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ARTICLE

Mass Spectrometry-based Sequencing of Venom Peptides (Conotoxins) from Vermivorous Cone Snail, Conus Loroisii: Toxicity of its Natural Venom

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ABSTRACT

Conus loroisii is a marine vermivorous snail found profusely in the southern seas of India. They harbor several toxic peptide components commonly called as 'conotoxins'. In this study, we have identified and sequenced five conotoxins using proteome based tandem mass spectrometry analysis through Data analysis 4.1 software. Among them, we found Lo959 as contryphan which is previously described. All other conotoxins Lo1702, Lo1410, Lo1385 and Lo1686 belong to M-Superfamily conotoxins and novel to C. loroisii. Lo1410 is completely novel to conotoxin research with 3 disulfides and the amino acid sequence is derived as CCSTNCAVCIPCCP. All the identified M-Superfamily conotoxins are sub categorised to mini M2 superfamily conotoxins. Lo1702 and Lo1686 possess C- terminal amidation which is the key feature in conotoxins. Moreover, we have screened the natural venom for the occurrence of toxicity in the zebrafish model and brine shrimp.

1. Introduction

Cone snails form the largest single genera of living marine invertebrates and include various carnivorous predators. Conidae, commonly known as 'cone snails', is a taxonomic family of predatory sea snails and marine gastropod molluscs belonging to the genus Conus established as a family by John Flemming in 1822. The Conidae along with the Turridae and Terebridae form the Superfamily Conidia [1]. These marine gastropod genus Conus (cone snails) found mainly in shallow waters around the world. The genus Conus is rich in diversity found in almost all parts of tropical seas which makes this as important in marine diversity and also play a major role in economy for its beautiful shells. As cone snails possess venom cocktail which majorly act in the prey's neuronal system,

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which primarily serves to capture prey is now a days developed as neurological drugs and tools. A venom peptide from Conus magus Ziconotide is the first approved drug from a marine gastropod which is used in chronic pail alleviation [2]. From that point several other library of conotoxins is continuously studied for their medicinal properties isolated from several conotoxins worldwide [3]. The venom of all classes of conotoxins from various cone snails are highly unique in their structure and function [4]. Despite cone snails are widely used through exploitation for their commercial, medical and scientific value, very less research has been focused towards the conservation of the genus Conus. Based on feeding habits conus is classified in to piscivorous which hunt fish, molluscivorous which feeds mollusk and vermivorous which hunts on worms. Among them piscivorous which hynt fishes is the remarkable group as these venom has potential to target vertebrate system and it normally used to rapidly immobilize fish [5].

Few conotoxins have reached human clinical trials; many are at preclinical stages of development for diverse potential therapeutic applications.

Only very few percentage of the whole conotoxins identified have been studied and developed for medical applications [6]. Cone snails which harbor cocktail of potential conotoxins, serve as a big reservoir for development of several marine drugs. Most of these toxins have been found to exhibit potential bioactivity in a diverse range of mammalian ion channels and receptors associated with pain-signaling pathways.

Typically the conotoxins are of small size, with well-defined stable structure, highly specific towards the target receptors make them attractive and potential pharmacologic agents.

Many conotoxins have shown promise and potential in preclinical models of pain, convulsive disorders, stroke, neuromuscular block, and cardioprotection.

Most conopeptides families studied to date target receptors and ion channels associated with muscle tissue and nervous system.

Some conotoxins which have specific function of alleviation of pain are developed as pain killers [7]. In this study, we have characterized few of the venom peptides using mass spectrometry based studies and biological characterization of the venom using the zebrafish model.

2. Materials and Methods

2.1 Collection and Identification of Cone Snail

Conus loroisii samples were collected from Kasimedu fishing harbor (13.1251° N, 80.2955° E), (Figure 1a), Tamil Nadu, India. A total of 22 alive C. loroisi samples were collected from the trawling fish waste littered in the boat jetty of Kasimedu fishing harbor in the month of January, 2019. The collected cone snail was identified following standard keys [8]. We selected C. loroisii for this study as is not enlisted under endangered or protected species.

