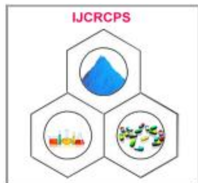

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RESEARCH ARTICLE

STUDIES ON BIOLOGICAL PROPERTIES AND *INSILICO* APPROACH OF *Plotosus canius*, FROM PARANGIPETTAI COASTAL WATERS

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Abstract

Catfishes, member of the order Siluriformes, are representative venomous fishes having venom glands in dorsal and pectoral spines that are locked into place when threatened. A certain *Plotosus canius* toxin is lethal and cardiotoxic, having neuromuscular blocking activity, envenomation causes immediate, local and intense pain, soft tissue edema, and a variable extent of bleeding. The present study carried out on studies on biological properties and *in silico* approach for *Plotosus canius*. The crude was extracted with saline and it is screened for antimicrobial properties and was tested against 6 pathogenic bacteria and 5 pathogenic fungi. The result of present investigation reported that mucus extract displayed significant antimicrobial activity. The toxic activities of crude extracts were then determined by hemolytic activities in chick, goat, and human blood erythrocytes. The result reported that mucus extracts showed a moderate hemolytic activity in chick and goat and no activity was recorded in human erythrocytes. Particularly, a previously isolated *Plotosus canius* protein (NADH - ubiquinone oxidoreductase chain 4) was characterized and reported to have proton and sodium ion transport in the electron transport chain in the inner mitochondrial membrane. This protein was highly homologous with other fish NADH - ubiquinone oxidoreductase proteins. Since, the unavailability of the 3D structure of NADH - ubiquinone oxidoreductase chain 4 subunit protein from catfishes, this attempt was carried out and Homology modeling was performed using Phyre3D-PSSM folding server. In the present study, subcellular localization prediction suggested that it is a inner mitochondrial membrane protein. Quality analysis for validation of the model determined that it is a reliable model. Furthermore, this protein (NADH - ubiquinone oxidoreductase chain 4) is a structural protein suggested to be localized in mitochondrial inner membrane of cristae involved in ubiquinone binding mechanism and oxidoreduction - drive active membrane transporter activity.
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Keywords: *Plotosus canius*, antimicrobial activity, hemolytic activity, homology modelling and NADH - ubiquinone oxidoreductase.

Introduction

Catfish of the family, Plotosidae are extensively rampant all the way through the watercourses of India that let liquid flow away into the Indian ocean. Catfish species from the coastal waters of the Parangipettai are known to possess a toxin with mucous - covered sting, responsible for most of the injuries, as well as a protective epidermal secretion. Venom produced by the animals consist indispensable multifarious concoction peptides and proteins. The venom of catfish (Plotosidae) is unintentional for the pharmacological expediency in India and constrainedly considered in foreign countries. The genus *Plotosus* consists of a large number of species, belonging to the family plotosidae and order siluriforms. More than 37 are found distributed throughout the tropical pacific and Indian ocean. Tamil Nadu coastline includes Parangipettai which is located (11° 29’ 33. 62” N79° 46’ 08. 86” E) on the banks of Vellar estuary and

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Bay of Bengal, where the distribution of catfish is more and their venom properties has to be studied to treat injuries and using it as a pharmacological tool for validating it as a lead for drug development. Although accidents caused by these animals are considered severe, it is surprising that only a few studies have ever examined this relationship, given all the pharmaceutical benefits and health threats posed by venomous aquatic animals (Rodriguez, 1972). In aquatic environment large numbers of venomous and poisonous animals were more than 200 species of marine fish including stingray, scorpion fish, zebrafish, stonefish, weever fish, toadfish and some species of shark, ratfish, catfish, surgeonfish are known otherwise suspected to be venomous (Russrell, F.E, 1996). Catfish are found in shallow tropical coasts worldwide to temperate waters including marine and fresh water species (Al - hassan 1986, 1987). In India fresh water catfishes include *Plotosidae* family, which comprise three valid genera *P. lineatus* (Shiomi., *et al* 1986), *P. canius* (Auddy *et al*., 1994) and *P. limbatus* (Haddad,. 2000). Fresh water catfish in India are very common in northern, central, western and southern rivers, and marine catfish are distributed throughout Indian ocean coastline (Nelson, 1984). Many catfish have three serrated bony stings on dorsal and pectoral fins, which was used for defence against predators (Haddad 2000, Halstead 1953). Venomous catfish have a sharp and stout sting immediately in front of the soft rayed portion of pectoral and dorsal fins, where stings are derived from fin rays and are covered by thin integumentary sheath (Rodrigues, R.J., 1972). In addition to venom, the skin of fish plays a passive role in protective immunity, serving as an anatomical and physiological barrier against the external environment (Cameron and Endean 1973; Shiomi 1988)

The information of 3D structure remains an indispensable fact for experimentally discovering the functionality of any protein. This is partly due to the considerable experimental challenge and manual inputs required to solve three-dimensional structures by methods such as X - ray diffraction and multi - dimensional nuclear magnetic resonance (NMR) spectroscopy in comparison to high-throughput sequencing (Sheehan and Sullivan, 2011). Moreover, the rate at which protein sequence data is accumulating is far more than the structural information available, thus creating a gap between available sequences and experimentally solved structures. Computational methods like homology modeling can help reduce this gap. It is known that existing proteins are result of continuous evolution of previously existing ones, thus proteins can be grouped into families (Giancarlo *et al.,* 2011). Homology modeling methods use the fact that evolutionary related proteins share a similar structure. Therefore, models of a protein with unknown structure (target) can rebuilt based on an alignment of a protein of known structure (template). This typically involves four steps (Sánchez and Sali, 1997; Marti-Renom *et al.,* 2000); (1) identification of homologs that can be used as template(s) for modeling; (2) alignment of the target sequence to the template(s); (3) building a model for the target based on the information from the alignment(s); and (4) evaluation of the model. Finally, all four steps can be repeated until a satisfactory model is obtained.

