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Isolation, Identification and in-Silico Characterization of Bioactive Peptide from the Venom Sac of *Conus inscriptus*

Vidyalatha Jakkana[1]* and Shanti Prabha Yamala[2]

[1]Department of Biology, Zilla Parishad High School, Vaddadi, Visakhapatnam, Andhra Pradesh, India;
[2]Department of Zoology, Dr. V.S. Krishana Govt. Degree & PG College (Autonomous), Visakhapatnam, Andhra Pradesh, India

E-mail/Orcid Id:

VJ, vidyashok5511@gmail.com, http://orcid.org/0009-0003-3397-7846;
SPY, shantiprabhay@gmail.com, http://orcid.org/0009-0006-9198-5969

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Abstract: Nature has been the source of new medications since ancient times, and at least half of all commercialised pharmaceuticals have been made from renewable resources to treat various illnesses, including pain, inflammation, and infection. In recent years, interest has risen in bioprospecting of new bioactive peptides, especially for medical and health-related applications. Therefore, the production of functional meals is starting to get recognition for its potential to enhance quality of life when combined with a healthy lifestyle. In view of this, the current work aimed to isolate, purify, and identify bioactive peptides from *Conus inscriptus* venom sac protein extracts, with an emphasis on antioxidant and anti-inflammatory properties. The findings reveal that the DPPH radical scavenging activity, albumin denaturation, and HRBC membrane stabilisation inhibition activities of *Conus inscriptus* venom sac crude protein extracts are 23.76±2.4, 66.23±3.1, and 57.32±2.6% respectively. Secondary screening of purified protein fractions obtained through sephadex G100 gel filtration exhibited increased anti-inflammatory efficacy from fractions 19 to 23. Electrophoresis and PMF analysis revealed that the purified anti-inflammatory peptide was homogenous with a molecular weight of 12 KDa, and it was identified as a conotoxin MI15b precursor. The 3D model of conotoxin exhibits the highest similarity with template c6nk9A with a confidence of 54.1%, and its function was predicted as a potassium ion channel inhibitor. The findings of the Insilco characterisation of the conotoxin MI15b precursor concluded that this protein might serve as an anti-inflammatory agent and may be responsible for therapeutic actions in the medical management of many inflammatory-related disorders.

Introduction

Nature has historically served as the source of novel medication, with a minimum of 50% of all commercialised therapeutics being derived from renewable resources or synthesised using natural compounds as prototypes or precursors. Early civilisations have utilised natural remedies to cure infection, inflammation, pain and a range of other health conditions since ancient times (Sarkar et al., 2016, 2024; Ghosh et al., 2022). Even now, natural remedies are the only therapies accessible in many regions of the world (Bhattacharjee, 2021; Kar et al., 2022). People currently desire diets that are nutritious and have the potential to enhance their health, extend their lifespan and lower their risk of disease occurrence (Banerjee et al., 2014; De et al., 2023). As a consequence, the association between nutrition and wellness has grown significantly in the past few years. Food is now recognised as a source of dietary vitamins, minerals, and bioactive molecules that promote human health. Thus, functional foods are becoming identified as having the potential to increase quality of life when combined with a healthy lifestyle. Furthermore, individuals who have a fascination with organic foods that are high in vitamins, minerals, and other bioactive

*Corresponding Author: vidyashok5511@gmail.com

metabolites such as polyphenols (Dziki et al., 2019), anthocyanins (Szymanowska and Baraniak, 2019), carotenoids (Wang et al., 2018), and peptides (Zaky et al., 2020) reflect their knowledge of the impact of diet on health. Some bioactive compounds are proteinaceous and contain proteins, peptides, and amino acids. Fortunately, proteins have been ignored in the search for bioactive properties for a long time, but in recent years, extensive research has focused on the structural, compositional and sequential features of bioactive peptides (Lordan et al., 2011).

Bioactive peptides are specific fragments of proteins that are capable of a wide range of possible therapeutic uses within the human system. The majority of bioactive peptides vary in length between 2 and 20 amino acid residues and their molecular weight ranges between 0.4 and 2 KDa. The therapeutic effects of bioactive peptides are often determined by their amino acid quantity and sequence (Philanto-Leppala, 2000), and the pharmacological properties of bioactive peptides include antioxidant, anti-inflammatory, antimicrobial, anticancer, immunomodulatory, and hypertensive activities (Murray and FitzGerald, 2007; Sarkar et al., 2021, 2022; Rami et al., 2023). Bioactive peptides derived from foodstuffs have significant potential for use in the development of functional foods and nutraceuticals to avoid or cure certain chronic diseases (Chakrabarti et al., 2014). Hence, there has been an increase in fascination with bio-prospecting of novel bioactive peptides from natural resources, particularly for health and medicinal uses.

The marine environment has been focused on searching for novel bioactive peptides due to the untapped species richness compared to terrestrial habitats. In addition, since marine species contain high protein content, several marine creatures are used as excellent raw materials for producing protein-based bioactive molecules. Among these organisms, marine gastropods are a prominent source of bioactive peptides. The bioactive constituents obtained from different snail materials, such as crude extracts, mucus, and slime, consist of various compounds including glycans, polypeptides, and proteins (Perpelek et al., 2021). The application of snail slime inhibits the expression of H_2O_2-induced COX-2 gene expression in human gingival fibroblasts, thereby exhibiting a potent anti-inflammatory effect (Ricci et al., 2023). The mucus extracts from *H. aspera* and *E. desertorum* showed greater efficacy in stabilising human RBC membranes, inhibiting proteinase activity, and denaturing albumin compared to the standard aspirin (EI-Zawawy et al., 2021). The molluscan-derived compounds demonstrated significant promise for anti-inflammatory and antioxidant effects, as evident by in vitro, in vivo, and clinical trials. Hence, the present study concentrated on the idenfication of bioactive peptides from marine gastropods, with an emphasis on therapeutic qualities. Among the marine gastropods, *Conus* is a genus with hundreds of species with varying degrees of venom, which is largely employed in prey trapping. Venom machinery may also be employed in some species for defence purposes (Saravanan et al., 2009). Cone snails use the combined effect of several conus peptides aimed at different targets to effectively paralyse their prey, and this mechanism of synergistic process can be employed for disease treatment (Wang and Chi, 2004).