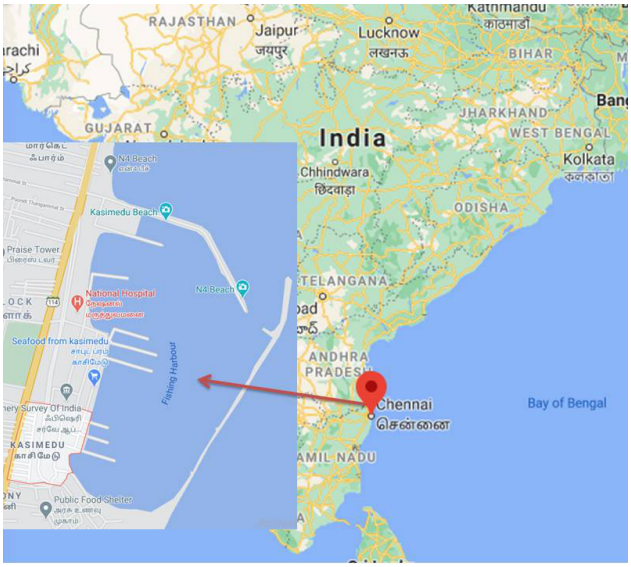

Figure 1a. Map showing sampling location of conus loroisii at Kasimedu fishing harbor

Figure 1b. Shell and venom duct of conus loroisii

2.2 Extraction of Natural Peptides

The venom ducts of C. loroisii specimens were dissected and stored in 50: 50 % HPLC grade acetonitrile: water at the collection site. The samples were transported to the laboratory and the crude natural extract was filtered through Whatman No.1 filter paper and the clean filtrate was concentrated using a rotary vacuum evaporator. The crude extract was then stored at -20 °C till further use [9].

2.3 LC-MS-MS of the Natural Extract

The crude extract of C. loroisii was filtered through a 0.2µM filter and diluted and subsequently, used for mass spectrometric analysis. The mass spectrometric data was acquired in LC-MS-MS (Bruker Daltonics, Bremen, Ger-

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many) to identify the number of peptide components in the crude mixture [9].

2.4 Global Reduction and Alkylation of Natural Venom and Analysis by LC-MS-MS

An aliquot of crude venom extract was treated with reducing agent TCEP (tris (2-carboxyethyl) phosphine) at a final concentration of 20mM and incubated at 37 °C for 1.5 h. After incubation double the concentration (40mM) of alkylating agent NEM (N-Ethyl maleimide) was added and incubated at room temperature for 45min. The reaction mixture was analyzed in LC-MS-MS to identify the number of disulfide-rich conopeptide [9]. Auto MS (n) experiments (CID fragmentation) was performed for the reduced and alkylated peptides. All the above experiments are carried after the peptide components were chromatographically separated based on their polarity using a reverse phase C18 column [9].

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 using Data analysis 4.1 software (Bruker Daltonics, Bremen, Germany).

2.6 Toxicity Testing of Conotoxin on Zebrafish Embryos

Adult and healthy zebrafish were obtained and maintained in a standalone system (Aquaneering, USA) 25-28˚C, under 14–10 h light/dark cycle photoperiod in 50 L housing tank. 6 hpf (hours post fertilization) healthy embryos were screened without any visible physical defects and developmental deformities. The zebrafish embryos were exposed to the Conotoxins (100, 200, 400, 600, 800, 1000µg/mL) for 6-72 hpf and then assessed for toxicity. The embryos were kept in sterile 24-well plates with 10 embryos per well-containing 1mL of the solution. The mortality and developmental deformities of the zebrafish larvae were recorded at 72 hpf (hour post-fertilization) [10, 11, 12].

2.7 Toxicity Assay of Crude Venom on Brine Shrimp

Artemia salina (brine shrimp) eggs were purchased from Ocean Star International O.S.I, USA. Dried cysts were placed in a separating funnel containing natural seawater. After 24-28 hours of incubation and strong aeration at room temperature (30-35º C) under continuous light supply, the nauplii (larvae) were hatched. The larvae were separated using a coffee filter and rinsed well in sterile seawater. The nauplii were then suspended in sterile seawater.

