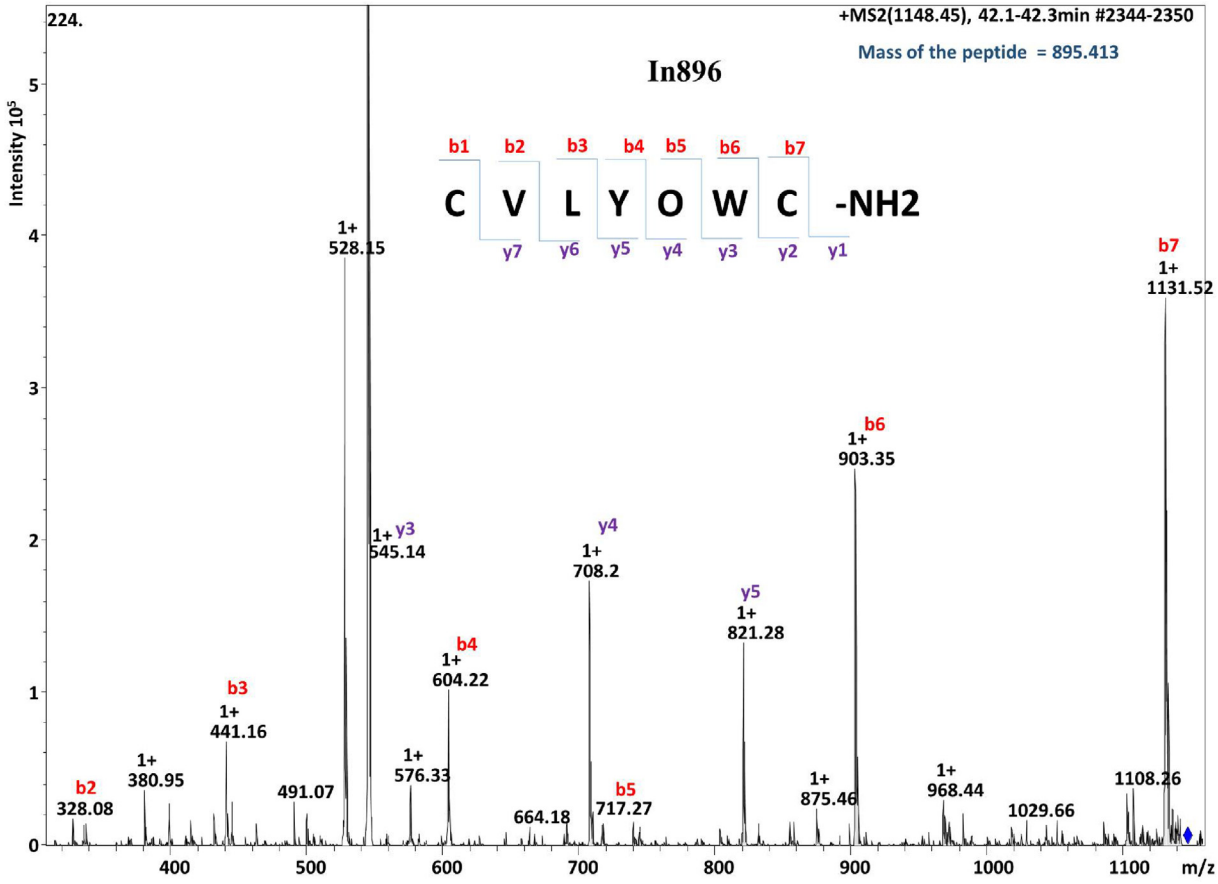

Fig. 3. Collision Induced Fragmentation of contryphan In896 from *C. inscriptus* demonstrating arrangements of 'y' and 'b' ions from the parent ion (reduced and alkylated) 1148.45 [M+H].

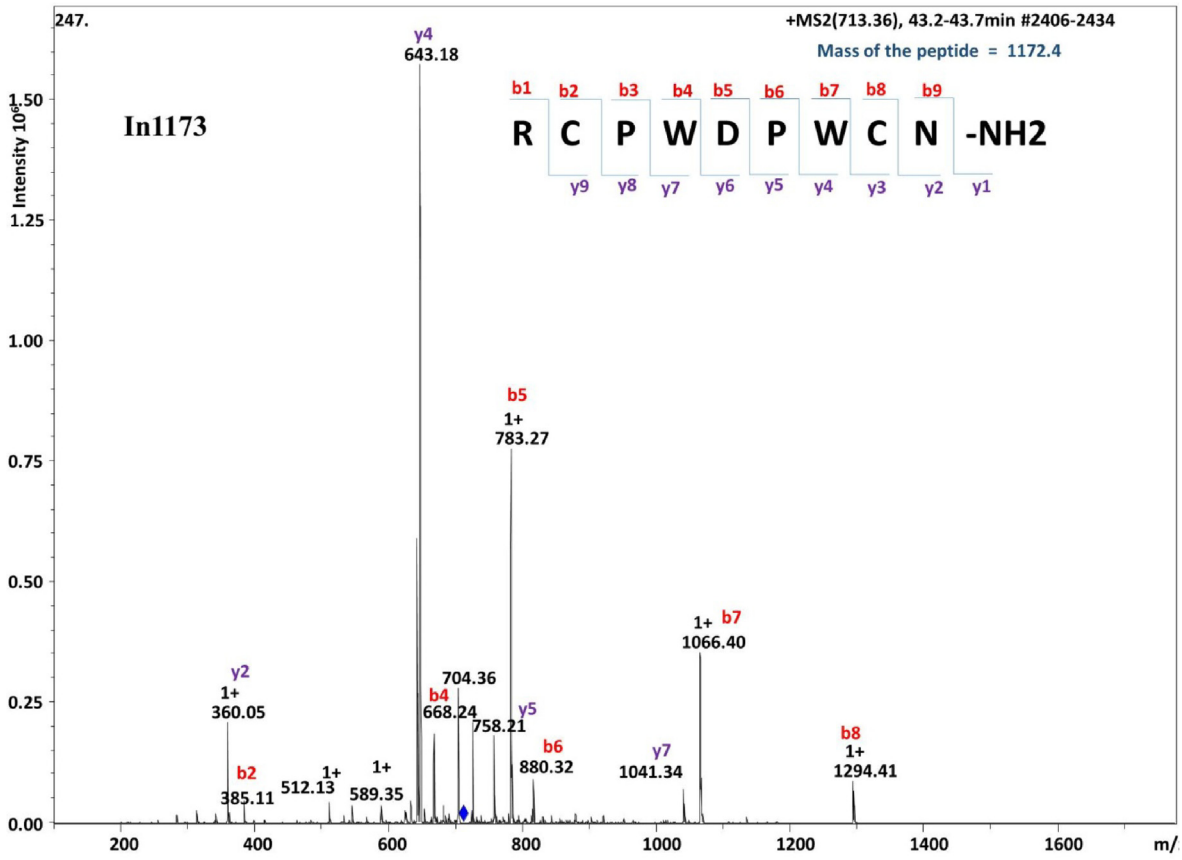

Fig. 4. Collision Induced Fragmentation of contryphan In1173.4 from *C. inscriptus* demonstrating arrangements of 'y' and 'b' ions from the parent ion (reduced and alkylated) 713.3 $[M+2H]^{+2}$.

on the extract to discover the peptide components in the venom complex (Rajesh, 2015; Rajesh et al., 2019). The crude venom components were separated in HPLC (Agilent 1100 series) using an analytical HPLC column [Agilent Zorbax analytical C18 column, 150 × 4.6 mm, 5 μm, 90 Å pore size] with a binary gradient solvent system (H_2O with 0.1% TFA): (acetonitrile with 0.1% TFA) at a flow rate of 0.2 mL min^{-1}. Data acquisition was performed over *m/z* 100–2000 in positive ion mode. All MALDI-TOF analysis was performed in Ultraflextreme MALDI-TOF-TOF (Bruker Daltonics, Germany) with CCA being the matrix (Rajesh, 2015; Rajesh et al., 2019).

2.3. Global reduction and alkylation of natural venom and analysis by LC-MS-MS

Since most of the conotoxins are disulfide-bonded peptides with multiple cysteine bonds, we linearised the bonded peptides for complete sequence determination. This is achieved by reducing the sample with TCEP (tris (2-carboxyethyl) phosphine) (20 mM) and incubated at 37 °C for 1.5 h. After linearization of the peptide, double the concentration of alkylating agent NEM (N-Ethylmaleimide) was added and incubated for 60 min at room temperature. ESI-MS is used to further analyse and find the number of cysteines present in individual conotoxins (Rajesh, 2015; Rajesh et al., 2019).

2.4. Acetylation of reduced and alkylated peptides

The reduced alkylated venom extract was incubated with acetic anhydride and incubated for 60 min at 25° C. After incubation this mixture was analysed in ESI-MS as explained, elsewhere to discover the conotoxins with free amino-terminus and lysine residues in the sequence (Rajesh, 2015; Rajesh et al., 2019).

2.5. Sequencing of venom peptides

Manual de novo sequencing strategy was followed to sequence the conotoxins from the raw data obtained from LC-MS-MS. Data were analysed in Data Analysis version 4.1 and Flex analysis (Bruker Daltonics). The daughter ions generated from singly and doubly charged parent ions were carefully analysed to derive the amino acid sequences of the venom complex (Rajesh, 2015; Rajesh et al., 2019).

2.6. Conotoxin superfamily prediction

Conotoxin superfamily was predicted using online servers ConoDictor and PredCSF (Fan et al., 2011; Koua et al., 2012).