Conus inscriptus is a member of the Conidae family in the phylum of molluscs, and all species in the genus *Conus* are predatory and poisonous. Several potentially active compounds capable of therapeutic uses have been discovered by studying the pharmacological features of cone snail-derived compounds. Additionally, cone snail venom comprises numerous distinct chemicals, and its precise composition differs greatly across species. These comprise multiple peptides, each targeting a different nerve channel or receptor (Terlau and Olivera, 2004). Certain cone snail venom additionally includes a pain-relieving toxin that the snail employs to pacify its prey before killing it (Olivera, 1997). Conotoxins are unique because of their disulfide bonding network and particular amino acids in inter-cysteine loops. This specificity is one of the characteristics that makes them ideal for use as diagnostics for characterising neurological circuits, therapeutics in healthcare, and possibly biodegradable toxic substances for agro-veterinary purposes (Adams et al., 1999). Hence, the current study sought to identify the bioactive peptides in the venom sac protein extracts of *Conus inscriptus* by measuring antioxidant and anti-inflammatory assays.

Materials and Methods

Sample collection

Healthy *Conus inscriptus* specimens were collected at Visakhapatnam fishing harbour (Long: 17º38'37.58" N; Lat: 83º07'15.36" E), which is located on the north-east coast of Andhra Pradesh, adjacent to the Bay of Bengal, Visakhapatnam, India. The collected samples were packed aseptically in polypropylene zip bags and transported to the laboratory. The species identification was done by the morphological characteristics of the shell, which include a solid and smooth shell with groves around the base. In addition, the shell colour is ash-white, with dark brown hieroglyphic characters divided by a

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rotating pattern of dots in the center and base (Tryon, 1879). The samples were first rinsed with tap water and then with distilled water. After washing, the shells of the samples were gently broken and the venom sac was separated.

Extraction and estimation of total protein

The protein content from the venom sac of *Conus inscriptus* was extracted using the Ferreira et al. (2002) methodology using polyvinylpyrrolidone. The isolated venom sac was homogenised in 50 mM sodium phosphate buffer with 10% PVP at 4ºC to isolate crude protein. The homogenate was centrifuged for 20 minutes at 4ºC at 14000 rpm, and the supernatant was collected. Then, the protein concentration in the supernatant was estimated using the Lowry et al. (1951) method. Protein quantification was carried out by taking 1 ml of supernatant, and 4 ml of the freshly prepared alkaline copper sulphate solution was added. Alkaline copper sulphate solution was prepared by mixing 50 ml of 2% Na_2CO_3 in 0.1 N NaOH and 1 ml of 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartrate. The reaction mixture was incubated for 10 min at room temperature. Then 0.5 ml of Folin-Ciocalteau reagent was added and then incubated for 30 min at room temperature. Following incubation, the absorbance of the developed colour was measured at 660 nm using a spectrophotometer against the blank. The quantity of total protein in the test sample was determined by comparing the ODs of the test samples with the BSA standard curve.

Primary screening of antioxidant potential

DPPH radical scavenging activity was used to assess the antioxidant capability of venom sac protein extract from *Conus inscriptus*. The methodology of Mensor et al. (2001) was used to screen the DPPH scavenging ability. To perform this experiment, 20µl of crude protein extract was mixed with 0.5 ml of 0.1 mM DPPH solution and 0.48 ml of phosphate buffer. The control was maintained without the protein extract. The reaction mixture was incubated at room temperature for 30 min in the dark. Subsequently, the colour intensity of the samples was measured against a blank using a spectrophotometer at 517 nm. The results were represented as a percentage of scavenging activity.

Primary screening of anti-inflammatory potential

The in-vitro anti-inflammatory potential of venom sac protein extract from *Conus inscriptus* was quantified by inhibition of albumin denaturation and heat-induced HRBC membrane hemolysis assays.

Inhibition of albumin denaturation

The inhibition of albumin denaturation was examined according to the Sakat et al. (2010) methodology, with slight changes. To perform this experiment, 1 ml of venom sac protein extracts were mixed with 1 ml of 1% aqueous BSA solution. The pH of the reaction mixture was adjusted to an acidic range by using a small quantity of 1N HCl. Then, the reaction mixture was incubated for 20 min at 37ºC and then heated for 20 min at 51ºC. The reaction mixture was allowed to cool, and the absorbance was measured at 660 nm.

Inhibition of heat induced HRBC membrane lysis

The red blood cell suspension was prepared using the Sakat et al. (2010) methodology. Blood was drawn into EDTA tubes from a volunteer who was in good health and had not taken any NSAIDs for two weeks before the experiment. The tubes were centrifuged for 10 min at 3000 rpm, and the obtained RBC pellet was washed thrice with phosphate-buffered saline. Then the RBC volume was measured, and a 10% RBC suspension was prepared by adding PBS. Subsequently, the Shinde et al. (1999) method was employed to perform heat-induced hemolysis. To carry out this experiment, 1 ml of venom sac crude protein extract was mixed with 1 ml of RBC suspension. The control and standards were maintained by adding PBS and aspirin instead of protein extract. The reaction mixture was incubated for 30 min at 56ºC by keeping them in a boiling water bath. Following incubation, the tubes were cooled and centrifuged for 5 min at 2500 rpm. The supernatant was collected, and the absorbance was measured at 560 nm.

Purification of anti-inflammatory peptide from crude venom sac extract

The crude protein extract exhibited significant anti-inflammatory activity among the tested assays. Hence, the subsequent studies concentrated on anti-inflammatory peptide isolation, identification, and characterisation. The purification of protein was performed by ammonium sulphate precipitation, followed by dialysis and gel filtration chromatography.

Ammonium sulphate precipitation

The crude protein extract was partially purified by differential precipitation of protein components through ammonium sulphate mediated salting-out according to Engarld and Seifter (1990) methodology. The salting-out process was initiated by adding 20% saturation of solid ammonium sulphate to the crude protein extract. Then, the solution was centrifuged for 20 min at 14,000 rpm and 4°C, and the pellet was collected as a 20% ammonium sulphate crude enzyme fraction. The supernatant was further subjected to ammonium sulphate precipitation to obtain 40, 60, and 80% saturation, and all the respective precipitates were collected by centrifugation at 14,000 rpm for 20 min at 4°C. Then, all

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the precipitate fractions were separately suspended in a minimum volume of 0.1M Tris-Hydrochloride buffer at pH-7.0 and carried out dialysis overnight against the 0.001M Tris-HCl buffer to remove ammonium sulphate. All the ammonium sulphate fractions were evaluated for anti-inflammatory activity by using inhibition of albumin denaturation and heat-induced haemolysis assays. Finally, the dialysed samples were further purified by gel filtration chromatography.

