ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Comparative Characterization of Toxins Isolated from *Conus loroisii* Kiener, 1845 and *Conus amadis* Gmelin, 1971.

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ABSTRACT

Cone snails are marine predators, which can synthesize potent venom known as conotoxins, to paralyze preys and deter aggressors. The main objective of this study was to compare the toxicity of venom from these species of *Conus* loroisii (vermivorous) and *Conus amadis* (molluscivorous) which have different feeding habitats. The protein content of crude toxin extract of *C. amadis* was less when compared to *C. loroisii* and was found to be 42.97 µg/mL and 238.59 µg/mL. The hemolytic activity of crude extracts of *C. amadis* showed higher level of toxicity than the crude extract of *C. loroisii.* The SDS - PAGE of crude toxin extract of these two species showed the peptides in the range of 20-60 kDa. The FTIR spectra of these two cone snails showed similar 13 major peaks. These two cone snails showed marked difference in toxicity, protein content and molecular weight, but showed the presence of similar functional groups in crude toxin extracts as evidenced from FTIR spectra. The different feeding habits of these snails could be attributed to the differences observed in the characteristics of the toxin.

Keywords: *Conus amadis, Conus loroisii,* conotoxins, hemolytic activity, FTIR spectrum.

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ISSN: 0975-8585

INTRODUCTION

The sea covers about the 71 % of the total earth's surface and is a vast area of living and non-living things. The marine organisms consist of a very rich source of proteins, polysaccharides, lipids and glycoproteins which show many diverse biological activities. The animal kingdom includes more than 100,000 venomous species spread through major phyla such as chordates (reptiles, fishes, amphibians and mammals), echinoderms (starfishes, seaurchins), molluscs (cone snails, octopuses), annelids (leeches), nemertines, arthropods (arachinids, insects, myriapods) and cnidarians (sea anemones, jellyfishes, corals).

Venoms are highly concentrated biofluids that provide animals with a variety of advantages, including an ability to subdue and digest prey efficiently and to defense themselves against predators. Venom is a unique cocktail of hundreds of different peptides and proteins, making venoms a source of millions of bioactives naturally tailored act on a myriad exogenous targets, ion channels, receptors and enzymes. Venoms thus constitute an unprecedented source of pharmacological tools, drug candidates and diagnostic reagents with at least five drugs derived from venom components that are already available in the market and dozens of related active pharmaceutical products that are currently being tested in preclinical or clinical trials [4].

Among venomous animals, the beautiful marine snails hold a peculiar place with their complex and efficient venomous system. Venomous marine gastropods are traditionally divided into three families: The Conidae (800 species), Turridae (15-20,000 mostly undescribed species) and Terebridae (300 species) families representing the Toxoglossate group conventionally called Conoidae family [10].

All family represents are characterized by the presence of venom apparatus. These animals paralyze their prey with specialized mouth that injects venom through sophisticated radular teeth. They are strictly carnivores that can be put into three categories, namely vermivorous, molluscivorous and piscivorous feeding on annelids, molluscs and fishes respectively. They can be found in any marine environments, preferably in the tropical waters. Venom mostly comprises linear peptides and strongly folded mini-proteins featured by a relatively small size (800-6000 Da). These are usually disulphide-constrained molecules and are commonly termed as conopeptides (zero or one disulphide bridge) or conotoxins (two to five disulphide bridges) [10].

The physical manifestations of cone shell stings are well documented concerning human fatalities, and can be traced back to reports from the mid-1850s [1,6]. Although humans are rarely stung by *Conus*, the event can be deadly as evidenced by an approximate fatality rate of 70% [12].

Access to novel screening methods and defined biological targets may result in increased pharmacological classification of conotoxins. Yet, this is one of the many bottlenecks faced in conotoxin discovery, compounded by the limited access to the natural resource.

Conus loroisii is a vermivorous species, feeding on glycerid and capitellid polychaetes [8]. The alimentary track of *C. amadis* (molluscivorous) was reported to contain remains of columbellid snail, probably *Pyrene testudinaria* Link, 1807 [8], observed this species feeds on neritid gastropods. The tooth configuration of *Conus amadis* was similar to *C. araneosus*, but the structure of the short blade distinguishes from other *Conus sp*.

In the present study, an attempt has been made for a comparative study of the biochemical activity of toxin with respect to feeding habits. In addition, determination of molecular weight through SDS-PAGE and structural analysis through Fourier Transform Infra-Red Spectrum of toxins produced by *Conus lorosii* and *Conus amadis*.