Materials and Methods

Collection and processing of sample

The live sample of *P. canius* were collected from Annankoil landing centre (Lat 11° 29 N: Long 79° 46 E), Parangipettai, southeast coast of India (figure 1 A, B). The collected animals were kept at - 2° C for 1 hour. Mucus was collected from surface of fish body by scrapping with dull blade (Al - Hassen, 1982). Further, mucus were placed in tubes and stored at - 4°$_C$ until use. Briefly, homogenization and all subsequent procedures were carried out at 4°$_C$. The homogenate was centrifuged at 8,000g x 15min. The pellets were collected, re - extracted with extraction buffer (0.005M sodium phosphate buffer pH 7.5 containing 0.14M NaCl) recentrifuged as before and the supernatant was subsequently called mucus respectively.

Extraction of Venom

Aqueous extraction

The aqueous extract of *P. canius* was prepared by squeezing the sand - free specimens in triple distilled water. The resultant solution was filtered and dialyzed by using Sigma dialysis membrane - 500 (Average Flat width - 24.26 mm, Average Diameter - 14.3 mm and capacity approx - 1.61ml/cm) against D - glucose to remove the excess water. The supernatant so obtained was lyophilized (Labcono Freeze Dry System) and stored at 4°C in a refrigerator for the further use as aqueous extract.

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Figure 1: a) Shows the study area

B) Shows the *Plotosus canius* (Hamilton – Buchanan, 1822)

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Partial purification of crude protein

Partial purification of the crude extract *P. canius* was carried out using DEAE Cellulose Anion Exchange chromatography according to the procedure of Stempion *et al.,* (1970).

Protein estimation

Protein content from crude extracts was estimated by Lowry and Lopaz method. (1946).

Microbial Strains Used

Antibacterial effect of *P. canius* was determined against 6 different bacterial strains viz. *Pseudomonas* sp*, Streptococcus aureus, Vibrio cholerae, Bacillus sp, E. coli* and *Lactobacillus brevis* similarly Antifungal effect was determined against 5 different fungal strains viz. *A. flavus, A. niger, Candida albicans A. oryzae and A. sojae* These pathogenic strains were obtained from the Department of Medical Microbiology (*Raja Muthiah Medical College Hospital*) Annamalai University, Annamalai Nagar.

Antimicrobial activity

Petri dishes with nutrient agar and Potato Dextrose Agar (PDA) were inoculated with five different species of bacteria and fungus. *P. canius* mucus extracts were sterilized by passing each through a 0.22 mm Millipore GV filter (Millipore, U.S.A). Round paper discs with a radius of 0.8 cm were dipped into each extract of different concentration of 5mg/ml and 10mg/ml and placed in the center on inoculated petridishes. The bacterial and fungal colonies were allowed to grow overnight at 37ºC and 20ºC respectively, and then the inhibition zone around the disc was measured.

Hemolytic assay

The hemolytic activity of crude extracts of *P. canius* were assayed on chick, goat and human erythrocytes followed by the method of Pani Prasad and Venkateshwaran (1997).

Ileal loop Assay in chick.

The toxicity was assayed using the chicken ileal loop method followed by Sedlock D.M. and Diebel R.H (1978) .

Protein retrieval and sequence analysis :

The aminoacid sequence of protein was retrieved from UniProt database using accession no. B2L8R2 which is then translated to amino acid by similarity search in blastx. Physiochemical properties of the predicted protein were computed by Prot Param tool (web.expasy.org/protparam). Sub-cellular localization of any protein is important in understanding protein function. Prediction of sub cellular localization of protein was carried out by CELLO V.2.5 (Yu *et al*., 2006; Yu *et al*., 2004).

Computational modelling

Predict Protein was employed for computing and analyzing the secondary structural features of NADH - ubiquinone oxidoreductase chain 4 amino acid sequence (Rost *et al.,* 2004). A three dimensional model of test protein was generated by homology modeling (Kelley *et al.,* 2000) (Phyre; 3D - PSSM folding server;). In brief, this method aligns a test sequence to one or more template structures with known structures as determined by crystallization/ X - ray diffraction, or NMR spectrometry.

Quality and reliability assessments

Once the 3D model was generated, energy minimization was performed by GROMOS96 force field in a Swiss - PdbViewer. Structural evaluation and stereochemical analyses were performed using ProSA - web (Wiederstein and Sippl, 2007; Sippl, 1993) displaying Zscores and Procheck (Morris *et al.,* 1992) visualising Ramachandran plot. Furthermore, superimposition of query and template structure, and visualization of generated models was performed using UCSF Chimera 1.5.3.