Gel-filtration chromatography

The partially purified protein fraction exhibiting anti-inflammatory activity was suspended in Tris-HCl buffer (0.05M; pH 7.0). The solution passed through a sephadex-G-100 gel chromatography column preequilibrated with Tris-HCl buffer. 3 ml volume fractions were collected at a flow rate of 24ml/hour and the eluted fractions were analysed for inhibition of albumin denaturation and heat-induced haemolysis. Then, the fractions showing anti-inflammatory activity were pooled together, concentrated by lyophilisation, and used for further characterization.

Assessment of purified protein homogeneity

SDS-PAGE was used to determine the molecular weight and purity of the isolated anti-inflammatory peptide, as according to by Sambrook et al. (2001) methodology. Purified protein sample was suspended in the sample loading buffer at a 1:3 w/v ratio. The sample loading buffer contains 0.5M Tris-HCl (pH 6.8), 20.2% glycerol, 0.001% bromophenol blue, 0.04% SDS, 0.03% Dithiothreitol, and 0.04% β-mercaptoethanol. The protein-containing sample loading buffer was heated at 95°C for 10 minutes, then cooled and run on SDS-PAGE along with a concurrent run of protein markers ranging from 8 to 65 KDa. The Laemmli (1970) method was adopted to prepare a 15% resolving gel that consists of 1.5 M Tris-HCl (pH 8.8), 20% SDS, 10% ammonium persulphate and TEMED. The stacking gel (5%) was prepared by using 0.5 M Tris-HCl (pH 6.8), 20% SDS, 10% ammonium persulfate and TEMED. The electrophoresis was performed initially at 50 V for 1 h and then run at 100 V using a vertical electrophoretic unit with running buffer containing 25 mM Tris-base, 192 mM glycine and 3 mM SDS. After running, the gel was stained with 0.5% coomassie brilliant blue R-250 in 45% methanol and 10% acetic acid for one hour. After staining, the gels were allowed to destain for 12 h in a solution containing 20% methanol and 10% acetic acid. Subsequently, the gel was rinsed with distilled water till the background was transparent. The gels were viewed and photographed using Gel-doc.

Protein identification by Peptide Mass Fingerprinting

The purified anti-inflammatory peptide fraction was subjected to peptide mass fingerprinting by using MALDI-TOF-MS for identification and molecular weight determination. For PMF, the purified peptide fraction (0.5µL) was deposited onto a matrix containing saturated α-cyano-4-hydroxycinnamic acid prepared in 50% acetonitrile and 5% trifluoroacetic acid. The ABI 4800 MALDI-TOF (Applied Biosystems, Foster City, CA) collected the mass spectrometric data of the digested protein sample. The spectral data were acquired in reflector mode from a 600-9000 Da mass range. The raw data provided by the ABI 4800 was utilised to build a peak list with GPS Explorer software. The obtained mass spectrum was subjected to sequence database searches using the MASCOT software. The score produced from the MASCOT software analysis was to reflect the possibility of a true positive recognition, which had a value of at least 50.

Insilco characterisation of anti-inflammatory peptide

Insilco characterisation of the identified peptide was performed by predicting physiochemical properties, secondary structure, tertiary structure, and gene ontologies for functional activities. The identified peptide sequence i.e., conotoxin was retrieved from NCBI, an open access protein database (Bairoch and Apweiler, 2000), and this sequence was subjected to further analysis in FASTA format. The Expasy's ProtParam server was applied to determine the physicochemical properties. The secondary structural elements of conotoxin were predicted by using SOPMA server. The 3D model of conotoxin was developed by the Ab-initio method with the help of the Phyre2 server (Kelley et al., 2015). After predicting the 3D structure, the model quality was evaluated by using the QMEAN scoring function.

Statistical analysis

All the data were statistically analysed and presented as mean ± standard deviation. The mean values were obtained from at least three independent experiments. SPSS 10.0 software was used for the one-way ANOVA.

Results and Discussion

Estimation of protein

The protein quantification of any organism is very important in assessing biochemical and medicinal significance, since protein quantity is directly associated with the nutritional and medicinal properties. In the present study, the protein concentration from the *Conus inscriptus* venom sac was 7.27±0.6 mg/gm. In contrast to this result, previous studies such as Nirmal (1995)

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reported that male and female Babylonia zeylanica exhibited a protein content in the 42.18 – 68.31% and 43.15 - 69.75% range, respectively. Similarly, Baskaran (2001) found that the protein content in male and female Lambis lambis ranged between 46.75-70.88%.

Screening of bioactive potentials

The results of bioactive potentials are shown in figure 1. These results showed that the venom sac protein extracts of *Conus inscriptus* show greater anti-inflammatory activity. The venom sac protein extracts of *Conus inscriptus* exhibited inhibition percentages of 23.76±2.4, 66.23±3.1, and 57.32±2.6% with respect to the DPPH radical scavenging activity, albumin denaturation, and HRBC membrane stabilisation activities.

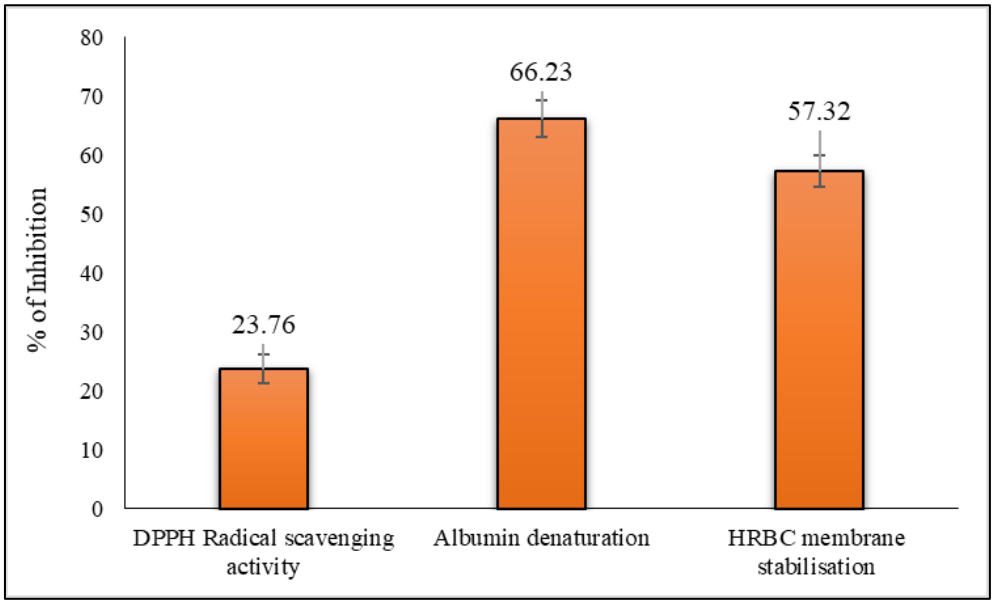

Figure 1. Bioactive potentials from the venom sac protein extracts of *Conus inscriptus*.