MATERIALS AND METHODS

The live specimen *Conus loroisii* and *Conus amadis* are commonly distributed along the Parangipettai coastal region, and Mudasalodai (Lat. 11°29'N; Long. 79°44'E), Tamil Nadu, South east coast of India and they were collected for the purpose of the present study.

Extraction of Crude Toxin

The crude extract was prepared from the venom duct as described by Cruz *et al.* [2]. The shells were carefully broken with the help of hammer and animals were removed gently. After dissection, the venom duct of each animal was removed. The anterior end of the duct was cut as its junction with pharynx and posterior end of the duct was cut at its junction with venom bulb. The pooled venom ducts were suspended in 50 ml of 1.1% acetic acid and was homogenized with manual tissue homogenizer. The mixture was centrifuged using 17,200 rpm for 10 minutes at 4°C. The supernatant was retained and lyophilized. The lyophilized samples were stored at -20°C.

Protein Estimation

The protein content of lyophilized samples was estimated by the method of Lowry *et al.* [9] using BSA as standard.

Hemolytic Activity

Preparation of Erythrocyte Suspension

The haemolytic activity of crude venom on goat blood was tested by micro haemolytic method [13]. After the blood was centrifuged at 5,000 rpm for 5 minutes, the supernatant was discarded and the pellet was suspended in normal saline (pH 7.4). The procedure was repeated thrice and 1% erythrocyte suspension was prepared by adding 99 mL normal saline to 1 mL of packed red blood cell (RBC).

Hemolytic Assay by Microtitre Plate

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

Determination of Molecular Weight

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) which utilized 5% stacking gel and 10% resolving polyacrylamide gels was carried out to estimate the protocol described [12]. The protein was analyzed by SDS- PAGE. The molecular marker of range 14.3 kDa to 97.4 kDa was used as a reference. Ten microlitre of the marker was loaded in the right well as marker and then lyophilized proteins were loaded in subsequently wells. Upon, completion of electrophoresis, the gel was washed gently with distilled water to remove excess SDS, stained in Coomassie Brilliant Blue R-250 (Coomassie brilliant blue R-250, 1.25g methanol, 227mL; glacial acetic acid 46mL distilled water to complete a volume of 500mL) for two hours at room temperature and de-stained (methanol, 7 mL; glacial acetic acid, 7 mL; and distilled water to reach 100 mL) for 48 hours. Protein bands were visualized as dark blue bands on a light blue background. Samples were denatured by boiling in buffer containing SDS and β – mercaptoethanol prior to loading onto the gel. Following electrophoresis at 30 mA for four hours, gels were stained by Coommassie blue staining. The bands were observed under gel documentation system.

Fourier Transform – Infra Red Spectral Analysis

FT-IR spectroscopy of powdered samples of extracted crude toxins relied on a Bio-Rad FTIR–40 model, USA. Sample (10 mg) was mixed with 100 mg of dried potassium bromide (KBr) and compressed further to prepare as a salt disc (10 mm in diameter) for reading the spectrum.

ISSN: 0975-8585

RESULTS AND DISCUSSION

Yield of Lyophilized Crude Venom

The total amount of 250 mg of lyophilized crude toxin was obtained from about 15 cones of *C. loroisii*. The total amount of 340 mg of lyophilized crude toxin was obtained from the same number of cones of *C. amadis* (Table: 1).

Table: 1 shows the yield of lyophilized crude toxin from *C. loroisii* and *C. amadis*.

Species	No: of samples	Yield of lyophilized crude toxin (mg)	Yield obtained/sample (mg)
C. loroisii	15	250	16.67
C. amadis	15	340	22.67

Protein Estimation

The total protein content in the lyophilized crude toxin of *C. loroisii* was found to be 42.97 µg/ml and in *C. amadis* was found to be 238.59 µg/ml.

Hemolytic Activity

The results of hemolytic test revealed hemolytic effect in the first five wells of goat blood with the hemolytic effect of 32 HU/gm in *C. loroisii* whereas, in *C. amadis,* first six wells of goat blood with the hemolytic effect of 64 HU/gm (Fig. 4). (One HU is defined as the quantity of protein catalyzing lysis of 50% of red blood cells present).