2.7. Toxicity testing of conotoxin in zebrafish embryos

Zebrafish were obtained from Tharun fish form, Chennai (Registration number: FWCS-80) and maintained in the standalone system (Aquaneering, USA) with standard conditions. Zebrafish embryos were collected after natural spawning from male and female in the ratio 2:1. Healthy embryos after Six hours post fertilization (hpf) were used for toxicity analysis. The embryos were exposed to different concentrations of conotoxin (100, 200, 400, 600, 800, 1000 μg/mL) based on OECD guidelines and evaluated for toxicity. 10 Embryos in each well were treated in sterile 24-well plates containing 1 mL of the solution. Developmental deformities and death of the larvae were evaluated at 72 h

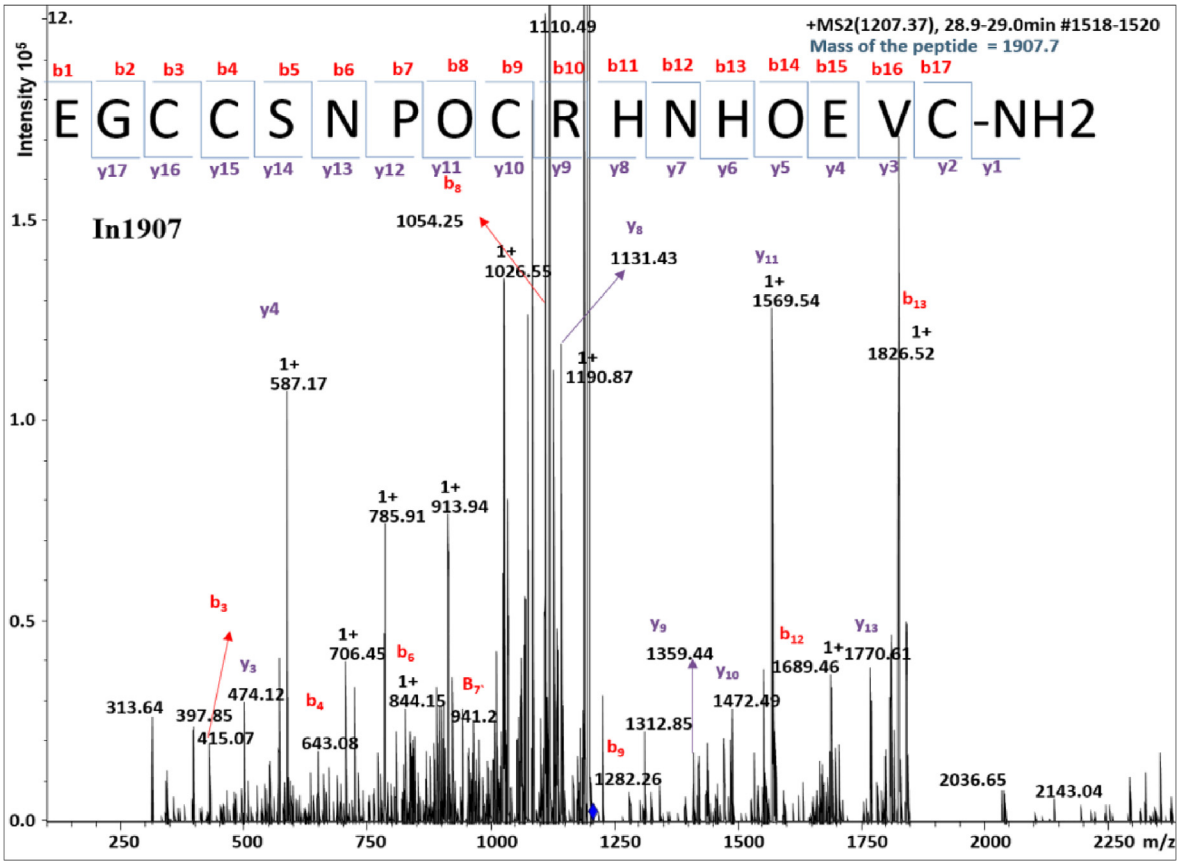

Fig. 5. Collision Induced Fragmentation of A superfamily conotoxin In1907 from *C. inscriptus* demonstrating arrangements of 'y' and 'b' ions from the parent ion (reduced and alkylated) 1207.37 $[M+2H]^{+2}$.

post-treatment (hpt) using stereomicroscope (Leica M165C) (Kumar et al., 2017; OECD, 2013; Rajesh et al., 2019; Schmidt, 1985).

2.8. Assay to ascertain the anticonvulsant property of conotoxin

In a 96-well plate, 6 days post fertilization (dpf) zebrafish larvae were placed with one larva per well. Following the toxicological evaluation, the zebrafish larvae (6 dpf) were then transferred to fresh 200 μL E3 medium with the experimental setup containing control, 20 mM pentylenetetrazole (PTZ); vehicle (1% DMSO); 200 μg/ml and 300 μg/ml concentrations of conotoxin without PTZ; 200 μg/ml and 300 μg/ml concentrations of conotoxin with PTZ (20 mM). Stereo microscopic monitoring of zebrafish larvae was started immediately by recording the real-time video of the treated and untreated wells. Videos were later analysed using the software TRACKER (version 5.0.6) to obtain the X-axis and Y-axis coordinates of the moving frequency and behavioural response by the fish. The interpretation of the specific behaviour with and without drug treatment were interpreted with the behaviours documented for zebrafish. Sodium valproate of 3 mM is used as a positive control in the study (Berghmans et al., 2007; Jackson and Scheideler, 1996; Rajesh et al., 2019).

2.9. Cytotoxicity of conotoxin on brine shrimp larvae

Artemia salina (Brine shrimp) eggs were purchased from Ocean Star International O.S.I, USA. Dried cysts were added to a separating funnel containing natural seawater. After 24–28 h of incubation and strong aeration at room temperature (28–32 °C) and under continuous light supply, the nauplii were hatched. The larvae were separated using a coffee filter and rinsed thrice in sterile seawater. The nauplii was then suspended in sterile seawater. The evaluation of cytotoxicity on brine shrimp embryo was performed by adding 14 larvae in each well containing 200 μL of sterile seawater. The test was performed in triplicates. The larvae were exposed to different concentrations of natural venom (10, 20, 40, 80, 160, 320, 640 μg/mL). The control well consisted of only nauplii and sterile seawater. After 24 h, the number of nauplii surviving were checked under a stereomicroscope (Leica M165C). The percentage of death was calculated by comparing the test and control wells (Rajabi et al., 2015).

2.10. Assay to assess the effect of conotoxin on acetylcholinesterase enzyme

Elman's method in 96-well plate was followed to analyse the acetylcholinesterase inhibitory assay. 50, 100, 200, 400 μg/ml concentration of conotoxins were mixed with 40 μL of acetylcholinesterase enzyme extract from zebrafish brain and made up to a final volume of 250 μL with PB buffer (pH 7). Controls were maintained separately. Tris HCL (pH 8) was added to arrest the reaction and 10 μL of 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) was added to each well. Following this 2 μL of acetylthiocholine iodide was added and the absorbance was measured at 412 nm in multi-mode plate reader (Perkinelmer) (Ferreira et al., 2006; Mathew and Subramanian, 2014).

2.11. Acetylcholinesterase enzyme agonist activity of natural venom

This is a similar experiment as presented above 'Assay to assess the inhibitory effect of conotoxin on acetylcholinesterase enzyme'.

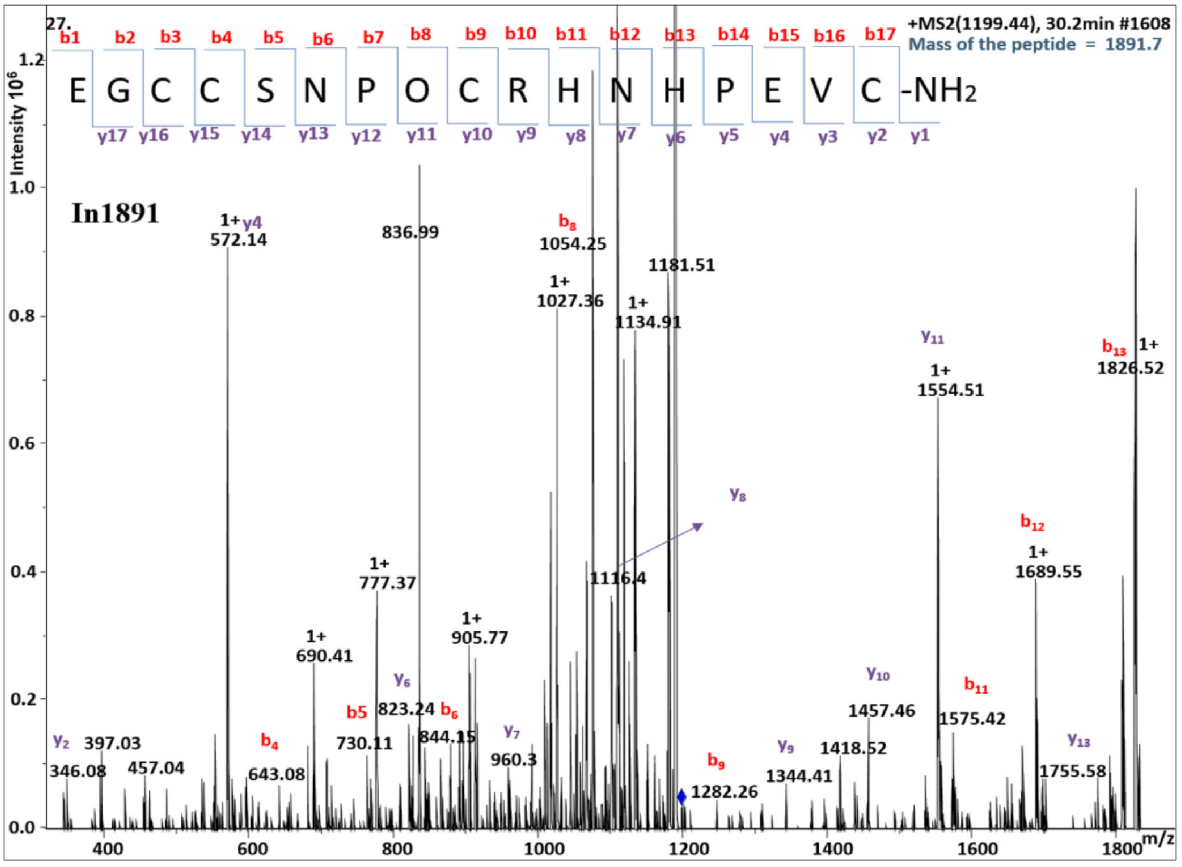

Fig. 6. Collision Induced Fragmentation of A superfamily conotoxin In1891 from *C. inscriptus* demonstrating arrangements of 'y' and 'b' ions from the parent ion (reduced and alkylated) 1199.44 $[M+2H]^{+2}$.