Function annotations of the protein

To functionally annotate the NADH - ubiquinone oxidoreductase chain 4 predicted protein, Profunc was used, and to find the conserved domains in protein to identify its family, it was searched against close orthologous family members. NCBI Conserved Domain Database (NCBI CDD) (Marchler - Bauer and Bryant, 2004) was used to find the conserved domains or ancient domains in the protein sequence. The present study intends to explore therapeutic characterization of crude extract and to perform sequence and structure analysis of *P. canius* NADH-ubiquinone oxidoreductase chain 4

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protein. The protein sequence was retrieved using accession no. B2L8R2 from Uniprot database

Statistical analysis

Tests were carried out in triplicates. The mean values were calculated from the triplicate values. Values are expressed as the mean ± SD and differences between groups were considered to be significant if $p<0.05$.

Results

Preparation of Crude Extracts

Aqueous extracts yield a total amount of 1.02g of crude extract from 50g of lyophilized mucus sample.

Protein Estimation

The protein content in crude extracts of aqueous exacts was found to be 1.05 mg/ml. The obtained results were represented in figure 2.

Antimicrobial activity

Antibacterial Activity

The crude of aqueous extracts were tested against 6 species of bacteria viz. *Pseudomonas* sp, *Streptococcus aureus, Vibrio cholerae, Bacillus sp, E. coli* and *Lactobacillus brevis.* The obtained results were represented in figure 3.

Antifungal Activity

The crude of aqueous extracts were tested against 5 species of fungai viz. *A. flavus, A. niger, Candida albicans, A. oryzae* and *A. sojae.* The obtained results were represented in figure 4.

Hemolytic Assay

The results of the hemolytic assay on chick, goat and human blood sample erythrocyte were done using aqueous crude extract. The crude extracts in mucus venom induced hemolysis in chick, goat blood sample. In chick blood aqueous extract it was found to be 4 and its specific hemolytic activity was estimated to be 2.6 HT/mg of protein. In goat blood sample, it was found to be 4 and its specific hemolytic activity was estimated to be 2.6 HT/mg of protein. In human blood sample no hemolysis were recorded. The results were shown in figure 5.

Ileal loop assay

In experiment chick illeal loop were used measuring 6 -10 cm were constricted each carrying seven loops, 1 ml of *P. canius* crude was injected into the loop, the last loop was inoculated with saline (control). The effect of drug at lower and higher doses in the intestine of the chick was found by injecting 25µl/ml and 100µl/ml to its intestinal wall. This was then stored in phosphate buffer saline. The extract would attach to the acetyl choline receptor and the sodium potassium pump will be blocked as a result it brings out the fluid secretion. In the present study the fluid secretion was observed which may be due to the effect of toxic protein present in the extracts **(**Figure 6 A**,** B**)**. These results indicated that presence of toxic substances in the extracts and the intensity of low dose can be used in therapeutic purpose .

Protein sequence analysis

ProtParam was used to find out the physiochemical properties from protein sequence. The hypothetical protein was predicted to have 317 amino acids, with molecular weight of 34737 Daltons and theoretical isoelectric point (PI) of 6.25. An isoelectric point below 7 indicates a negatively charged protein. The instability index (II) is computed to be 23.04. This classifies the protein as stable. The N - terminal of the sequence considered is M (Methionine). Therefore estimated half-life is 30 hours (mammalian reticulocytes, in vitro), >20 hours (yeast, in vivo) and >10 hours (*Escherichia coli*, in vivo). The negative Grand average of hydropathicity (GRAVY) of value - 0.292 indicates that the protein is hydrophilic and soluble in nature. Threonine and glycine were found in rich amounts in the protein. Cellular functions are often localized in specific compartments; therefore, predicting the sub-cellular localization of unknown proteins can give information about their functions and can also help in understanding disease mechanisms and developing drugs. The sub-cellular localization prediction using CELLO predicted that our protein is a cytoplasmic protein and this protein does not contain a nuclear localization signal. Predict Protein was used to predict the secondary structure of the protein. Results showed that protein is a mixed protein having composition of Strand = 64.04%, Loop = 35.96%, the obtained results were represented in (figure 7A, B, C). Ramachandran plot and Errat analysis revealed that the tested hypothetical protein consists rich amount of amino acids which possess significance for the predicted protein would be highly flexible (Figure 9 A, B, C, D).

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Figure 2: Estimation of Protein (Lowry method) *P. canius* crude mucus extract