DPPH is a stable diamagnetic free radical with significant absorption at 517 nm and can take either an electron or a proton during the free radical scavenging reaction, and it transformed into a 2, 2-diphenyl-dipicrylhydrazine which leads to the gradual decrease of its colour and reflects the sample free radical scavenging potential (Ayoola et al., 2008). Krishnaraju et al. (2009) stated that the efficacy of antioxidants is frequently connected with their capacity to scavenging highly reactive free radicals. In the present study, the venom sac protein extracts of *Conus inscriptus* exhibit good free radical scavenging activity. The scavenging ability of extracts may reflect the antioxidant-related proteins and enzymes present in the extracts. Rizzi et al. (2021) reported that the slime extract of *Helix aspera* exhibited significant DPPH and ABTS scavenging activities. In addition, the slime extract of Helix aspera also have potent tyrosinase inhibitory activity, thereby exhibiting significant anti-inflammatory properties. Ekin and Sesen (2018) reported that the mucus of slugs was used in ancient Greece to reduce stress-related disorders and inflammation. Similarly, Lang et al. (2016) found that the slime of garden snails can used to treat erythema and wound healing by suppressing free radicals.

Inflammation is a typical reaction to harmful agents like allergens, infections, or damaged cells; inflammatory reactions result in allergens removal and tissue healing (Ferrero et al., 2007). The inhibition percentages of albumin denaturation and HRBC membrane damage are directly proportional to the anti-inflammatory activity. The present results show greater anti-inflammatory activities than the previous studies such as Wiya et al. (2020) reported that the aqueous and ethanolic slime extracts of *Lissachatina fulica* exhibited an inhibition percentage of protein denaturation as 41.99±2.37 and 12.76±3.61 respectively at 1000 µg/ml concentration. In addition, the inhibition percentages of RBC hemolysis are found to be 7.01±0.54 and 13.42±0.28 with the aqueous and ethanolic slime extracts of *Lissachatina fulica*. Padmanaban and Jangle (2012) reported that the alcoholic extract exhibited a significant inhibition percentage of albumin denaturation. The process of inflammatory reactions encompasses a compiled series of incidents, such as the discharge of cytokines and enzymes, as well as leukocyte movement and extravascular fluid accumulation (Vane and Botting, 1998). The result of this study indicated that the venom sac protein extract from *Conus inscriptus* might alleviate inflammatory events due to the presence of anti-inflammatory peptides. In addition, the anti-inflammatory peptides from venom sac protein extracts exhibit their

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action in multiple ways, including inhibition of protein denaturation and inflammatory enzymes and membrane stabilisation.

Bioactive potentials of partially purified ammonium sulphate protein fractions

The crude venom sac protein extracts exhibited greater anti-inflammatory activity than the other tested bioactive potentials. Hence, subsequential studies focused on isolating and identifying anti-inflammatory bioactive peptides. Substantial difference was found in the activities of inhibition percentages of albumin denaturation and HRBC membrane stabilisation among the four ammonium sulphate fractions (20, 40, 60, and 80%) of venom sac crude protein extracts. The results are presented in figure 2. From these results, the 60 and 80% fractions of crude protein had the highest anti-inflammatory activity, whereas 20 and 40% crude enzyme fractions didn't exhibit anti-inflammatory activity.

The inhibition percentages of albumin denature in the samples containing crude enzyme extracts of 20, 40, 60, and 80% ammonium sulphate fractions were found to be 0.00, 0.00, 73.66±6, and 8.43±2%, respectively. As well as the inhibition percentages of HRBC membrane haemolysis from the 20, 40, 60, and 80% ammonium sulphate fractions were found as 0.00, 0.00, 62.24±4 and 7.23±2%, respectively. According to these results, the greatest anti-inflammatory activity was found with the 80% crude protein fraction. While the anti-inflammatory activity of pectinase was not found in the test samples containing 20 and 40% ammonium sulphate crude fractions. As a result, the 80 and 40% ammonium sulphate fraction of crude protein extract were pooled and subsequently subjected to purification by gel-filtration chromatography.

The current study sought to identify and isolate the anti-inflammatory peptide from the venom sac of *Conus inscriptus*. Purification of proteins by precipitation technique using ammonium sulphate salt is generally known as "salting-out". Ammonium sulphate is the most often used salt because it is inexpensive, highly soluble, and it is considerably more hydrated than other ionic solvents. This is one of the well-known and commonly used methods in laboratories for partial purification of proteins by fractionation. In the present study, the crude protein extracts were exposed to ammonium salt saturations ranging from 20 to 80%, and the results showed that the crude protein fraction containing 80% ammonium sulphate saturation had the greatest anti-inflammatory activity. Hence, the 80% crude enzyme fraction was further investigated to purify the anti-inflammatory peptide. The present study shows that high ammonium sulphate saturated fractions give higher anti-inflammatory activity that indicates molecular weight peptides precipitated at high salt saturations. The current findings are consistent with the observations of Green and Hughes (1955), who stated that lower salt concentrations reduce protein solubility and cause precipitation.