The only modification in this assay is that the enzyme extract is not added to evaluate the agonist activity of natural venom.

3. Results

Cone snail sample were identified as *Conus inscriptus* following standard keys (Fig. 1). The dissected venom duct is displayed in Supplementary Fig. 1.

3.1. Mass spectrometric analysis

The total ion chromatogram (TIC) of the reduced and subsequently alkylated spectrum unveils the diverse nature of venom components from the venom duct of *C. inscriptus* (Fig. 2). The elution of most of the venom components ranged from 25 min to 50 min, which indicates the hydrophilic nature of venom peptide components. Few hydrophobic venom components were also traced from 70 to 100 min elution. MALDI-TOF investigation of the venom revealed the presence of conopeptides in the masses ranging from 500 to 5500 Da. A total of 53 *m/z* traces were identified (Table 1 and Supplementary Figs. 2–18) from analysing the MALDI-TOF spectrum of the HPLC fractions. Reduced and alkylated fractions revealed 37 of this 53 *m/z* produced an explanatory shift in increased molecular mass proving to have modified cysteines with N-Ethylmaleimide attached to it. We have identified, 4 peptides which showed a 252 Da increase inferring the presence of two cysteine-containing single disulphides, similarly 20 peptides showed a 504 Da increase inferring 4 cysteine-containing two disulfides. 11 peptides with a 756 Da increase in mass infers the presence of 6 cysteine-containing three disulphide peptides (Supplementary Table 1 and Supplementary Figs. 2–18).

3.2. De-nova peptide sequencing of venom

3.2.1. Single disulfide peptides

Two novel single disulfide-bonded contryphan with molecular mass 896 Da (CVLYOWC-NH_2) and 1173.4 Da (RCPWDPWCN-NH_2) were sequenced from the fragmented spectrum of the reduced and alkylated parent ions 713.3$[M+2H]^{+2}$ and 1148.45 [M+H] respectively (Table 1 and Figs. 3 and 4).

3.2.2. Double disulphide conotoxins

Six A-superfamily conotoxins- In1907, In1891, In1874, In1857, In1878 and In1761 were unambiguously sequenced to its amino acid level. All six conotoxins possess C-terminal amidation. Both In1857 and In1874 possess modified pyroglutamic acid in 1st position. Except for In1857 all the other five conotoxins possess modified hydroxyl proline at 8th position, while In1907 possess two hydroxyl proline, the second modification at 14th position.

The CID fragmentation spectrum of doubly charged ion 1207.37 *m/z* $[M+2H]^{+2}$ is presented in Fig. 5. The series of 'b' and 'y' ions (Table 1) were carefully analysed which derived us with the sequence of In1907 as EGCCSNPOCRHNHOEVC-NH_2.

MS_2 fragmentation data of doubly charged ion 1199.44 *m/z* $[M+2H]^{+2}$ is presented in Fig. 6. The sequence of 'b' and 'y' ions (Table 1) were carefully analysed, which derived us with the sequence of In1891 as EGCCSNPOCRHNHPEVC-NH_2. Both In1907 and In1891 are identical but In1907 possess a second hydroxylation of proline residue at 14th position.

In1874 is sequenced by inspecting the 'b' and 'y' ions obtained from the daughter ion spectrum of the doubly charged ion 1190.6 *m/z* $[M+2H]^{+2}$ (Table 1 & Fig. 7) and triply charged ion 794.03 *m/z* $[M+3H]^{+3}$. Since it contains 3 charged amino acid

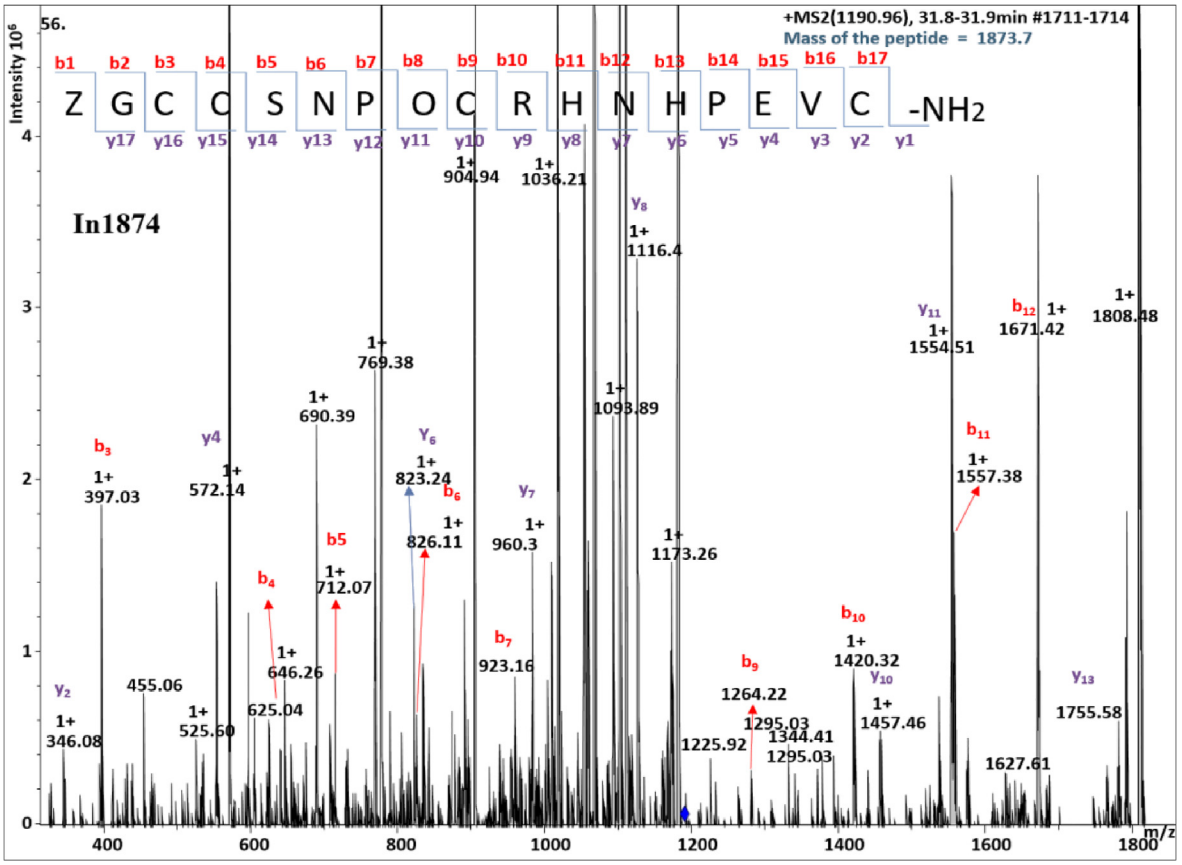

Fig. 7. Collision Induced Fragmentation of A superfamily conotoxin In1874 from *C. inscriptus* demonstrating arrangements of 'y' and 'b' ions from the parent ion (reduced and alkylated) 1190.6 $[M+2H]^{+2}$.

residues like 'R', 'E' and 'Z' we observed 3 charged state ions viz... doubly charged ion 1190.6 *m/z* $[M+2H]^{+2}$, triply charged ion 794.03 *m/z* $[M+3H]^{+3}$ and quadruply charged ion 596.4 *m/z* $[M+4H]^{+4}$. Moreover, the first residue observed is a post-translationally modified cyclised pyroglutamic acid residue (Table 1).

Fig. 8 shows the fragmented spectrum attained by the fragmentation of the doubly charged reduced alkylated ion 1182.53 *m/z* $[M+2H]^{+2}$. The series of 'b' and 'y' ions were presented in Table 1, which unambiguously deduced the sequence of In1857. Both In1874 and In1857 is identical except the 8th residue is proline, whereas In1874 contains the posttranslationally modified hydroxyproline in its 8th position. The derived sequence of In1857 is presented in Table 1.

Almost all "b" and "y" sequence ions (Table 1 and Fig. 9) are observed from the fragmented doubly charged ion 1192.9 *m/z* $[M+2H]^{+2}$. The sequence of In1878 thus derived unambiguously as EGCCSNPOCRHTHPEVC-NH_2.

The aminoacid sequence of In1761 is GCCSHPOCNVNNPHICG-NH_2 which is derived by examining the 'b' and 'y' ions of doubly charged ion 1134.4 *m/z* $[M+2H]^{+2}$ (Table 1 and Fig. 10). This conotoxin is quite different from otherA- Superfamily conotoxin derived from this study.