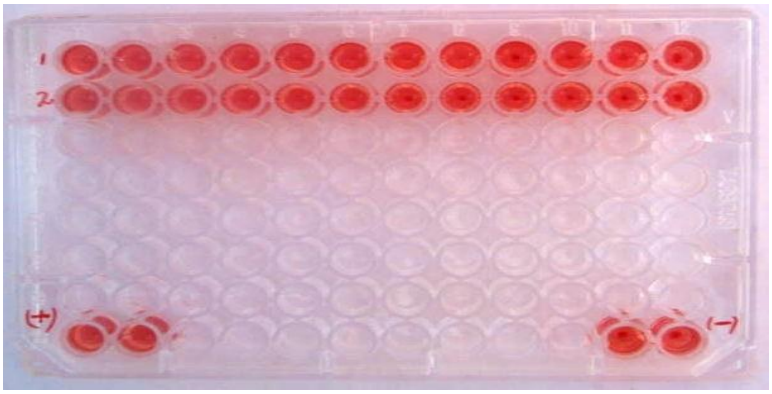

Figure 1: Showing hemolytic activity of toxin from *C. loroisii* (row 1) and *C. amadis* (row 2)

Molecular Weight Determination

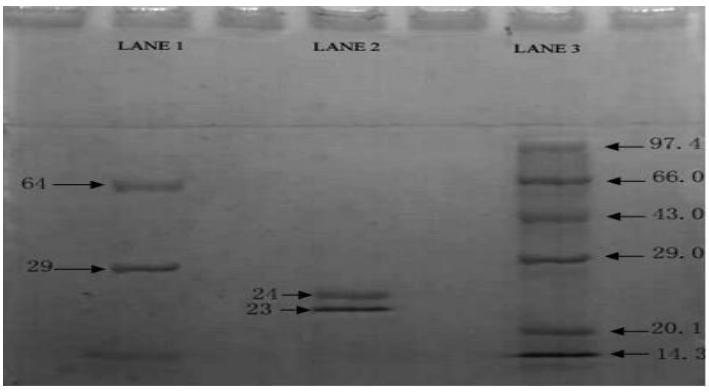

Figure 2: SDS-PAGE profiles of toxin from *C. loroisii* and *C. amadis* (Lane 1- *C. loroisii*, lane 2- *C. amadis* and lane 3- Protein marker)

ISSN: 0975-8585

In the SDS-PAGE, the crude protein of *C. loroisii* and *C. amadis* showed distinct bands each lying in between 20.1 to 66.0 kDa molecular weight. The toxin from *C. loroisii* showed two bands of molecular wieght 64 kDa and 29 kDa. The molecular weight of two bands from *C. amadis* was found to be 24 kDa and 23 kDa through gel documentation system (Fig. 2).

Fourier Transform-Infra Red Spectra Analysis

The FT-IR spectrum of crude toxin was measured between 400-4000 spectral ranges. A total 13 peaks were observed for *C. loroisii* (Fig. 3) and *C. amadis* (Fig. 4). The list of functional groups assigned for the wave number was presented in Table 2.