Purification of anti-inflammatory peptide by gel filtration and activity evaluation

All the collected fractions from gel-filtration

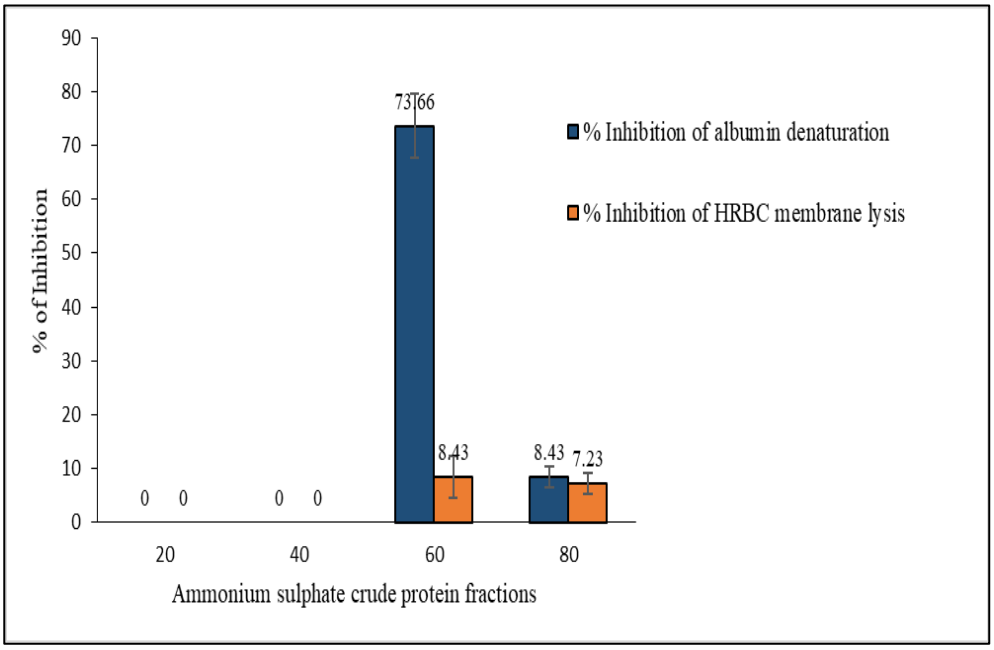

Figure 2. Antiinflammatory activity with the ammonium sulphate fractions (20, 40, 60, and 80%) crude protein extract from venom sac of *Conus inscriptus.*

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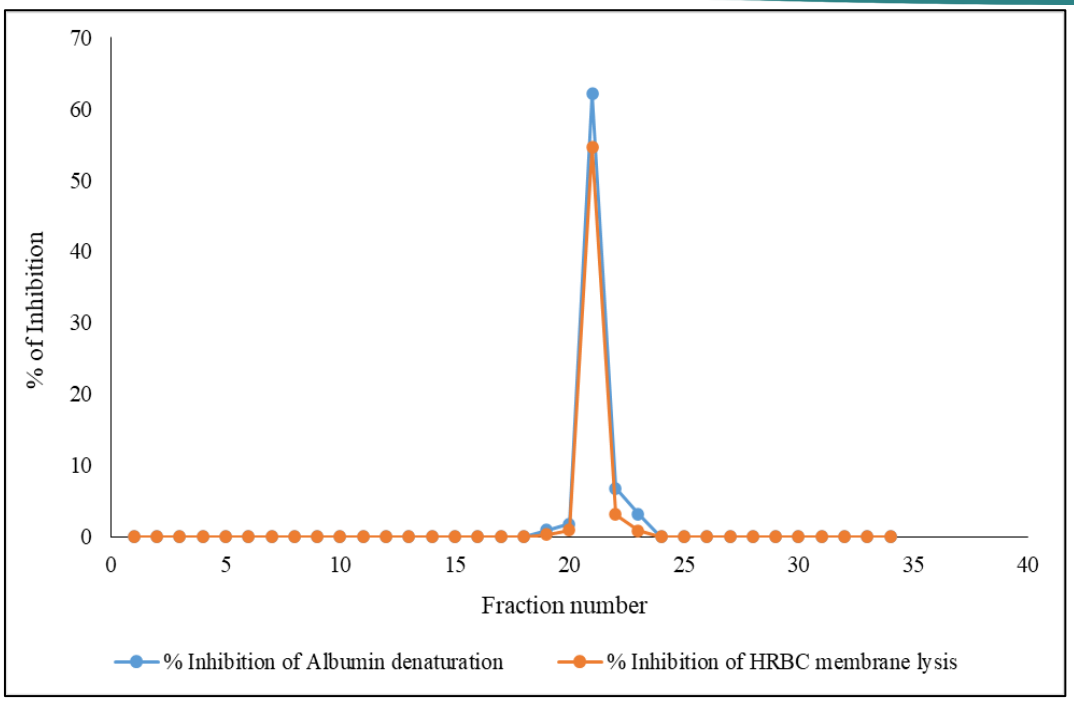

Figure 3. Anti-inflammatory activity of eluted peptide fractions through Sephadex G-100 column.

chromatography was evaluated for anti-inflammatory activity. Figure 3 illustrates the inhibition percentages of albumin denaturation and HRBC membrane lysis of the eluted protein fractions. From fig 3, it was observed that, out of 34 fractions, only five fractions exhibited anti-inflammatory activity.

Sephadex G100 gel filtration elution profile exhibited an elevated anti-inflammatory activity from fraction 19 to 23. These results showed that fraction 21 exhibits the highest inhibition percentages of albumin denaturation and HRBC membrane lysis, whereas fraction 19 exhibits the lowest activity. The purified fractions of anti-inflammatory activities begin at 19, reach to maximum at 21 and then decline until the activity is absent at fraction 24. The inhibition percentages of albumin denaturation from the purified fractions of 19, 20, 21, 22, and 23 were found to be 0.98±0.03, 1.81±0.9, 62.12±2.3, 6.82±1.2, and 3.21±0.9% respectively. As well as the inhibition percentages of HRBC membrane lysis from the purified fractions of 19, 20, 21, 22 and 23 were found to be 0.31±0.03, 0.92±0.02, 54.66±4.2, 3.12±0.9, and 0.86±0.2% respectively. Finally, all the anti-inflammatory activity fractions were pooled and evaluated for protein homogeneity. The present results are evident from earlier reports such as Dhevagi and Poorani (2016), who reported that the enzymes exhibit increased activity by 2.18% due to purification with a Sephadex-100 column by 85 folds. Dharmaraj (2011) purified the bacterial enzymes with 98.23% homogeneity and 78.88 U/mg activity using Sephadex G-100 gel filtration chromatography. This study observed that the purification of proteins using the sephadex G-100 column chromatography technique gives a good and significant yield.

Homogeneity of purified protein

The electrophoresis analysis demonstrated that the SDS-gel contains only one protein band, indicating that the purified anti-inflammatory peptide was homogeneous and that the anti-inflammatory peptide was a monomeric protein. Furthermore, utilising molecular weight markers in gel, the purified protein molecular weight was confirmed to be 12 KDa. Figure 4 depicts the gel image.