3.2.3. Sequences of three disulfide conotoxin

Three M-superfamily conotoxins In1696 (CCEWPCSHGCIPCCY), In1746(42-CCEWPCHHGCIPCCY-NH2) and In1762(CCEWOCHHGCIPCCY) comprising 15 amino acids residues with 6 cysteines (3 disulfides) each, are sequenced from the fragments acquired from doubly charged parent ions 1228.2, 1252.8 and 1281.8 $[M+2H]^{+2}$ respectively (Table 1 & Figs. 11–13). In1745 has histidine in the 7th position instead of serine as in In1696 and In1762. In1762 was sequenced from the acetylated parent ion1281.8 $[M+2H]^{+2}$, so a shift of 42 Da is observed from b_1 ion throughout the series of 'b' ions. This is similar to In1745 but the $b_4$ion (788.14) is 16 Da higher than the $b_4$ion (772.14) of In1745, which portrays the hydroxylation of proline, one of the major post-translational modification observed in conotoxins. The y_{11} ion also displays a sharp intense peak with *m/z* 1748.42, which is sixteen dalton higher than the y_{11} ion of In1745 (*m/z* 1732.42) endorsing the hydroxylation of proline. Succeeding ions y_{12} – y_{15} also displays a series of 16 Da increase.

3.3. Conotoxin superfamily

The conotoxin superfamilies were predicted using ConoDictor and PredCSF (Table 1). Both the single disulphide conopeptides were predicted as contryphan, all the double disulphide conopeptides were found to be positive for A-superfamily and the three disulphide conopeptides were found to be positive for M-superfamily.

3.4. Toxicity assessment of C. inscriptus natural venom on zebrafish embryo

Six hpf zebrafish embryos were subjected to different concentrations of natural venom of *C. inscriptus* for 72 h. The embryos were checked at regular intervals for any deformities or death due to the venom and its survival rate was calculated based on the dosage and time. For the first 44–48 h, no major changes are observed. This demonstrates that the natural venom of *C. inscriptus* is impermeable to the chorion of the zebrafish larvae. Once the

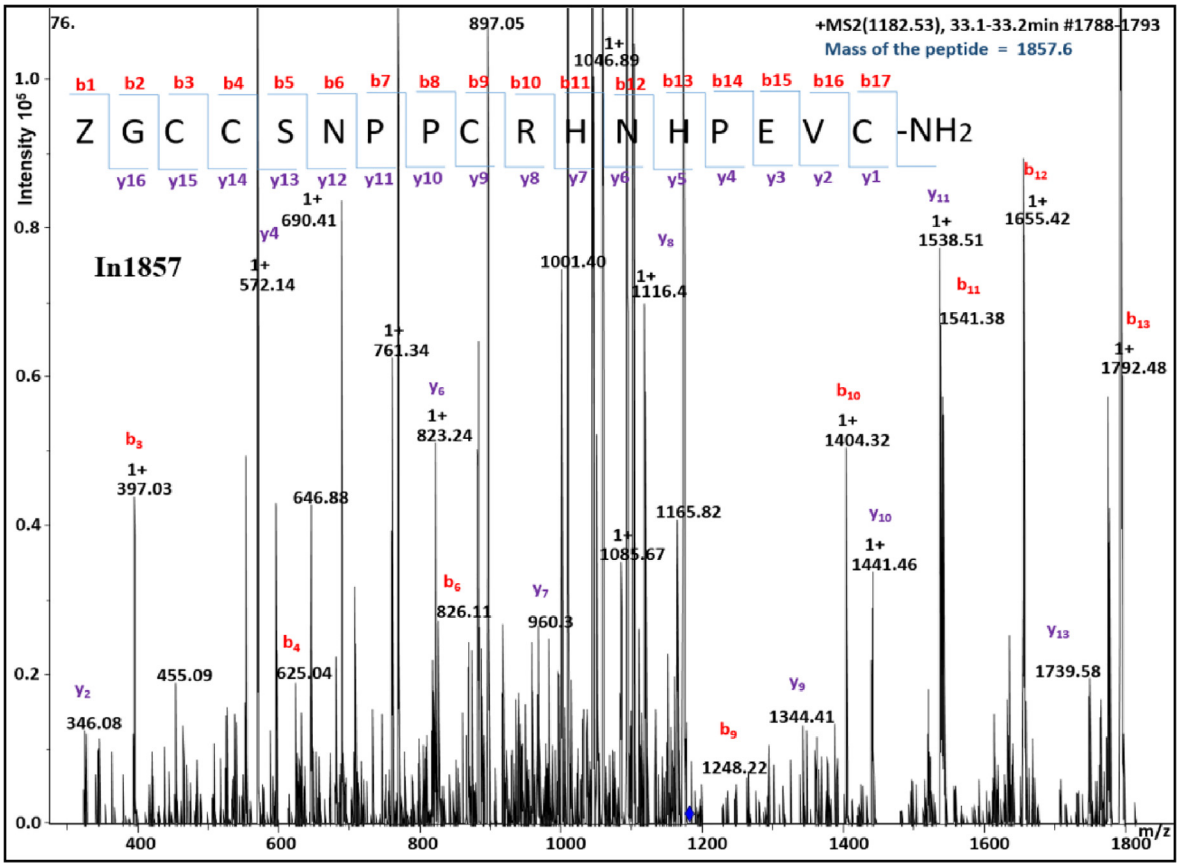

Fig. 8. Collision Induced Fragmentation of A superfamily conotoxin In1857 from *C. inscriptus* demonstrating arrangements of 'y' and 'b' ions from the parent ion (reduced and alkylated) 1182.53 $[M+2H]^{+2}$.

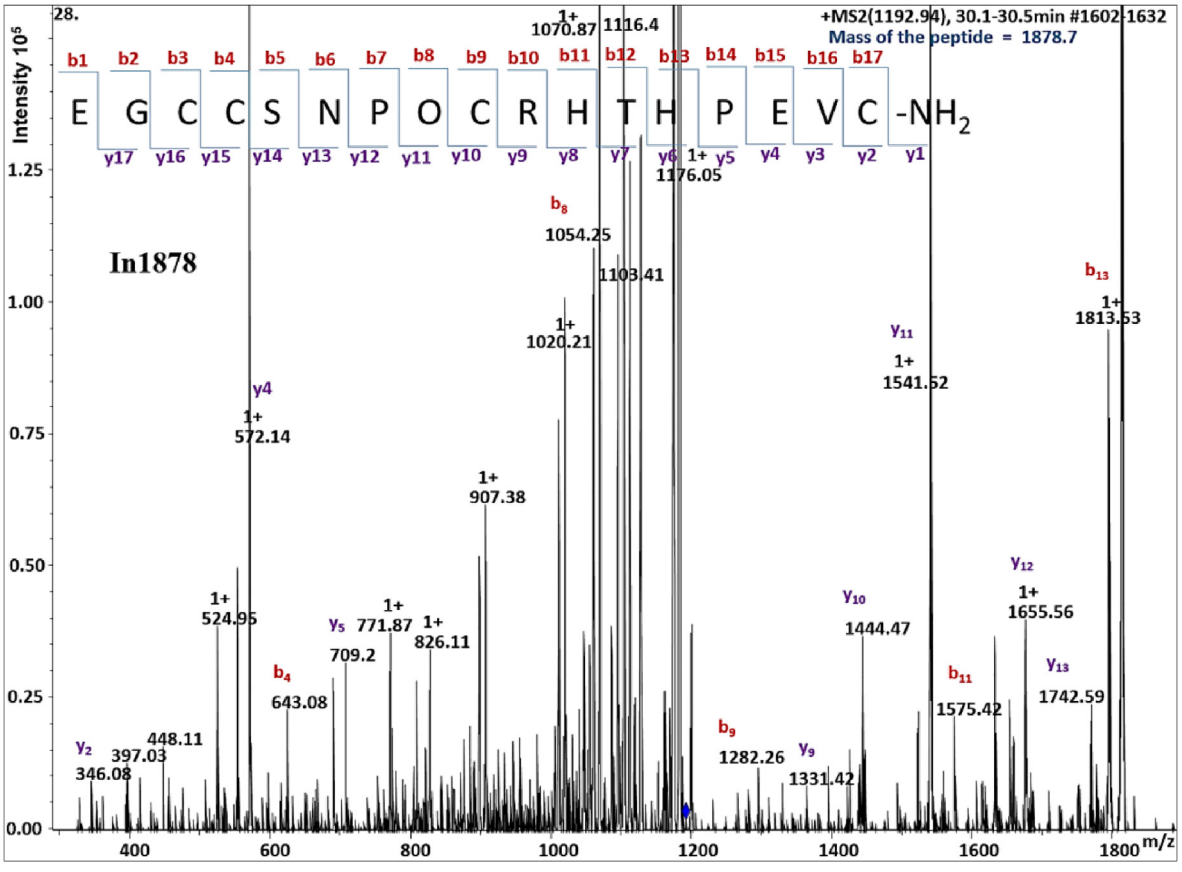

Fig. 9. Collision Induced Fragmentation of A superfamily conotoxin In1878 from *C. inscriptus* demonstrating arrangements of 'y' and 'b' ions from the parent ion (reduced and alkylated) 1192.9 $[M+2H]^{+2}$.