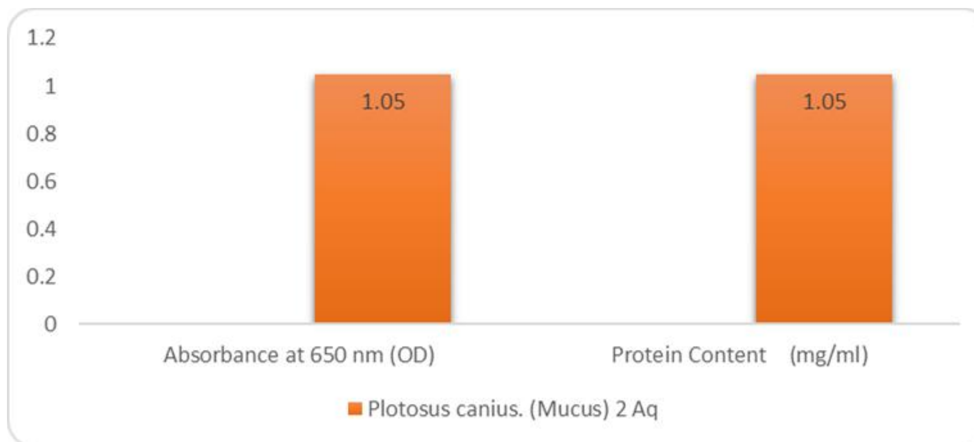

Figure 3: Antibacterial activity of of *P. canius* crude mucus extract

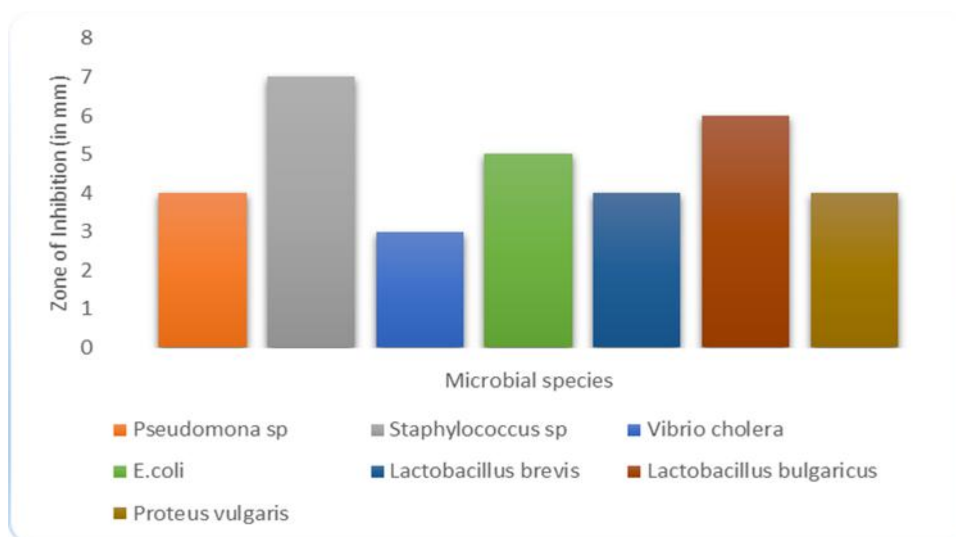

Figure 4. Antifungal activity of *P. canius* crude mucus extract

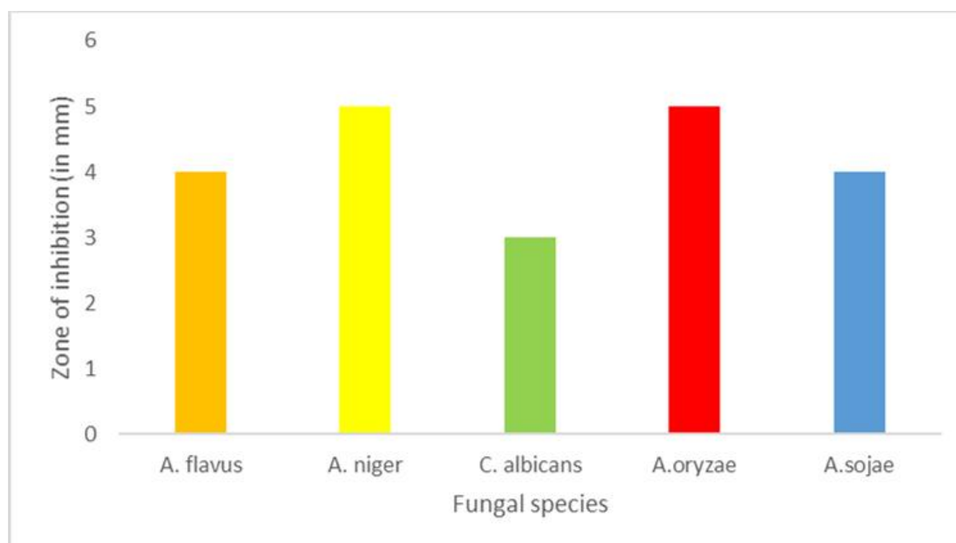

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Figure 5: Hemolytic assay of *P. canius* crude mucus extract

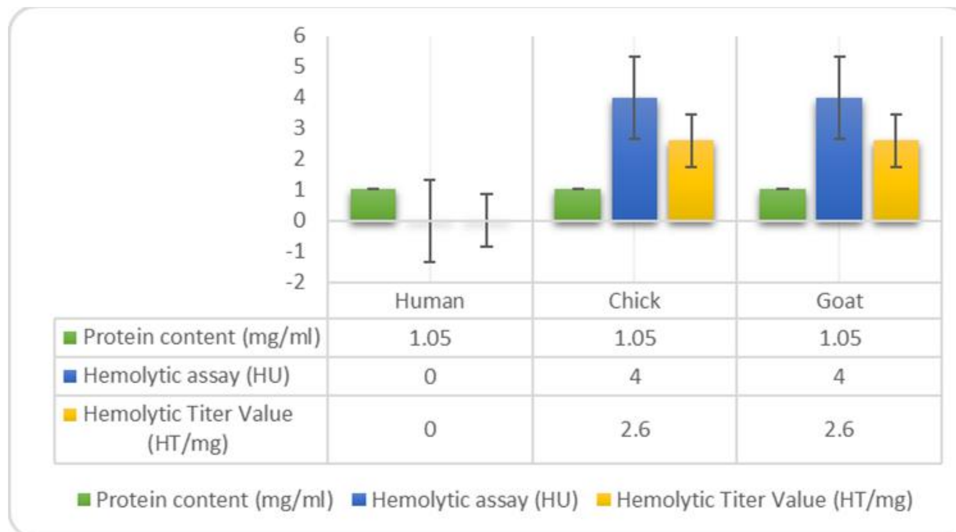

Figure 6. A) Illeal loop assy in chick intestine loop B) Shows the accumulation in crude *P. canius* in chick loop