Proteomic research has enabled the identification of bioactive peptides with therapeutic uses. Hence, the current study was conducted to find anti-inflammatory peptides from the venom sac of *Conus inscriptus*. Protein purification techniques generally begin with one of the fundamental steps, which is to remove at least part of the unwanted protein fractions prior to identification. Zambare et al. (2004) proposed that differential solubility is one of the most often used techniques for crude protein purification. In the differential solubility technique, proteins are precipitated with increasing levels of ammonium sulphate, with the majority of proteins precipitating between 60 and 80% ammonium sulphate saturation, which enables the simple partial purification of a protein. In the current study, the protein fraction at 60 and 80% ammonium sulphate saturation demonstrated anti-inflammatory effects. Several prior investigations, including Reda (2015) and Wakayama et al. (2005), isolated and purified bacterial enzymes and examined their homogeneity using SDS-PAGE. According to

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Sohail et al. (2011) molecular weight markers may be used to assess the migration of different-sized proteins on the gel.

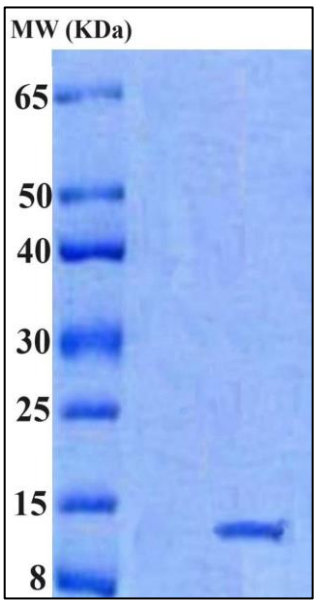

Figure 4. Molecular weight and homogeneity determination of purified anti-inflammatory peptide.

Protein identification

Peptide mass fingerprinting is a systematic method for identifying unknown proteins based on their peptide masses, and it is strongly reliant on high-quality protein databases (Mann et al., 2001). The PMF findings demonstrated that the 12 KDa purified anti-inflammatory protein fraction was identical to the precursor of conotoxin MI15b. Furthermore, the mass spectrum data indicate that the conotoxin of *Conus inscriptus* has the greatest similarity to the conotoxin of *Conus miles* (Accession number CO2FB_CONMI), with a predicted identity score of 31%. Table 1 summarises the peptide mass fingerprinting identification result of purified anti-inflammatory protein.

life, instability index, aliphatic index, and GRAVY of conotoxin were computed as 85, 9582.34 Da, 9.32, 3480 M^{-1} cm^{-1}, 30 hrs in mammalian reticulocytes, 58.99, 78, and -0.365 respectively.

The current findings showed that conotoxin is a basic protein, and the predicted pI may be used to develop a proper buffer solution for purification through isoelectric focusing. In this study, for both reduced and oxidised forms of cysteine, the extinction coefficient of the conotoxin was calculated to be 3480 and 2980 M-1 cm-1. According to Gill and Von Hippel (1989) a higher extinction coefficient indicates a higher concentration of tyrosine, tryptophan, and cystine. Guruprasad et al. (1990) demonstrated that proteins with an instability index of less than 40 are thought to be stable, whereas those with an index greater than 40 are thought to be unstable. Hence, under physiological conditions, conotoxin is regarded as unstable. Rogers et al. (1986) found that proteins with an instability index above 40 have an in vivo half-life of fewer than five hours, whereas proteins with an instability index below 40 have a half-life of more than sixteen hours. Ikai (1980) stated that the aliphatic index of a protein directly relates to its stability and may be used to estimate protein stability across a broad temperature range. Kyte and Doolottle (1982) proposed that positive and negative GRAVY show proteins' hydrophobic and hydrophilic characteristics.

Secondary structure of conotoxin

SOPMA predicted the secondary structural elements for the given protein sequence with an accuracy of 69.5% (Geourjon and Deleage, 1995). In the secondary structure of conotoxin, the random coils were found to be 54.12%, followed by alpha helices (35.29%) and extended strands (10.59%). Secondary structural elements of conotoxin are

Table 1. Identification result of the purified protein by peptide mass fingerprinting

Source Organism	Protein Gel Band	Max. homology (protein name)	Best match organism	Expt/Theor. Mw (KD)	Score (MS/MS)	Accession No.
Conus inscriptus	12 KDa	ConotoxinMI15b precursor	*Conus miles*	12/9.35	31	ACV07668

Insilco characterisation of conotoxin

Physicochemical characterisation

The primary sequence of the conotoxin from the beat match organism *Conus miles* was retrieved from GenBank through homology searching with the BLAST P suite by choosing the protein data base. The results of the physicochemical properties are shown in table 2. From these results, it was observed that the length, molecular weight, pI, Extinction Coefficient (EC), half-

shown in figure 7 and table 3. These results indicated that, among secondary structural elements, random coils and alpha helices are dominant.

The prediction of secondary structural elements of a protein is used to determine the protein's structural and functional effectiveness as well as the hydrogen bonding pattern (Krishnasamy et al., 2015). Generally, the proportion of random coils affects the confirmational changes and the adaptability of proteins (Buxbaum,

2007). The abundance of more flexible glycine and hydrophobic proline amino acids is the cause of the high coil proportion. Neelamathi et al. (2009) suggested that the prevalence of coiled domains indicates that the protein structure is extremely stable and conserved. Bansal et al. (2014) proposed that the functions of α-helices in proteins vary depending on the protein topology, including ligands, receptors, transporters, channels, and energy translocators. Additionally, a greater content of extended strands could aid in the dynamics and sliding action, which are essential for function.