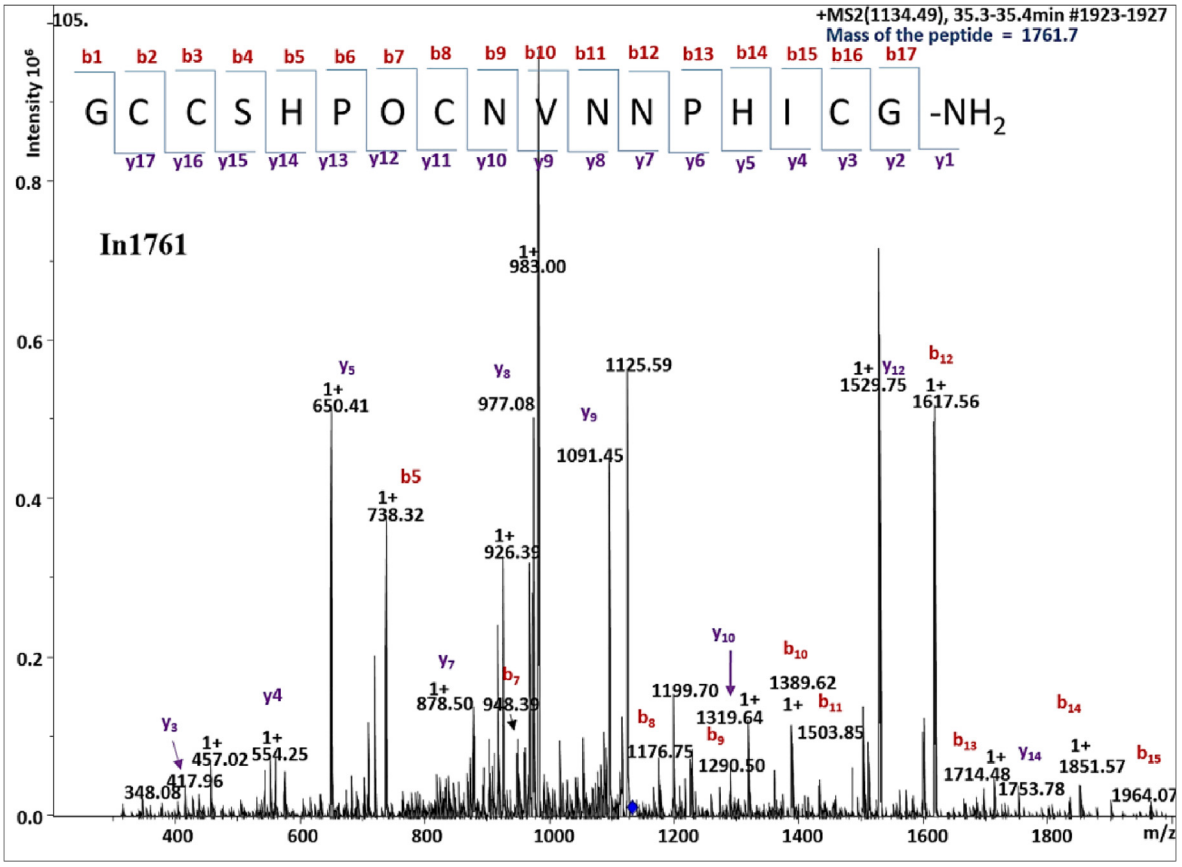

Fig. 10. Collision Induced Fragmentation of A superfamily conotoxin In1761 from *C. inscriptus* demonstrating arrangements of 'y' and 'b' ions from the parent ion (reduced and alkylated) 1134.4 $[M+2H]^{+2}$.

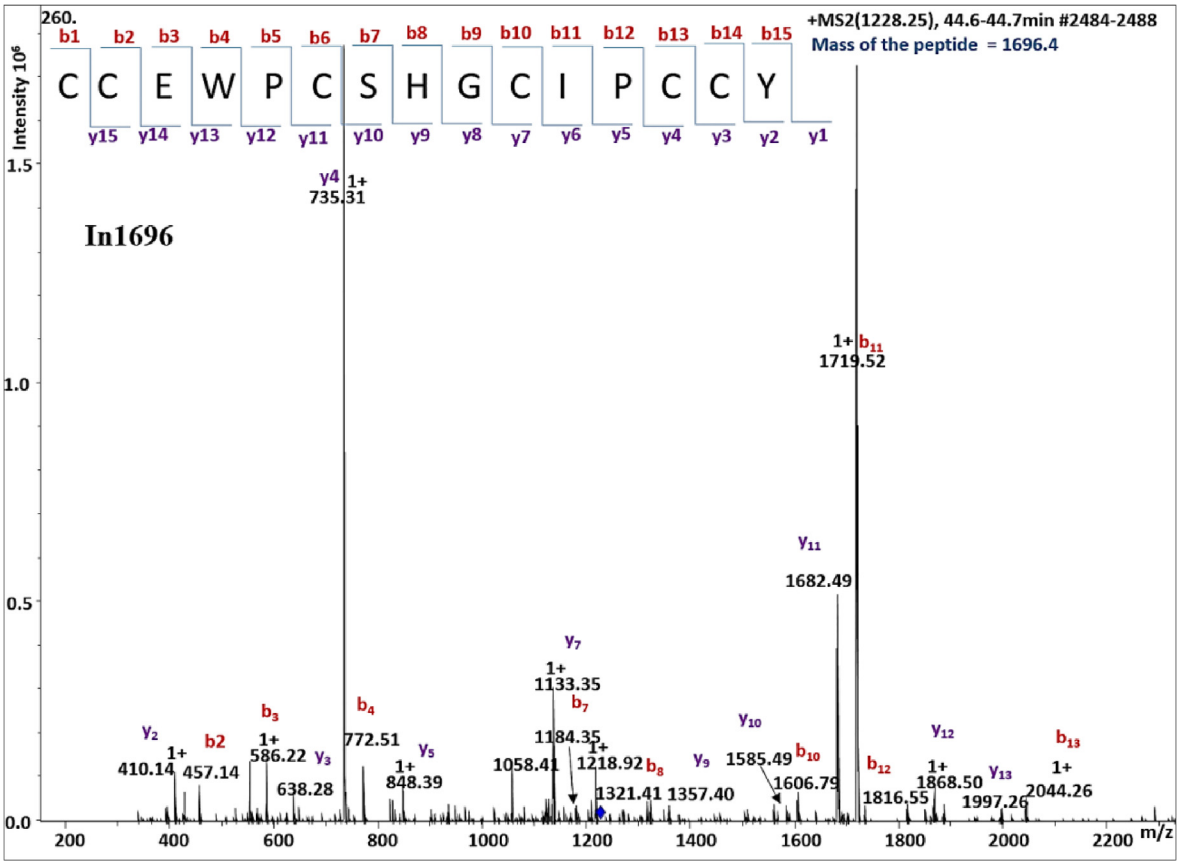

Fig. 11. Collision Induced Fragmentation of A superfamily conotoxin In1696 from *C. inscriptus* demonstrating arrangements of 'y' and 'b' ions from the parent ion (reduced and alkylated) 1228.2 $[M+2H]^{+2}$.

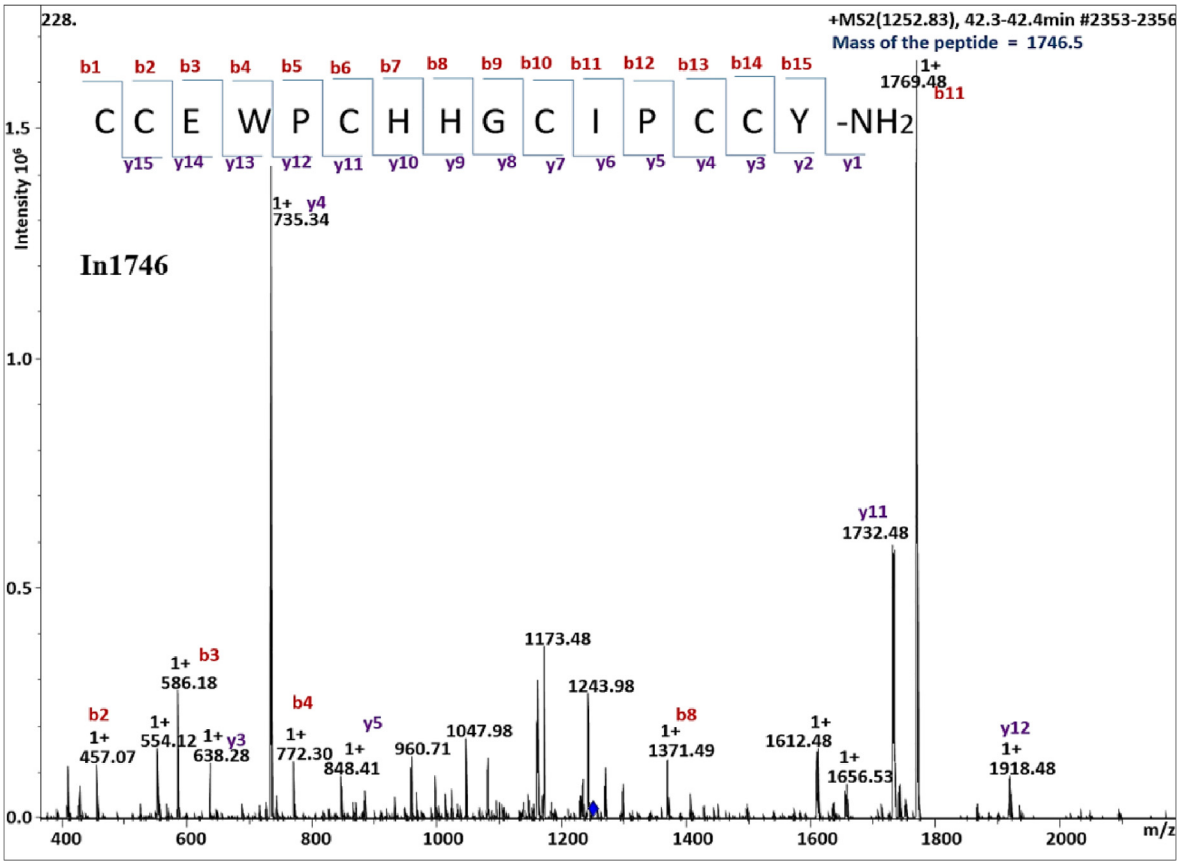

Fig. 12. Collision Induced Fragmentation of M superfamily conotoxin In1746 from *C. inscriptus* demonstrating arrangements of 'y' and 'b' ions from the parent ion (reduced and alkylated) 1252.8 $[M+2H]^{+2}$.

embryos hatched, within the next 24 h deformities such as spinal kyphosis, pericardial edema, hemorrhage are observed in the larvae (from 250 μg/ml onwards) and at higher concentration (500 μg/ml onwards) 100% death is observed (Fig. 14). LC_{50} was calculated as 304.82 μg/mL (Supplementary Fig. 19).