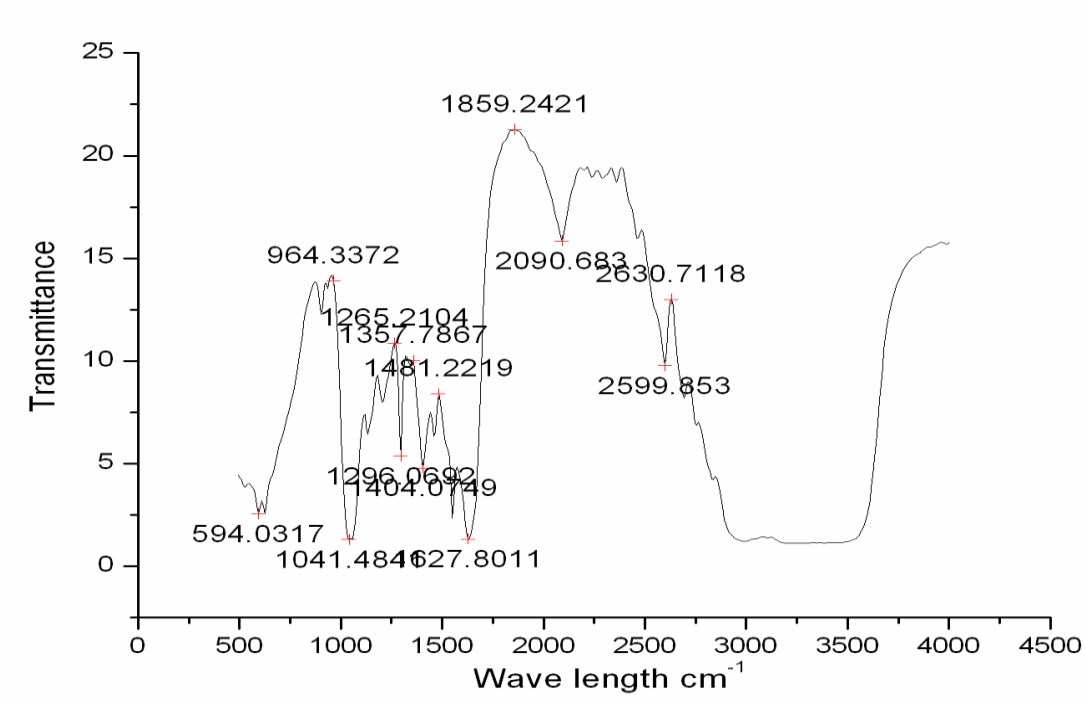

Figure 3: Showing FTIR spectrum of toxin from *C. loroisii*

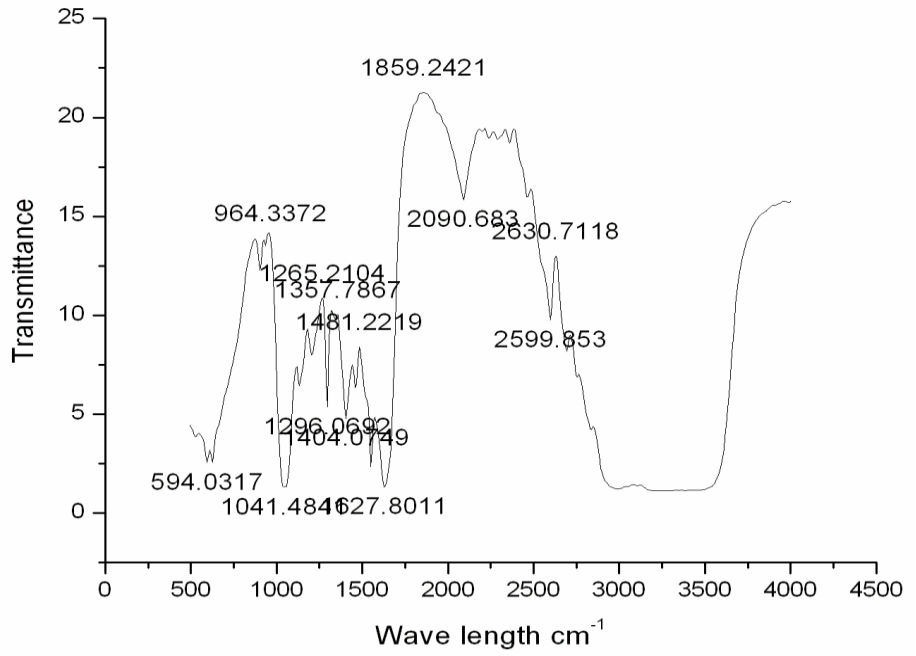

Figure 4: Showing FTIR spectrum of toxin from *C. amadis*

ISSN: 0975-8585

Table 2: Similar Functional groups assigned for *C. amadis* and *C. loroisii* after analysis through FTIR.

Sl.no:	wave length (cm^{-1})	Range (cm^{-1})	Intensity	Group and Class	Assignment and Remarks
1	594.0317	537-606	Strong	Amide VI, N-C=O in amides	out of plane C=O bending, N-C=O bend
2	964.3372	960-980	very strong	CH=CH- in trans disubstituted alkenes	=CH out of plane deformation
3	1041.484	1080-1040	Strong	SO_3H in sulfonic acids	SO_3 sym stretch
4	1265.2104	1229-1301	Strong	Amide III, aromatic amines	CN stretching, NH bending
5	1296.0692	1229-1301	very strong	Amide III, SO_2 in sulfones	CN stretching, NH bending, SO_2 antisym stretch
6	1357.7867	1360-1335	very strong	SO_2 in sulfones	SO_2 antisym stretch
7	1404.0749	1420-1400	Medium	Amide III C-N in primary amides	C-N Stretch
8	1481.2219	1480-1575	medium-strong	Amide II N=N-O in azoxy compounds	CN stretching, NH bending, N=N-O antisym stretch
9	1627.8011	1600-1690	Strong	Amide I, NH_3^+ in aminoacids	C=O stretching, NH_3 deformation
10	1859.2421	1870-1830	Strong	C=O in β lactones	C=O stetch
11	2090.683	2160-2080	Medium	$N=N^+=N^-$ in azides	N=N=N antisym stretch
12	2599.853	2600-2540	Weak	-SH in alkyl mercaptens	S-H stretch; strong in Raman
13	2630.7118	2750-2350	Broad	$-NH_3^+$ in amine hydrohalides	NH stretching modes