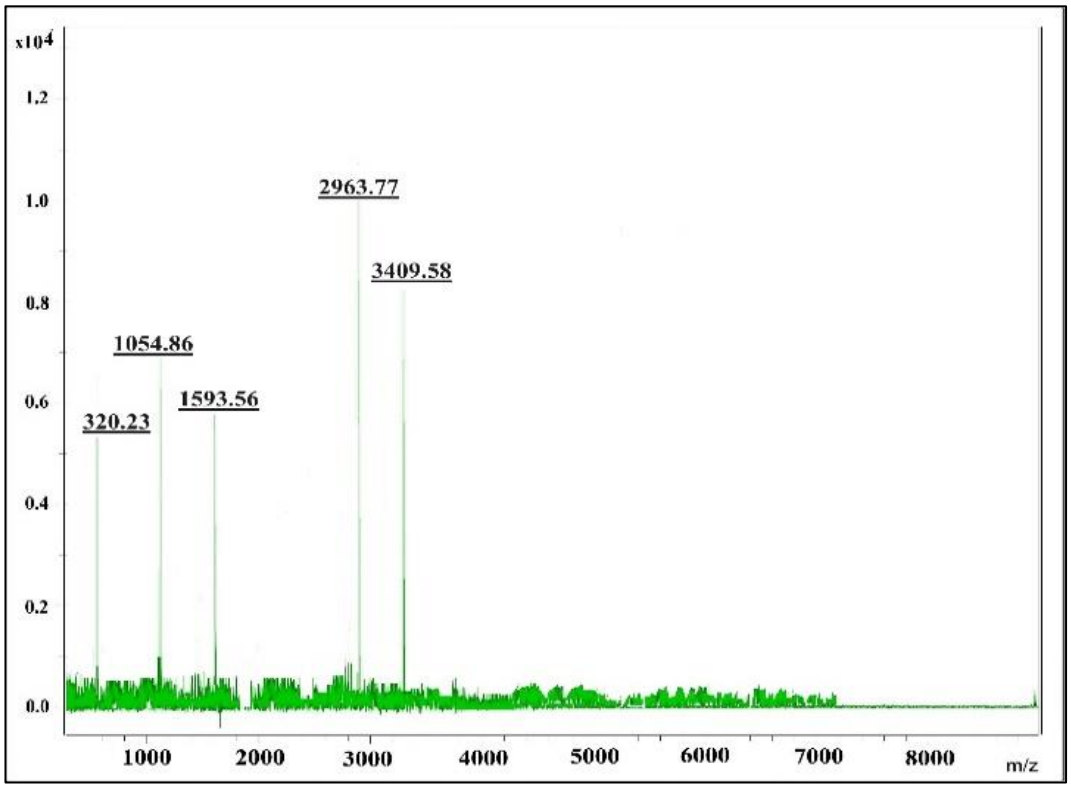

Figure 5. MALDI-TOF/MS spectrum of purified 12 KDa purified protein fraction. Purified peptide was subjected to in-gel trypsin digestion, then analysed by MALDI-TOF MS in reflector mode over mass range of 500-8000 Da.

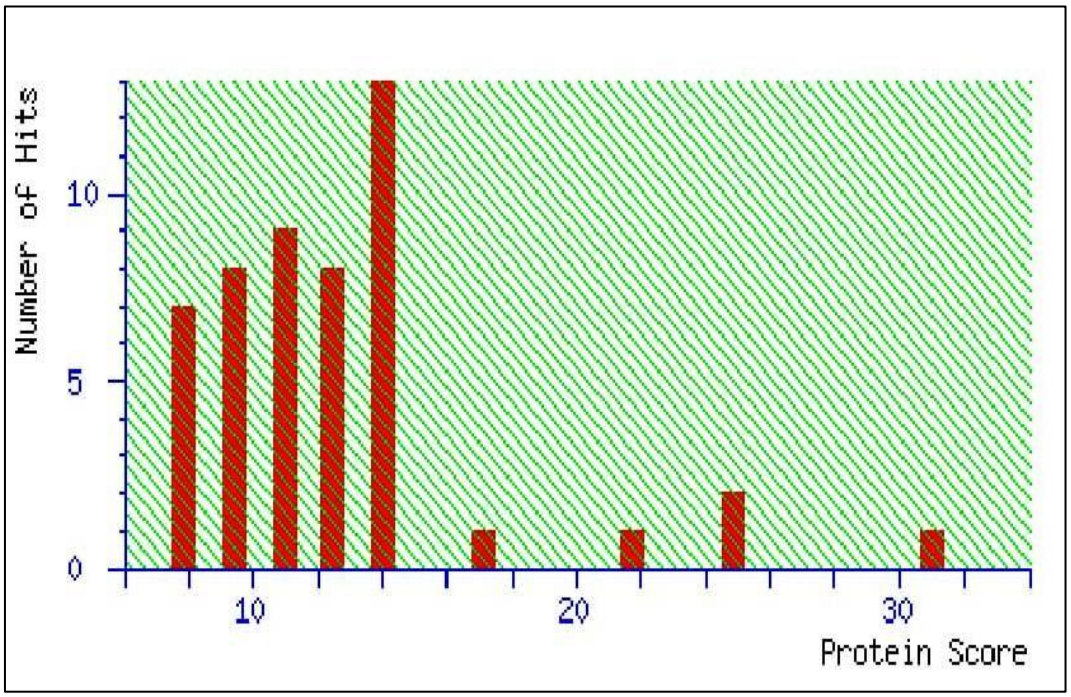

Figure 6. Mascot score histogram of purified anti-inflammatroy peptide from *Conus inscriptus.*

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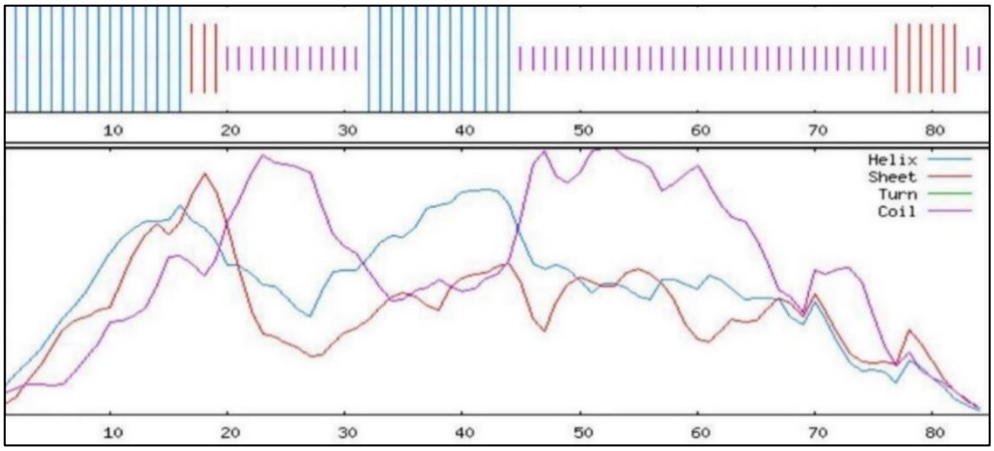