3.5. Anticonvulsant activity of natural C. inscriptus venom on zebrafish larvae

Sodium Valproate (SV) is a commercially available anticonvulsant drug and is used as a control for this assay. The recovering effect of sodium valproate is confirmed by testing against Pentylenetetrazole (PTZ) (chemo-convulsant) treated zebrafish larvae. The graphs indicate a significant reduction of epileptic seizures in the larval zebrafish when treated with SV. The prophylactic activity of conotoxins also showed significantly less locomotor activity when compared to only PTZ treated larvae (Fig. 15). The change in speed (Supplementary Fig. 20) was also calculated.

3.6. Cytotoxicity effect of conotoxin on brine shrimp larvae

30 h post-hatching, the brine shrimp nauplii were subjected to various concentrations of *C. inscriptus* venom. The nauplii were visualized after 24 h for toxicity. 100% survival was observed until the concentration of 20 μg/mL, after which a gradual decrease was observed (Fig. 16). LC_{50} was calculated as 130.31 μg/mL (Supplementary Fig. 21).

3.7. Acetylcholinesterase agonist activity

An assay was performed to determine the acetylcholinesterase inhibiting property of *C. inscriptus* crude venom based on Ellman method (as defined in materials and methods). The result shows a lack of acetylcholinesterase inhibition (Fig. 17). Given that, the venom lacks acetylcholinesterase inhibition activity, we also found that the test values were higher than the control values, suggesting that the conotoxin itself contains acetylcholinesterase. Based on the previous results of increased acetylcholinesterase activity, we tried to determine the presence of acetylcholinesterase in the crude venom. The results showed that the conotoxin lacks acetylcholinesterase. This suggests that the crude venom of *C. inscriptus* helps in the enhancement of acetylcholinesterase activity (Fig. 18).

4. Discussion

Very fewer studies have been done on *Conus inscriptus*. So far, there is only one notable conopeptide that have been reported from this species (Conoserver and NCBI). This peptide In936 is categorised as a single disulfide-bonded contryphan group. Structural studies, Aromatic/Proline interactions and Cis-Trans Isomerisation were widely studied using this conotoxin (Jimenez et al., 2001; Sonti et al., 2013). Most of the alpha conotoxins characterised in this study are identical except with few post-translational modifications and amino acid mutations. Amino acid sequences of In1907, In1891, In1874, In1857 and In1878 are almost similar and identical to Lo1a (Lebbe et al., 2014). In1907 possess hydroxyproline in 8th and 14th position. In1891 is made of proline in 14th position. In1874 and In1857 possess pyroglutamic acid at

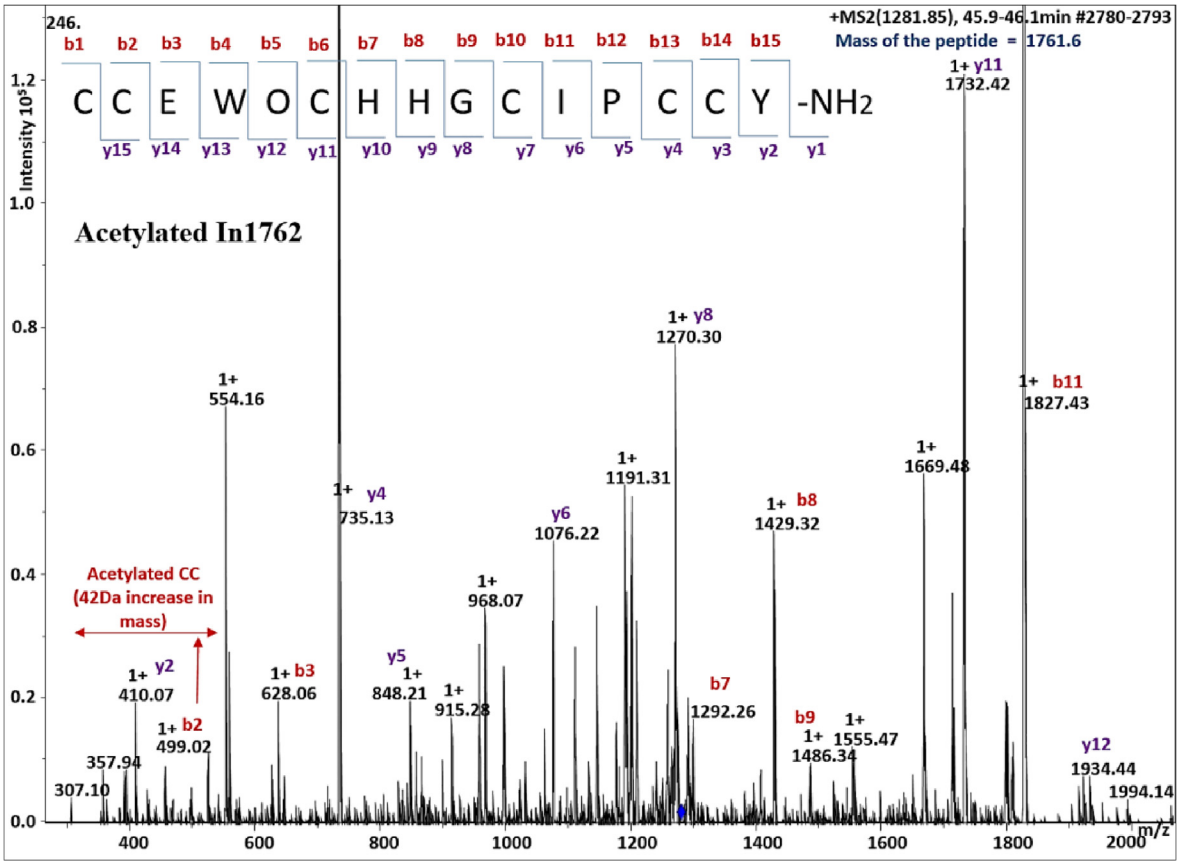

Fig. 13. Collision Induced Fragmentation of M superfamily conotoxin In1762 from *C. inscriptus* demonstrating arrangements of 'y' and 'b' ions from the parent ion (reduced, alkylated and acetylated) 1281.8 $[M+2H]^{+2}$.

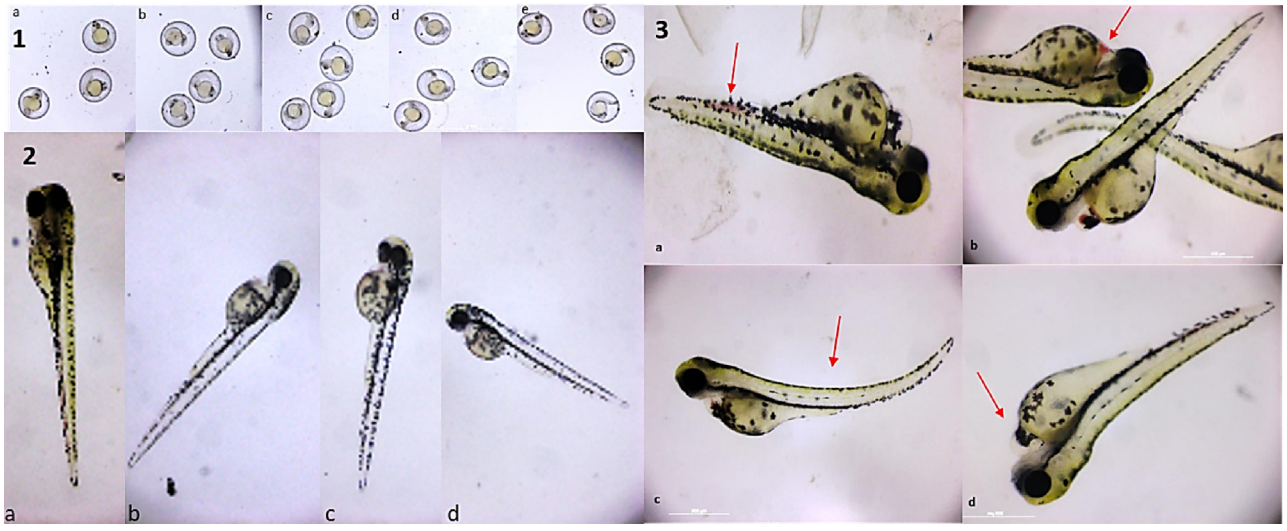

Fig. 14. Toxicity assessment of Zebrafish- 1. At 0 h post treatment (hpt) (a) Control, (b) 200 μg, (c) 300 μg, (d) 400 μg, (e) 500 μg (magnification 2 mm); 2. At 24 hpt (a) Control, (b) 100 μg, (c) 200 μg, (d) 300 μg (magnification 500 μg); 3. Deformities observed at the end of 72 h (a) Blood clot; (b) Hemorrhage; (c) Spinal khyphosis; (d) Pericardial edema (magnification 500 μg).

the first position. The amino acid sequence of In1761 is similar to that of Om1a, previously sequenced from *C. omaria* (Talley et al., 2006), except for the presence of hydroxyl proline in the 7th position. All the α-conotoxin possess a C-terminal amidation, contryphan with molecular mass 880 Da (CVLYPWC-NH_2) previously sequenced from *C. inscriptus* (Hanumae Gowd et al., 2005) and *C. textile* (Jimenez et al., 2001) was also derived by *de novo* sequencing. The contryphan In896 possess modified hydroxyl proline at the 5th position, also all the three contryphans identified in this study possess a C-terminal amidation.