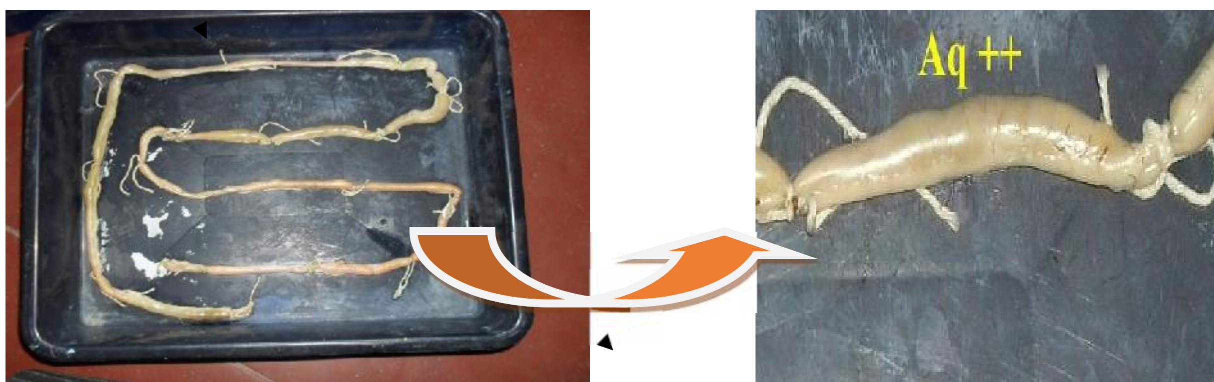

Figure 7. A) Amino acid frequency and composition B) Secondary structure composition C) Solvent Acessibility of test protein