The evaluation of cytotoxicity on the brine shrimp embryo was performed by adding 10 larvae in each well containing 100 µl of sterile seawater. The test was performed in triplicates. The larvae were exposed to different concentration of drug (5, 10, 20, 40, 80, 160, 320µg/mL). After 24 hours of incubation at room temperature, the number of nauplii surviving was checked under a stereo microscope. The control well consisted of only nauplii and sterile seawater. The percentage of deaths was calculated by comparing the test and control wells. The percentage of lethality was calculated by means of Abbott's formula: % Lethality= ((Test-Control)/ Control) * 100 [13].

2.8 Acetylcholinesterase Quantification

The acetylcholinesterase quantification assay was performed in 96-well plate to which different concentrations of conotoxin was added and made up to 250µl with PB buffer (pH 7) for 10 mins at room temperature. After incubation, the reaction was stopped by addition of Tris HCl (pH 8). Then 10 µl of 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) was added to each well and absorbance was taken at 412 nm. 2 µl of acetyl thiocholine iodide was added to each well to measure the hydrolysis of ATCI by formation of yellow reaction of DTNP with thiocholine and the reaction was measured by 412 nm in multi-mode plate reader (PerkinElmer) [10, 11, 12].

3. Results

3.1 Identification, Dissection and Isolation of Crude Venom from Conus Loroisii

The cone snail was identified following standard keys as Conus loroisii (Figure 1). The venom duct was dissected from the live specimen (Figure 1). Venom is extracted from the venom duct by following the protocol as given in materials and methods and stored in the deep freezer (-20°C) for preservation until further use. The presence of protein in the crude venom extract was confirmed using NanoDrop (Thermo Scientific™ NanoDrop 2000 and 2000c) and its concentration was 3 mg/ml.

3.2 Sequencing of Venom Peptides

The total ion chromatogram of C. loroisii venom yielded a spectrum showing various peptide components. Among them, we identified and sequenced five peptides Lo959, Lo1702, Lo1686, Lo1410 and Lo1385 respectively. The fragmented spectrum of Lo959, Lo1702, Lo1686, Lo1410, and Lo1385 with daughter ions which are exclusively used for deriving the peptide sequences are shown in figure 2-6 respectively. The individual masses

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of both the 'b' and 'y' series where determined manually by de-novo sequencing and are presented in the tables (2-6). Complete analysis of the daughter ions yielded the sequences Lo959- GCPWDPWC-NH2, Lo1702-CCSQD-CRVCIOCCPY-NH2, Lo1686- CCSQDCRVCIPC-CPY-NH2, Lo1410- CCSTNCAVCIPCCP, and Lo1385-CCKVLCESCTPCC. ExceptLo959 all other peptide toxins are novel to be reported from C. loroisii. Peptide Lo959 belongs to the contryphan family. The other four sequences Lo1702, Lo1686, Lo1410 and Lo1385 belong to the M superfamily of conotoxins. The MALDI-TOF spectrum of two conotoxins Lo1686 and Lo1702, after alkylation, was determined and it indicates the post translational modification that takes place between Lo1686 and Lo1702. A difference of 16 Dalton between both the peptides is noted.

Table 1. Conopeptides of conus loroisii

Sl. No.	Gene Superfamily	Name	Sequence	Mass	Notes
1	Contryphan	Lo959	GCPWDPWC-NH2	959	This Work and Gowd,K.H. et al. (2005) Sabareesh,V. et al. (2006) Sonti,R. et al. (2013)
2	M-superfamily	Lo1702	CCSQDCRVCIOCCPY-NH2	1701	This Work and Rajesh 2014
3	M-superfamily	Lo1410	CCSTNCAVCIPCCP	1409	Novel to conotoxins history
4	M-superfamily	Lo1385	CCKVLCESCTPCC	1384	Peng C et al., 2016
5	M-superfamily	Lo1686	CCSQDCRVCIPCCPY-NH2	1685	Conticello et al 2001 and Rajesh 2014

Table 2. Determined m/z values of b and y ions for the sequence Lo959

b ions	Theoretical Mass	Founded Mass	y ions	Theoretical Mass	Founded Mass
b1	59		y1	246	
b2	287		y2	432	
b3	384		y3	529.35	529.35
b4	570	570	y4	644.3	644.3
b5	685	685	y5	830	
b6	781		y6	927.47	927.47
b7	967.47	967.47	y7	1155	