In this study, the venom of predatory cone snails consist of a diverse library of peptides, with multiple disulfide bonded conotoxins being the best-studied constituents. It is estimated that venoms of marine cone snails (genus *Conus*) contain more than 100 000 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. The present effort was an underway to investigate the crude venom of *C. amadis* and *C. loroisii* venom.

The total protein content in the crude toxin of *C. loroisii* was lesser when compared to *C. amadis*. Hence the higher level of protein content in *C. amadis* than in *C. loroisii* indicated that *C. amadis* might have high toxicity since toxin comprises majorly proteins. Furthermore, the toxin of *C. loroisii* and *C. amadis* showed hemolytic activity which was found to be higher in *C. amadis* when compared to *C. loroisii*. The quantity of crude toxin was also found to be present in higher level in *C. amadis* than in *C. loroisii* (Table 1). In the present study, the hemolytic activity of *C. loroisii* showed lesser activity when compared to *C. amadis* against goat blood. The high hemolytic activity values could be attributed to the different feeding habits of both species as well as their protein contents.

In SDS-PAGE analysis, two distinct bands were observed in the venom extract of *C. loroissi* and the molecular weight of protein was found to be 64, and 29KDa, whereas the venom extract of *C. amadis* revealed two distinct bands with molecular weight of 24, and 23KDa. These variations in the molecular weight of venom extracts could be due to disulphide bonding and hydrophobic amino acids present in their toxin and also due to their feeding habits. The results of the present study revealed that there may be some similarity between structural and functional activity of low molecular weight proteins found in both Conus species.

The FT-IR results of toxins from *C. loroissi* and *C. amadis* showed similar 13 major peaks at wavelength of 594.0317 cm^{-1}, 964.3372 cm^{-1}, 1041.4841 cm^{-1}, 1265.2104 cm^{-1}, 1296.0692 cm^{-1}, 1357.7867 cm^{-1}, 1404.0749 cm^{-1}, 1481.2219 cm^{-1}, 1627.8011 cm^{-1}, 1859.2421 cm^{-1}, 2090.683 cm^{-1}, 2599.853 cm^{-1} and 2630.7118 cm^{-1}. From the similarity between the FT-IR peaks, it could be noted that similar functional groups and disulphide bonding between the cysteine residues of peptides from both species, *C. loroisii C. amadis* were prevalent. The qualitative aspect of infrared spectroscopy is one of the most powerful attributes of this diverse and versatile analytical technique. The frequency of 594 is responsible for amide VI band, strongly influence the chemistry and the relativity of the nitrogen and the N-C=O group. Frequency 964 is responsible for trans-distributed alkenes forms the =CH out of plane deformation. Frequency 1041 and 1404 are amide III bands which are

medium spectral activity and are having C-N in primary amides. Group frequency 1265 and 1296 are responsible for the amide III having aromatic amines shows strong spectral activity in CN stretch and NH bend. Frequency 1357 corresponds to the presence of SO_2 in sulfones shows a very strong spectral activity in SO_2 antisym stretch in the sample. Frequency 2090 form the $N=N^+=N^-$ in azides shows medium spectral activity in N=N=N antisym stretch. Group frequencies including 2630,2599,1859,1627 responsible for presence of -SH in alkyl mercaptens, -SH in alkyl mercaptens, C=O in β lactones shows medium to broad spectral activity.

CONCLUSION

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

"Poisons kill the poison" the famous proverb is the basis for researchers in finding the biomedical and metabolites from living organisms. Sea has got plenty of metabolites and other resources in living and dead form.. More research works for metabolites would result in further novel metabolites with pharmacodynamic properties. Presently, drug derivatives of conotoxins testify the important contribution to medicine. Furthermore, knowledge of venoms of conus species could facilitate peptides with novel or particular high bioactivity.

ACKNOWLEDGEMENT

The authors are thankful to the authorities of CLRI, Chennai Annamalai University, Chidambaram and VIT University, Vellore for the facilities provided to carry out this work. One of the authors (AJ) is thankful to the Department of Biotechnology, Government of India for the financial support.

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