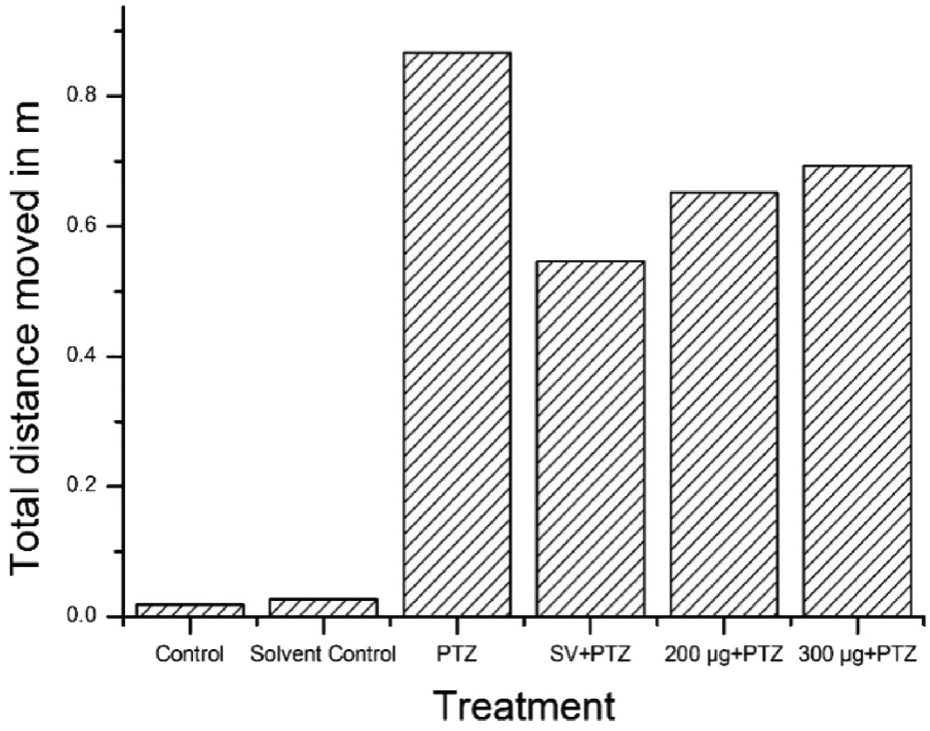

Fig. 15. A schematic representation of the total distance moved (in metres) by the zebrafish larvae.

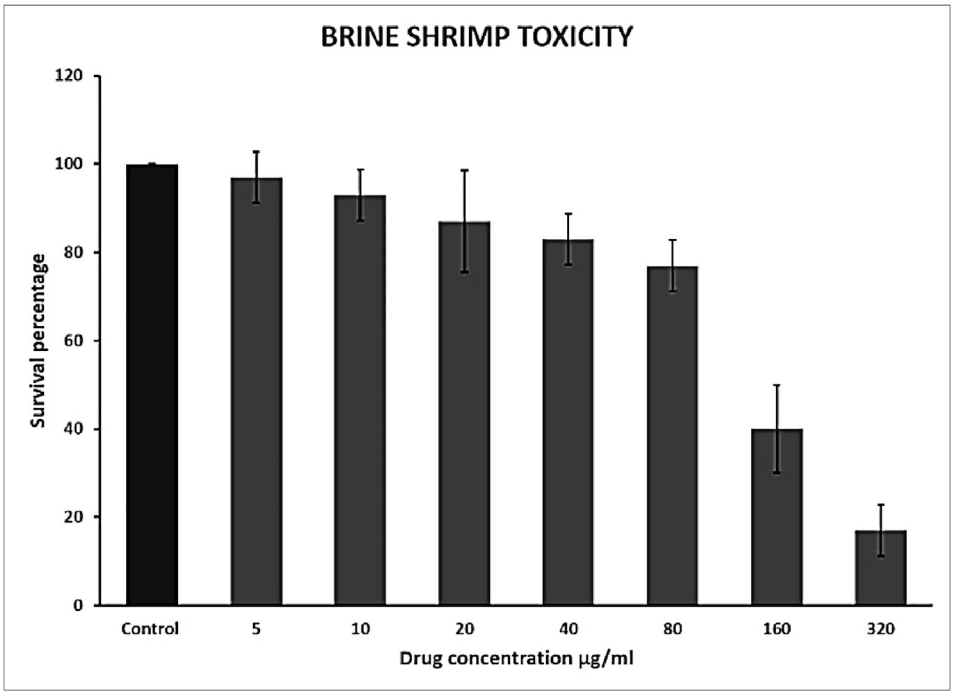

Fig. 16. A schematic representation of *C. inscriptus* crude venom toxicity on Brine shrimp.

Natural venom of *C. inscriptus* seems to be effective as an anticonvulsant agent. There is a significant reduction in the number of twitches and the distance moved by the zebrafish larvae when exposed to PTZ (convulsant agent) and the natural venom. We also found that the natural venom of *C. inscriptus* enhances the activity of acetylcholinesterase. Acetylcholinesterase hydrolyses acetylcholine that are released by the motor neurons to terminate neuronal signaling rapidly. This helps in maintaining homeostasis in normal physiology. The natural venom enhances the activity of AChE, thus destroying the neurotransmitter and resulting in paralysing the victim.

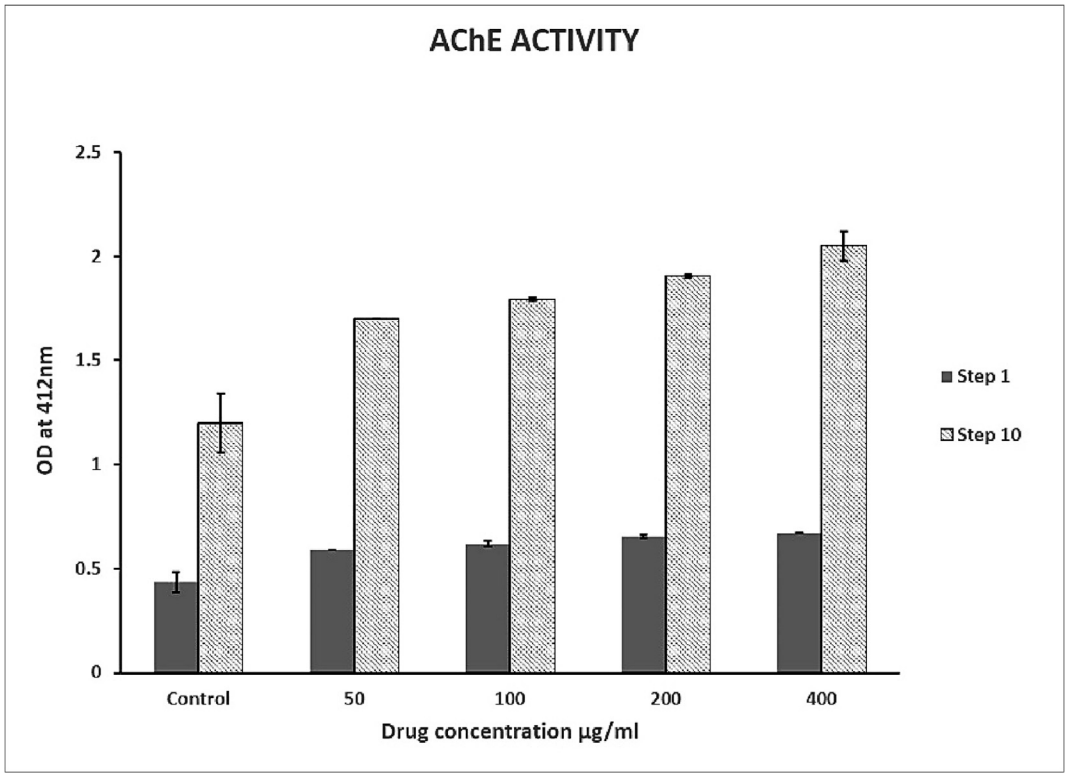

Fig. 17. A schematic representation of enhancement of acetylcholinesterase activity by different concentrations of conotoxin.

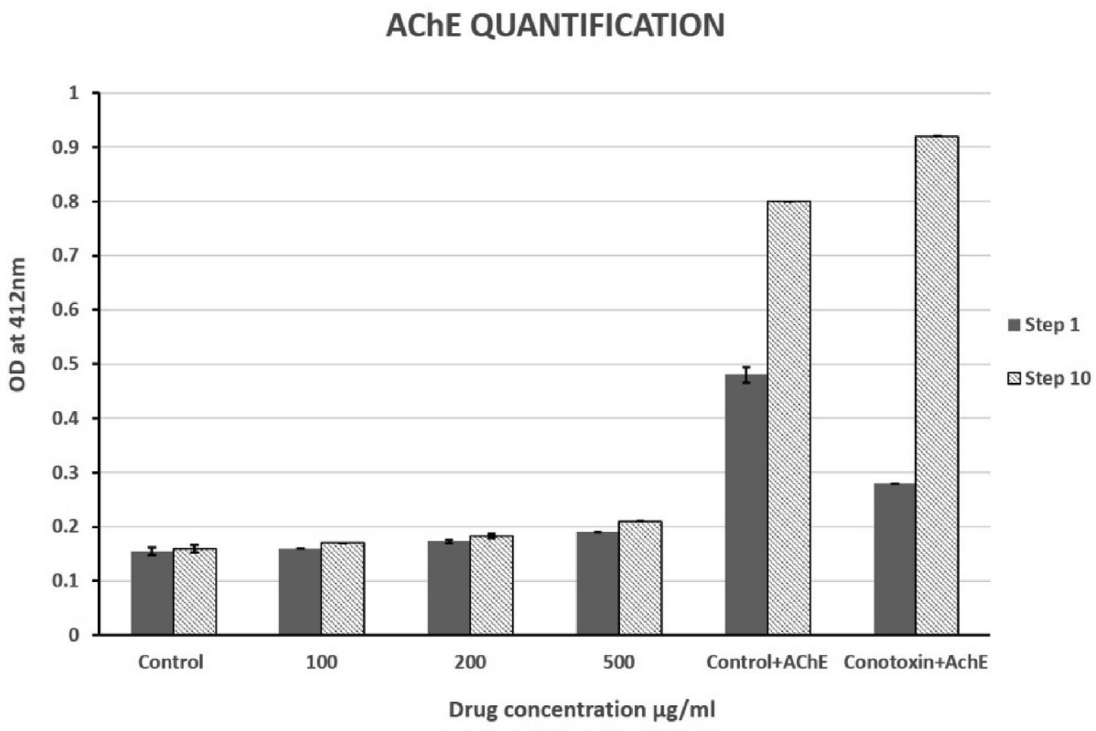

Fig. 18. A schematic representation of acetylcholinesterase agonist activityof the *C. inscriptus* crude venom.