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Table 3. Determined m/z values of b and y ions for the sequence Lo1702

b ions	Theoretical Mass	Founded Mass	y ions	Theoretical Mass	Founded Mass
b1	229		y1	182	
b2	457.3	457.3	y2	279	
b3	544.1		y3	507.2	507.2
b4	672.2		y4	735.1	735.1
b5	787.4	787.4	y5	848.3	848.3
b6	1015.3	1015.3	y6	961.4	961.4
b7	1171.3		y7	1189.5	1189.5
b8	1270.5	1270.5	y8	1104	1104
b9	1498.5	1498.5	y9	1445.4	1445.4
b10	1611.6		y10	1672.7	1672.7
b11	1724.9	1724.9	y11	1787.6	
b12	1952.6	1952.6	y12	1915.7	
b13	2180.7	2180.7	y13	2002.5	2002.5
b14	2277.6		y14	2230.5	

Table 4. Determined m/z values of b and y ions for the sequence Lo1410

b ions	Theoretical Mass	Founded Mass	y ions	Theoretical Mass	Founded Mass
b1	229		y1	116	
b2	457	457	y2	344	344
b3	544	544	y3	572	572
b4	645	645	y4	669	669
b5	759.4	759.4	y5	782	782
b6	987.3	987.3	y6	1010	1010
b7	1058.4	1058.4	y7	1109	1109
b8	1157.4	1157.4	y8	1180	1180
b9	1385.5	1385.5	y9	1408	1408
b10	1498	1498	y10	1522	1522
b11	1595.6	1595.6	y11	1623	
b12	1823.7	1823.7	y12	1710	
b13	2051.7		y13	1938	

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Table 5. Determined m/z values of b and y ions for the sequence Lo1384

b ions	Theoretical Mass	Founded Mass	y ions	Theoretical Mass	Founded Mass
b1	229		y1	475.2	475.2
b2	457	457	y2	572.3	572.3
b3	585	585	y3	673.3	673.3
b4	684.3	684.3	y4	901	901
b5	797.5	797.5	y5	988.4	988.4
b6	1025.5	1025.5	y6	1117	
b7	1154	1154	y7	1345.5	1345.5
b8	1241.6	1241.6	y8	1458	1458
b9	1469.6	1469.6	y9	1557	
b10	1570.7	1570.7	y10	1656	
b11	1667.7	1667.7	y11	1784	
b12	1895.7	1895.7	y12	2012	

Table 6. Determined m/z values of b and y ions for the sequence Lo1686

b ions	Theoretical Mass	Founded Mass	y ions	Theoretical Mass	Founded Mass
b1	229		y1	182	
b2	457	457	y2	279	
b3	544		y3	507	507
b4	672	672	y4	735	
b5	787	787	y5	832	832
b6	1015		y6	945	945
b7	1171	1171	y7	1173	1173
b8	1270	1270	y8	1272	1272
b9	1498	1498	y9	1428	1428
b10	1611	1611	y10	1656	1656
b11	1708	1708	y11	1771	1771
b12	1936	1936	y12	1899	
b13	2164	2164	y13	1986	
b14	2067		y14	2214	

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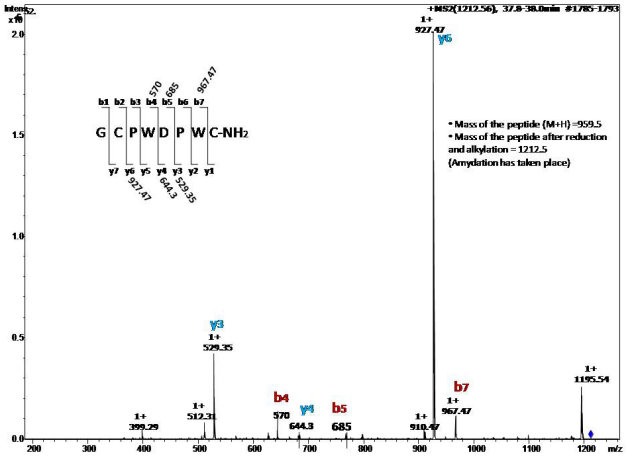