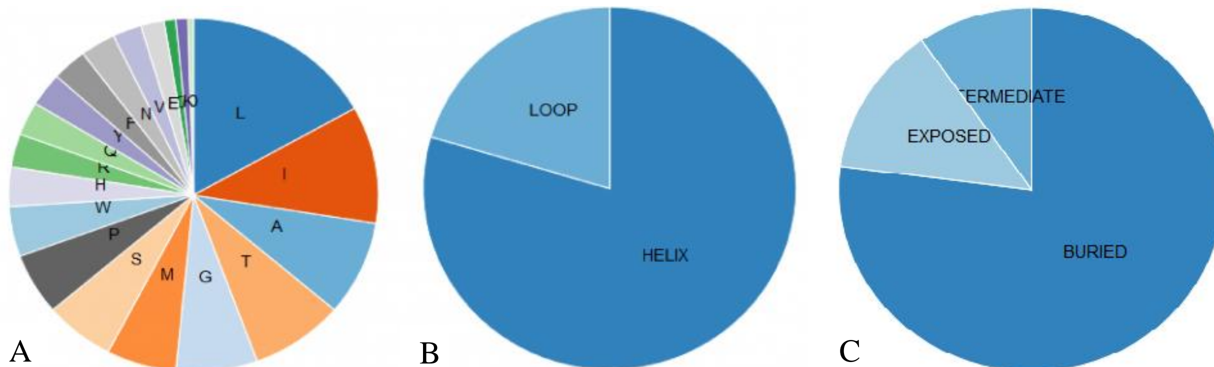

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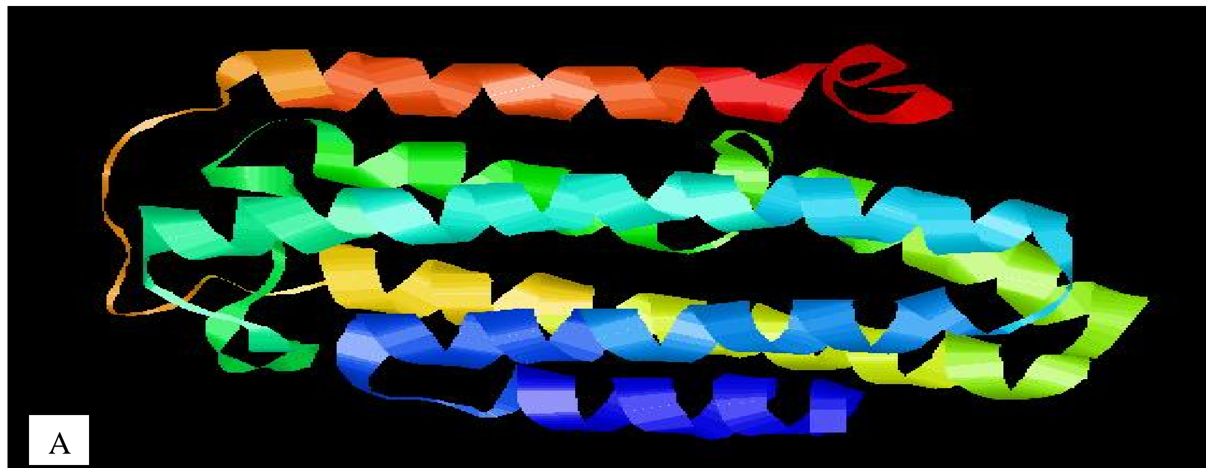
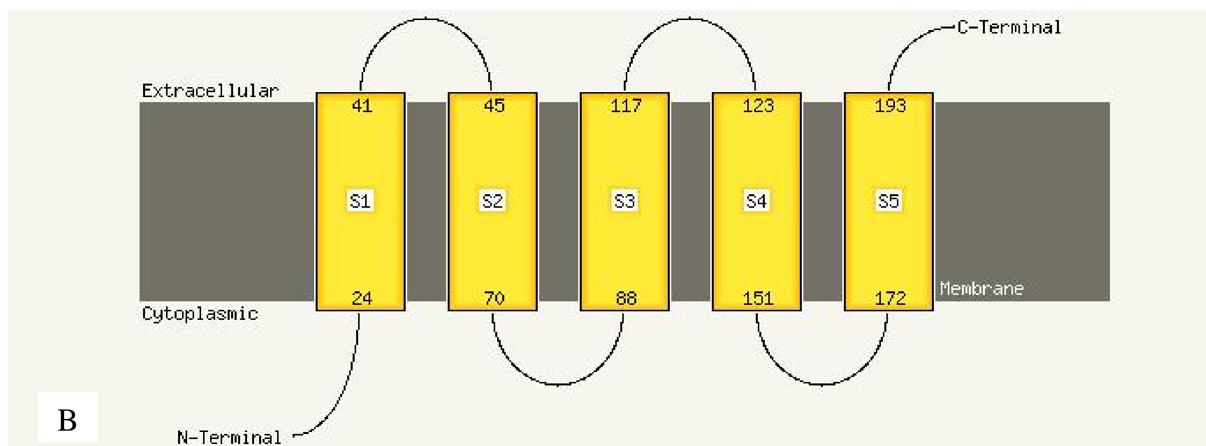
Figure 8. A) Predicted 3D structure of *P. canius* NADH-ubiquinone oxidoreductase chain 4 and B) Schematic representation of the predicted 3D structure.





3D structure prediction using homology modeling approach

Phyre3D method allows for the identification of homology based on PSI - BLAST alignments in combination with a profile–profile matching algorithm (Rost *et al.,* 2004) which adjusts for secondary structure alignments. Along with this hypothetical protein, non-self identifying (i.e., non - sequence identify) comparator templates with greatest homology to the target were NADH - quinone oxidoreductase subunit M (*Escherichia coli*; 30% sequence coverage; PDB template: C3rkoM) and NADH - quinone oxidoreductase subunit 13 (*Thermus thermophilus* 29% sequence coverage; PDB template: c4he8M). These structures each had 100% confidence and served as controls for homology modeling of test protein (Figure 8 A, B).

Functional annotation of the protein

To hypothetically annotate the function of the *P. canius* test protein B2L8R2, ProFunc was used. It was discovered that protein is involved in two biological processes, pathogenesis and inter-species interaction between the organisms and the biochemical function of the protein is dehydrogenase activity, acting on the transfer of protons for reducing potetntial in electron transport chain. To further investigate about the function of protein by finding its family; it was searched in the NCBI Conserved Domain Database (NCBI CDD) to find conserved domains so that its family can be identified. The results showed that *P. canius* NADH -ubiquinone oxidoreductase chain 4 has ubiqinone domain and belongs to electron transport chain of mitochondria as evident from protein annotation (Figure 10A, B).

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Figure 9. A) Knowledge-based energy of test protein using PROSA web, B) Z-score of test protein using PROSA web C) Overall quality factor checked by ERRAT and D) Ramachandran plot assessment for defined regions of flexibility