Figure 7. Spectrum of conotoxin secondary structural elements

Table 2. Physicochemical properties of conotoxin

Parameter	Conotoxin
Total no. of amino acids	85
Molecular weight	9582.34 Da
Theoretical pI	9.32
Negatively charged residues	7
Positively charged residues	15
Extinction coefficient	3480 M^{-1} cm^{-1}
Instability index	58.99
Aliphatic index	78
GRAVY	-0.365
Formula	$C_{409}H_{689}N_{125}O_{119}S_{10}$
Total number of atoms	1352
Carbon	409
Hydrogen	689
Nitrogen	125
Oxygen	119
Sulphur	10

Table 3. Secondary structural elements of conotoxin

Structural elements	Number of residues	Percentage of residues
Alpha helix	30	35.29%
310 helix	0	0.00%
Pi helix	0	0.00%
Beta bridge	0	0.00%
Extended strand	9	10.59%
Beta turn	0	0.00%
Bend region	0	0.00%
Random coils	46	54.12%
Ambiguous states	0	0.00%
Other states	0	0.00%

3D structure and quality assessment

Protein 3D structure provides reliable information on their interactions and localization with stable confirmation. In addition, understanding protein molecular activity requires an awareness of their 3D structural characteristics. In this study, the predicted 3D model of conotoxin exhibits c6nk9A as the best acceptable template with a confidence of 54.1% and coverage of 36%. The PDB collection's highest scoring template (c6nk9A) is described as acatoxin1, isolated from the sea anemone *Antopleura cascaia*, which has potassium ion channel inhibitory action. Figure 7 depicts an anticipated 3D model of conotoxin. From these results, it was identified that out of the complete protein sequence of 85 residues, 31 residues, which represent approximately 36% of the protein sequence, have been modelled using c6nk9A. According to Biasini et al. (2014), modelling and assessment approaches must also be able to justify protein flexibility because proteins are not static entities but may exist in fundamentally varied functional states.

In the current study, the predicted model QMEAN score was found to be 0.64±0.12. According to the density plot of the QMEAN score, the reliability of the predicted model was determined to be in the range of 0 to 1. The predicted conotoxin 3D model QMEAN score was extremely close to 0, indicating the quality of the model. Figure 8 depicts the density map of the QMEAN score. Sippl (1993) defined QMEAN as a complete measure that combines the statistical possibilities of mean energy and model accuracy with structural properties anticipated from the target protein sequence. Benkert et al. (2011) suggested that the QMEAN score determines the anticipated model effectiveness and is standardised in terms of the number of interactions.

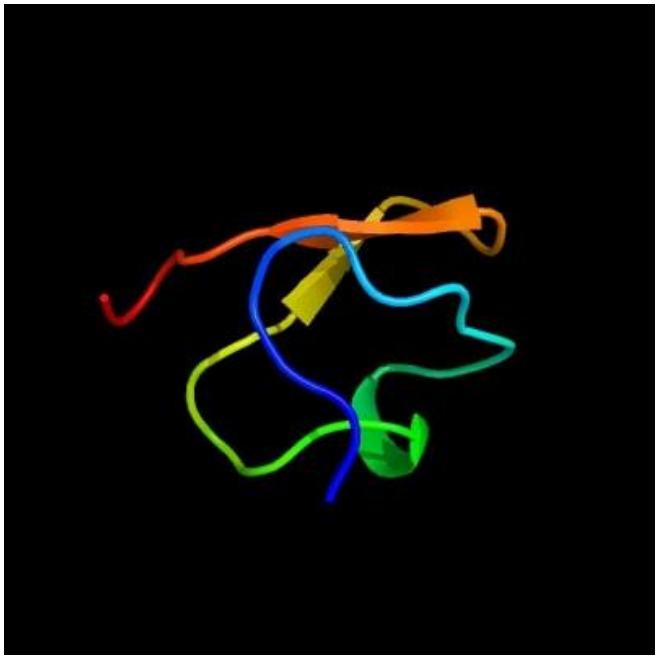

Figure 8. 3D structure of conotoxin

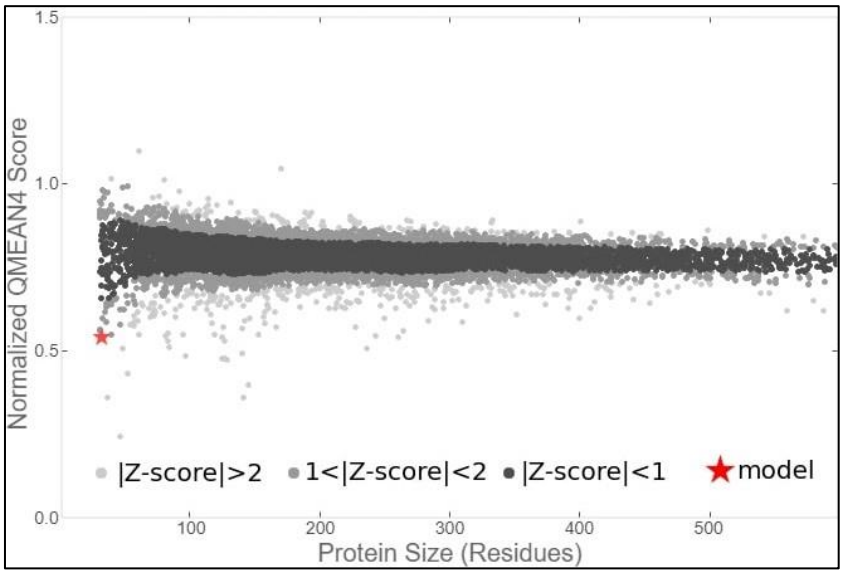

Figure 9. The plot shows the QMEAN score of the predicted 3D model.

Conclusion

The present results concluded that the protein extract from the venom sac of *Conus inscriptus* has a substantial anti-inflammatory effect by preventing albumin denaturation and HRBC membrane lysis. Secondary screening of the isolated anti-inflammatory protein fraction reveals that the 12 KDa protein fraction significantly inhibits albumin denaturation and HRBC membrane lysis. Furthermore, the 12 KDa purified peptide was identified as a conotoxin MI15b. The findings of the Insilco characterisation of the conotoxin MI15b precursor indicated that this protein might serve as an anti-inflammatory agent and may be responsible for therapeutic actions in the medical management of many inflammatory-related disorders. Further research to isolate and characterise the additional functional groups and toxicological investigations may improve our understanding of its medical applications.

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Conflict of interest

Authors declare no conflict of interest.

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