Figure 2. Spectrum showing sequence of Lo959 obtained from de novo tandem Mass Spectrometry

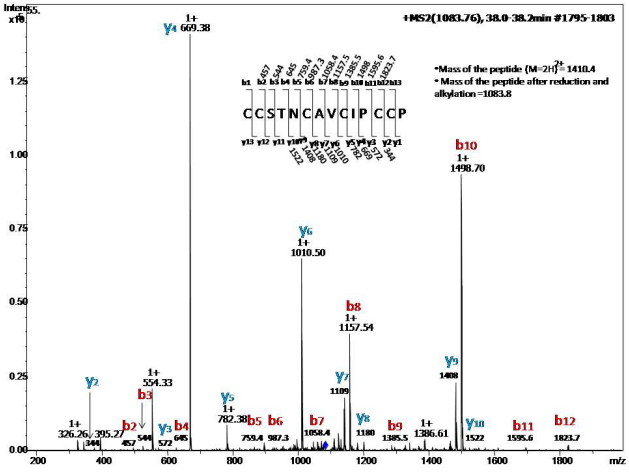

Figure 5. Spectrum showing sequence of Lo 1410 obtained from de novo tandem Mass Spectrometry

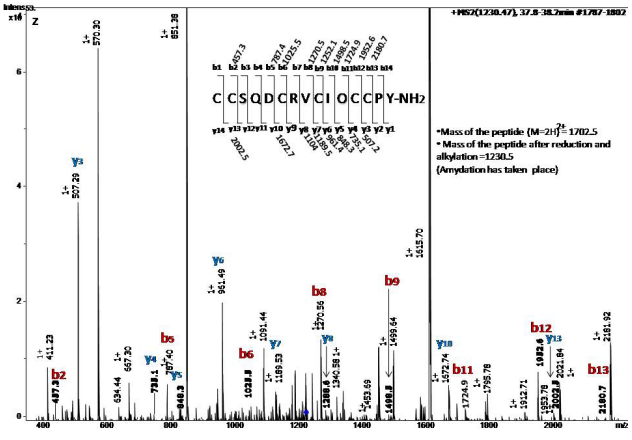

Figure 3. Spectrum showing sequence of Lo 1702 obtained from de novo tandem Mass Spectrometry (O= 4-trans-hydroxyproline: Hyp)

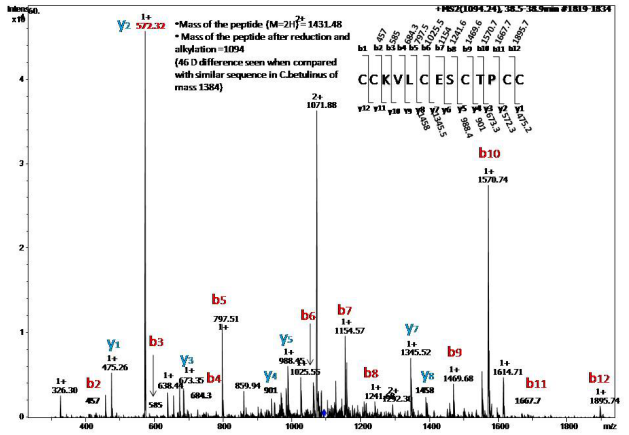

Figure 6. Spectrum showing sequence of Lo 1385 obtained from de novo tandem Mass Spectrometry

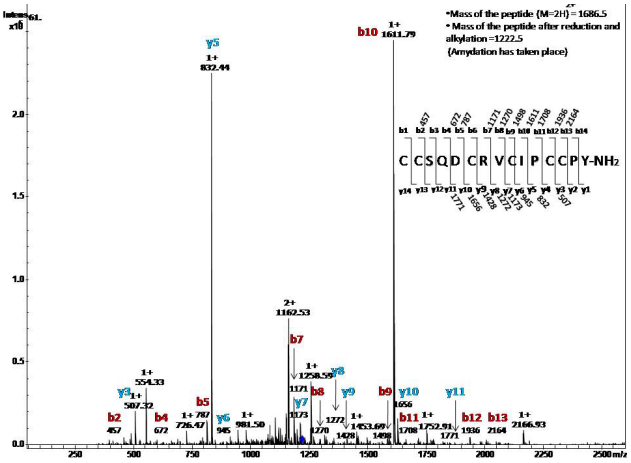

Figure 4. Spectrum showing sequence of Lo 1686 obtained from de novo tandem Mass Spectrometry