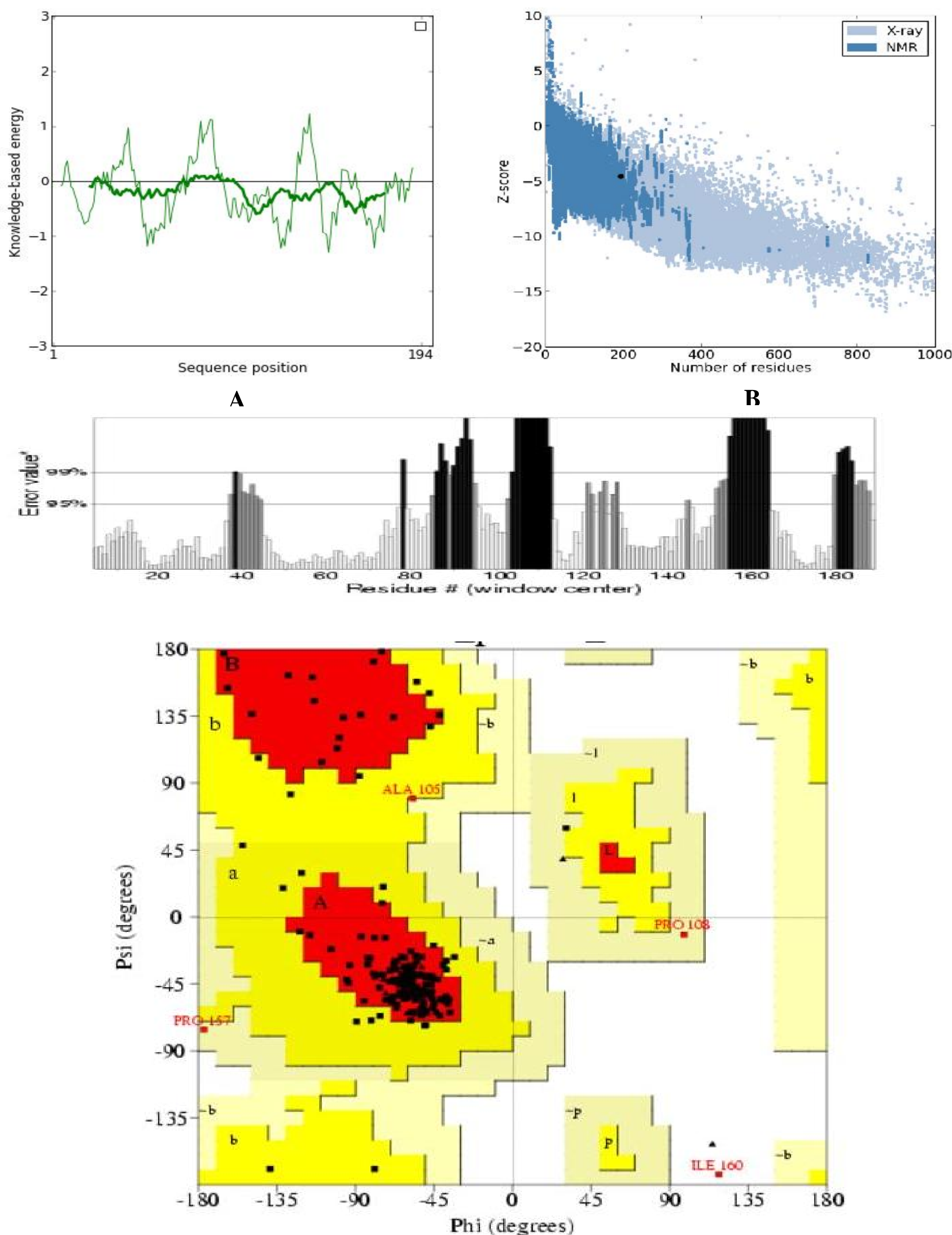
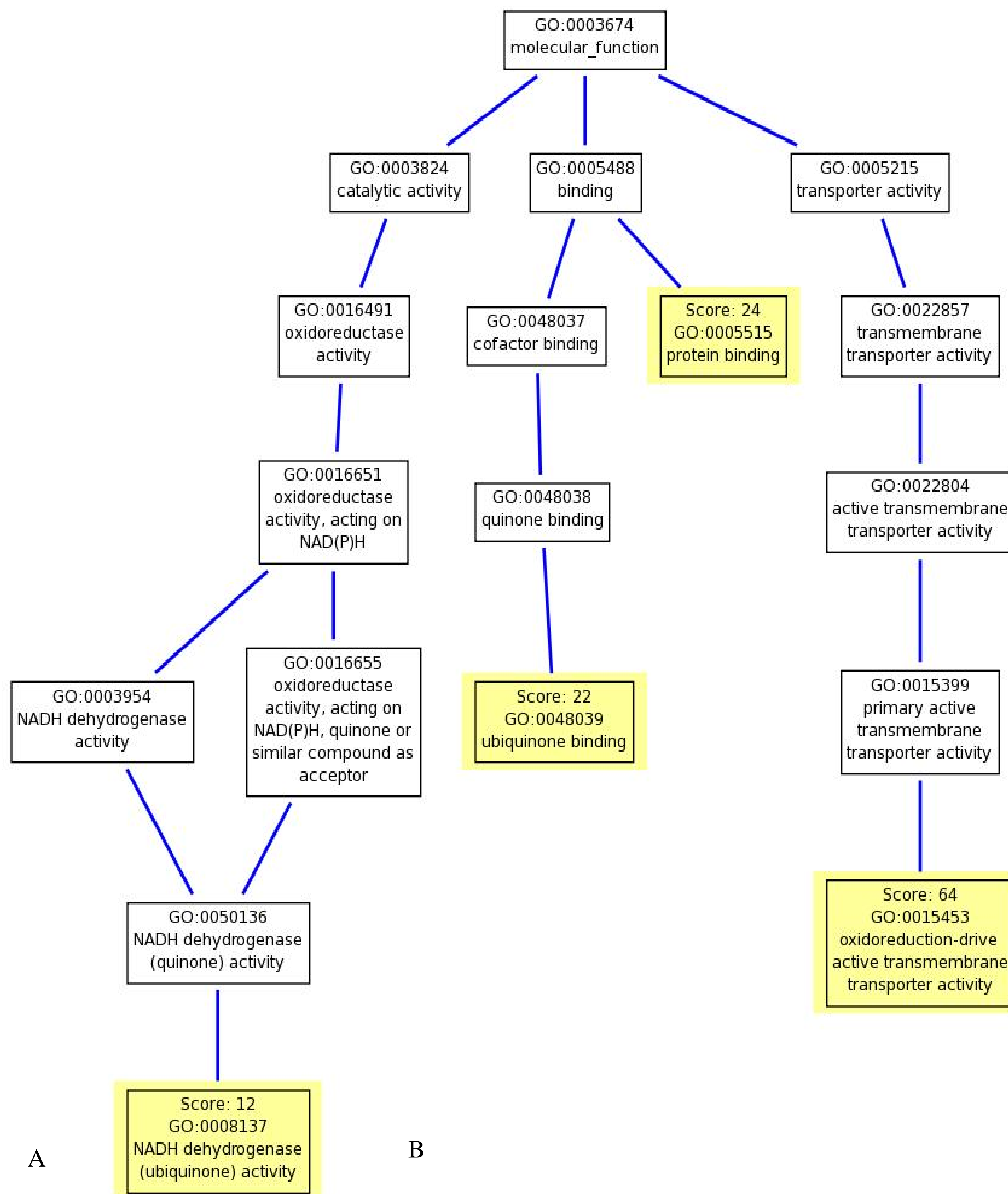