5. Conclusion

A total of 37 novel conotoxins was identified from the natural venom of marine vermivorous cone snail *C. inscriptus*. Among the, 11 conotoxins were characterised to its aminoacid level by following manual *denovo* sequencing strategies. 1, 2 and 3 disulphide conopeptides belonging to different classes have been identified adding to the knowledge of venom peptides from this species. Nat-

ural venom significantly reduced twitches that were induced by pentylenetetrazole in larval zebrafish. Acetylcholinesterase activity was also observed in venom. Further, studies on purified peptide will help in developing new potential antiepileptic peptide drugs and other also as sources as therapeutic agents.

Ethics approval statement and participation consent

The *Conus inscriptus* samples was collected from fish landing sites in Rayapuram (Fishing Harbour) trash fish waste. The species used (*C. inscriptus*) are not listed under endangered or protected species. Hence, the study is conducted without prior permission with the wild life authorities.

Human and animal rights

No human and animal are used that are basis of this research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2020.12.032.

References

Afrikanova, T., Serruys, A.S.K., Buenafe, O.E.M., Clinckers, R., Smolders, I., de Witte, P. A.M., Crawford, A.D., Esguerra, C.V., 2013. Validation of the Zebrafish Pentylenetetrazol seizure model: locomotor versus electrographic responses to antiepileptic drugs. PLoS One. https://doi.org/10.1371/journal.pone.0054166.

Berghmans, S., Hunt, J., Roach, A., Goldsmith, P., 2007. Zebrafish offer the potential for a primary screen to identify a wide variety of potential anticonvulsants. Epilepsy Res. https://doi.org/10.1016/j.eplepsyres.2007.03.015.

Dave, K., Lahiry, A., 2012. Conotoxins: review and docking studies to determine potentials of conotoxin as an anticancer drug molecule. Curr. Top. Med. Chem. https://doi.org/10.2174/156802612800166765.

Fan, Y.-X., Song, J., Kong, X., Shen, H.-B., 2011. PredCSF: an integrated feature-based approach for predicting conotoxin superfamily. Protein Pept. Lett. https://doi.org/10.2174/092986611794578341.

Ferreira, A., Proença, C., Serralheiro, M.L.M., Araújo, M.E.M., 2006. The in vitro screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. J. Ethnopharmacol. https://doi.org/10.1016/j.jep.2006.04.010.

Goessling, W., North, T.E., 2014. Repairing quite swimmingly: advances in regenerative medicine using zebrafish. DMM Dis. Model. Mech. https://doi.org/10.1242/dmm.016352.

Hanumae Gowd, K., Sabareesh, V., Sudarslal, S., Iengar, P., Franklin, B., Fernando, A., Dewan, K., Ramaswami, M., Sarma, S.P., Sikdar, S., Balaram, P., Krishnan, K.S., 2005. Novel peptides of therapeutic promise from Indian Conidae. Ann. the New York Acad. Sci. https://doi.org/10.1196/annals.1352.022.

Jackson, H.C., Scheideler, M.A., 1996. Behavioural and anticonvulsant effects of Ca2+ channel toxins in DBA/2 mice. Psychopharmacology (Berl) 126, 85–90. https://doi.org/10.1007/bf02246415.

Jacob, R.B., McDougal, O.M., 2010. The M-superfamily of conotoxins: a review. Cell. Mol. Life Sci. https://doi.org/10.1007/s00018-009-0125-0.

Jimenez, E.C., Olivera, B.M., 2010. Divergent M- and O-superfamily peptides from venom of fish-hunting Conus parius. Peptides 31. https://doi.org/10.1016/j.peptides.2010.05.020.

Jimenez, E.C., Watkins, M., Juszczak, L.J., Cruz, L.J., Olivera, B.M., 2001. Contryphans from Conus textile venom ducts. Toxicon. https://doi.org/10.1016/S0041-0101(00)00210-5.

Kaas, Q., Yu, R., Jin, A.H., Dutertre, S., Craik, D.J., 2012. ConoServer: updated content, knowledge, and discovery tools in the conopeptide database. Nucleic Acids Res. https://doi.org/10.1093/nar/gkr886.

Koua, D., Brauer, A., Laht, S., Kaplinski, L., Favreau, P., Remm, M., Lisacek, F., Stöcklin, R., 2012. ConoDictor: a tool for prediction of conopeptide superfamilies. Nucleic Acids Res. https://doi.org/10.1093/nar/gks337.

Kumar, A., Bhardwaj, M., Kaur, H., 2017. Possible role of neuroprotectants and natural products in epilepsy: clinical aspects and mode of action. In: Neuroprotective Natural Products: Clinical Aspects and Mode of Action. https://doi.org/10.1002/9783527803781.ch9.

Lebbe, E.K.M., Peigneur, S., Maiti, M., Devi, P., Ravichandran, S., Lescrinier, E., Ulens, C., Waelkens, E., D'Souza, L., Herdewijn, P., Tytgat, J., 2014. Structure-function elucidation of a new α-Conotoxin, Lo1a, from conus longurionis. J. Biol. Chem. https://doi.org/10.1074/jbc.M114.556175.

Leffler, A.E., Kuryatov, A., Zebroski, H.A., Powell, S.R., Filipenko, P., Hussein, A.K., Gorson, J., Heizmann, A., Lyskov, S., Tsien, R.W., Poget, S.F., Nicke, A., Lindstrom, J., Rudy, B., Bonneau, R., Holford, M., 2017. Discovery of peptide ligands through docking and virtual screening at nicotinic acetylcholine receptor homology models. Proc. Natl. Acad. Sci. U.S.A. https://doi.org/10.1073/pnas.1703952114.

Mathew, M., Subramanian, S., 2014. In vitro screening for anti-cholinesterase and antioxidant activity of methanolic extracts of ayurvedic medicinal plants used for cognitive disorders. PLoS One. https://doi.org/10.1371/journal.pone.0086804.

McDonough, S.I., Boland, L.M., Mintz, I.M., Bean, B.P., 2002. Interactions among toxins that inhibit N-type and P-type calcium channels. J. Gen. Physiol. https://doi.org/10.1085/jgp.20028560.

Norton, R.S., Olivera, B.M., 2006. Conotoxins down under. Toxicon. https://doi.org/10.1016/j.toxicon.2006.07.022.

OECD, 2013. Test No. 236: Fish Embryo Acute Toxicity (FET) Test. OECD Guidel. Test. Chem. Sect. 2, OECD Publ.

Rajabi, S., Ramazani, A., Hamidi, M., Naji, T., 2015. Artemia salina as a model organism in toxicity assessment of nanoparticles. DARU. J. Pharm. Sci. https://doi.org/10.1186/s40199-015-0105-x.

Rajesh, R.P., 2015. Novel M-superfamily and T-superfamily conotoxins and contryphans from the vermivorous snail Conus figulinus. J. Pept. Sci. 21, 29–39. https://doi.org/10.1002/psc.2715.

Rajesh, R.P., Franklin, J.B., Badsha, I., Arjun, P., Jain, R.P., Vignesh, M.S., Kannan, R.R., 2019. Proteome based de novo sequencing of novel conotoxins from marine molluscivorous cone snail Conus amadis and neurological activities of its natural venom in Zebrafish model. Protein Pept. Lett. https://doi.org/10.2174/0929866526666190614144006.

Robinson, S.D., Norton, R.S., 2014. Conotoxin gene superfamilies. Mar. Drugs. https://doi.org/10.3390/md12126058.

Schmidt, 1985. Antiepileptic Drugs. Springer, Berlin, Heidelberg, Springer, Berlin. https://doi.org/http://doi-org-443.webvpn.fjmu.edu.cn/10.1007/978-3-642-69518-6_27.

Sonti, R., Gowd, K.H., Rao, K.N.S., Ragothama, S., Rodriguez, A., Perez, J.J., Balaram, P., 2013. Conformational diversity in contryphans from conus venom: Cis-trans isomerisation and aromatic/proline interactions in the 23-membered ring of a 7-residue peptide disulfide loop. Chem. - A Eur. J. https://doi.org/10.1002/chem.201301722.

Talley, T.T., Olivera, B.M., Han, K.H., Christensen, S.B., Dowell, C., Tsigelny, I., Ho, K.Y., Taylor, P., McIntosh, J.M., 2006. α-conotoxin OmIA is a potent ligand for the acetylcholine-binding protein as well as α3β2 and α7 nicotinic acetylcholine receptors. J. Biol. Chem. https://doi.org/10.1074/jbc.M602969200.

Terlau, H., Olivera, B.M., 2004. Conus venoms: a rich source of novel ion channel-targeted peptides. Physiol. Rev. https://doi.org/10.1152/physrev.00020.2003.