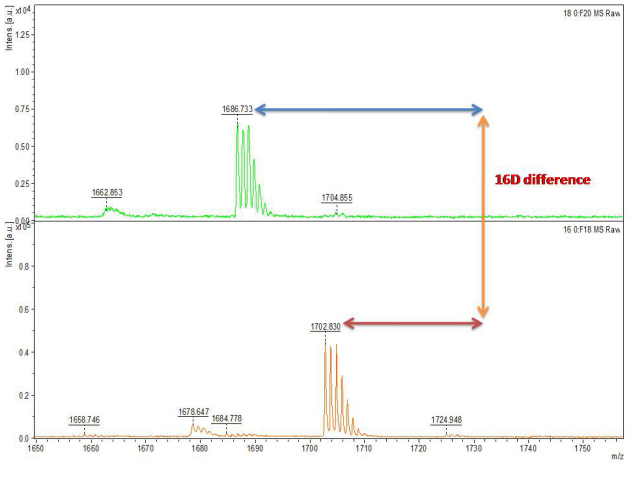

Figure 7. Spectrum showing sequence of Lo 1686 and Lo1702 with 16D more indicating the presence of hydroxyl proline in Lo1702

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3.3 Estimation of Acetylcholinesterase Activity

The venom sample was tested for the presence of acetylcholinesterase enzyme using the Ellman's assay (as described in detail in materials and methods). We observed that the venom sample of Conus loroisii contains the enzyme acetylcholinesterase as shown in Figure 8.

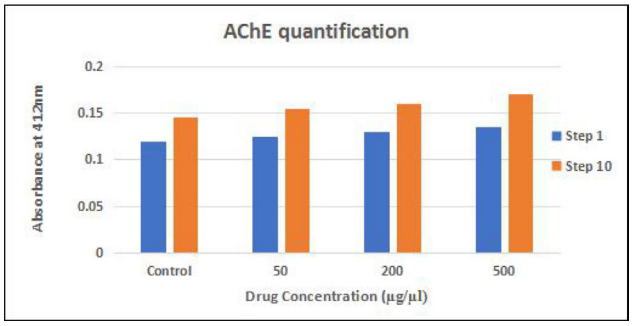

Figure 8. Quantification of the enzyme acetylcholinesterase

3.4 Toxicity Testing of Conotoxin on Zebrafish Embryos

Zebrafish embryos were subjected to varying concentrations of conotoxins for 72 h.p.f based on OECD guidelines to determine the LC50 value. It was observed that death was initiated at a concentration of 400 µg/µl at 24 hrs. Between 50-65 hours after treatment, various deformities were observed in higher concentrations such as pericardial edema, blood clot, yolk sac edema, spinal kyphosis, etc. 100% death was observed in concentration 800 µg and above (Figure 9&10). The LC50 was determined to be 700 µg/µl with 50% death.

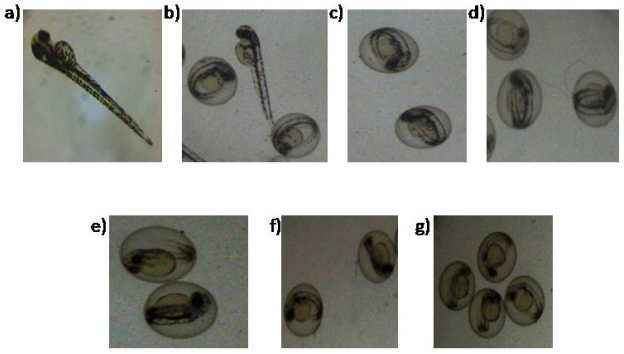

Figure 9. Toxicity assessment of zebrafish embryo at 24 hours post treatment (hpt) a) Control; b) 100µg; c) 200µg; d) 400µg; e) 600µg; f) 800µg; g) 1000µg

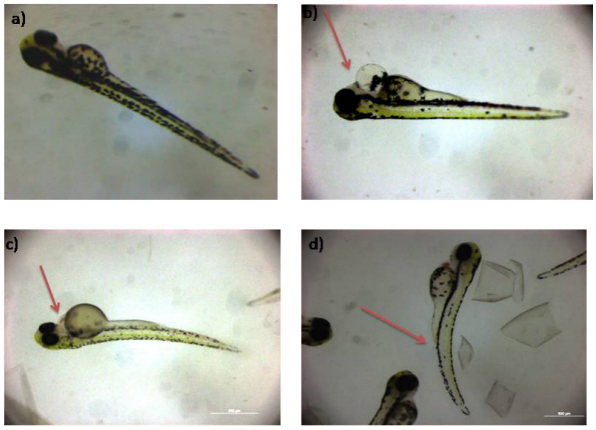

Figure 10. Different deformities observed in the zebrafish larvae during toxicity assessment vs control. a)Control b) Pericardial edema; c) Hemorrhage; d) Spinal kyphosis (Deformities seen at 48 & 72 hrs.)

3.5 Toxicity Assay of Crude Venom on Brine Shrimp

Brine shrimp toxicity was performed on 24hrs nauplii at varying concentrations of conotoxins for 24 h.p.f as mentioned in materials and methods. It was observed that death was initiated at a concentration of 80 µg/µl. The LC50 value was found to be 320µg/µl at 24 hours.

4. Discussion

Conus loroisii is abundantly distributed along the coast of the Southeastern state of Tamil Nadu in India. Despite its abundance, the venom components of this species have not been studied extensively. The de-novo sequencing done with the help of mass spectrometry has led to yield five peptide sequences. Among these peptides, one was found to be a contryphan and the other four belong to the M-superfamily conotoxin. One contryphan (Lo959) and two peptides from the M-superfamily Lo1385 [13] and Lo1686 [9] identified in this study have been identified earlier in other cone snails (Conus betulinus and Conus figulinus).Two peptides from the M-superfamily (Lo1702 and Lo1410) are novel toxins to the conopeptide library. Several conotoxins with from Conus amadis displayed almost similar sequence similarity with the peptide sequence of the present study (Vijayasarathy et al., 2019). Among the 5 conotoxins identified 3 peptides possess C terminal amidation which is the major hallmark of conotoxins. Lo1702 possess the hydroxylation of proline which is another major post-translational modification which also found in Conus figulinus as reported earlier [9]. The LC50 value was determined to be 700 µg/µl by zebrafish embryo toxicity and 320 µg/µl by brine shrimp toxicity. The conotoxin lacked acetylcholinesterase inhibitory activity but helps in increasing the activity of acetylcholinesterase. Based on the preliminary evidence for the occurrence of

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novel conotoxins and the toxicity studies, if this study is extended further and trace out and separate the toxigenic molecules, which would provide a lead for discovering biologically active molecules.

5. Conclusion

In this study the vermivorous cone snail Conus loroisi which is less explored for its venom based peptidomic research is analysed using mass spectrometry based de-novo sequencing and tested for its toxicity in zebrafish model and brine shrimp. We found 5 peptides and derived their aminoacid sequences, which belong to single disulfide contryphan group and 3 disulfide bonded M-superfamily conotoxins. Among them Lo1410 is completely novel to conotoxin research. This preliminary research paved the way to continue research in biological and structural characterisation of individual peptide molecules which would possibly yield us with drug leads against various human ailments in near future.

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Abbreviations

LC-MS-MS, Liquid Chromatography-Mass Spectrometry- Mass Spectrometry;

TCEP, tris (2-carboxyethyl) phosphine [Pierce Scientific, United States];

NEM, N-Ethylmaleimide [Sigma-Aldrich, United States];

CID, Collision-induced dissociation;

MS, Mass Spectrometry;

hpf, Hours post fertilization;

AChE, Acetylcholinesterase;

dpf, days post fertilization;

E3, Embryo medium;

DMSO, Dimethyl sulfoxide;

TIC, Total Ion Chromatogram;

Da, Dalton

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