Figure 10. A) Molecular pathway analysis from Gene ontology annotation and B) Predictive system for Protein spatial localization



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Discussion

Discoveries of toxins from venoms, for the most part from marine resources that are racing ahead because of their extremely complex and notable action on various mammalian physiological systems (G. Sivan *et al.,* 2007). Toxic proteins serve in a number of adaptive roles such as immobilizing paralyzing, killing, liquefying competitions. Other venom proteins may act synergistically by enhancing the activity or spreading of toxins (Garnier *et al.,* 1965). In the usual course of events, epidermal toxins, spine venom and poisonous fishes are produced by protein elaborating cells (Al - hassan *et al.,* 1982). Thus the epidermal secretions contain a mixture of highly active biochemical and pharmacological components that are different from typical fish mucus and spine, which is composed of catfish proteinaceous secretions (Al - hassan *et al.,* 1985). Thus knowing importance of toxins and venom in this present study was carried out to evaluate the bioactive properties of catfish *Plotosus canius* epidermal mucus. The present investigation strived to isolate mucus of *Plotosus canius* samples that were collected from Annankoil, South east coast of India while this species was identified by Hamilton, 1822. The aqueous extracts of mucus venom were purified by DEAE - anion exchange chromatography. In purified mucus of catfish (*Plotosus canius*)*,* aqueous extract yielded 1.02 g, of mucus extract from 2 kg fish. Poh *et al.,* 1991 had also done the same extraction in stone fish *Synanceja horrida.* SNTX was purified from crude venom by atleast two step procedure on Sephacryl S - 200 high resolution gel - permeation and DEAE bio gel an anion - exchange chromatography.

The catfish has proteinaceous secretory cells in its epidermis (Al - Hassan *et al.,* 1982). In the present study, the protein content of epidermal mucus, was found to be 1.05mg/ml in aqueous extract Similarly, the protein concentration of *Synanceja horrida* were estimated in the native and modified SNTX with a concentration of 1 mg/ml which showed an absorbance at 280 nm by (Chen *et al.,* 1997).

Protein 3D structure is very important in understanding the protein interactions, functions and their localization. Homology modeling is the most common structure prediction method. The multidimensional NADH ubiquinone complex signature consists of sequence and structural elements distributed in a hallmark pattern in 3-dimensional space. At the structural level, the test protein is typically characterized by the presence of a strand including loops. To assess whether hypothetical protein contained these structural components, molecular modeling analyses were carried out to generate a predictive model of test protein protein secondary structure. For this analysis, NADH dehydrogenase chains were identified as the template for 3-dimensional modeling. Several physicochemical features of the predicted 3 - dimensional structure of target protein were homologous to those in NADH dehydrogenase. The molecule appears to consist of two distinct regions, and an extended region that is relatively unstructured. From these perspectives, the predicted overall fold of target protein is consistent with that of ubiquinone domain. ProSA uses knowledge based potentials of CA atoms. The Z-score of - 4.58 indicates the overall model quality of target protein (Figure 9 B). Z - score also measures the deviation of total energy of the structure with respect to an energy distribution derived from random conformations. The scores indicate a highly reliable structure and are well within the range of scores typically found for proteins of similar size. The energy plot shows the local model quality by plotting knowledge-based energies as a function of amino acid sequence position (Figure 9 A).

The results of hemolytic assay showed good activity in chick and good blood samples. epidermal mucus of *Plotosus canius,* crude extracts showed maximum hemolytic activity in chick and goat blood sample no hemolysis were recorded in human blood. Hemolysis of human and sheep red blood cells was studied by Al - Hassan *et al.,* 1982. In earlier study reported by Al - ladhan *et al*., 1987, they studied the specific activity of catfish epidermal factor of 20.6 mg^{-1} protein. The Illeal loop assay showed positive results with fluid accumulation in mucus extracts which may due to the effect of toxic protein present in above mentioned extracts. The minimal and maximal dose extracts developed inflammation and accumulation of fluid. But the intensity of inflammation was less in 25µg/ml of the extracts and high fluid accumulation was observed in higher dose of 100µg/ml. The intestinal loop assay found the toxicity levels in mammalian tissues and it was identified by the accumulation of fluid in the intestinal loop. These results indicated that presence of active compounds substances in the extracts and it was observed from previous study (Pundha Subash Chandra Bose, 2010).

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Conclusion

Our main objective of this study was to perform sequence analysis, structure analysis and homology modeling on *P. canius* target protein B2L8R2. We have used various sequence and structure analysis tools that helped in understanding of the sequence and its structure. Furthermore, protein was functionally annotated by using ProFunc and by searching conserved domain of the protein. As a part of present study, we used homology modeling approach to propose the first 3D structure of the *P. canius* hypothetical protein. The predicted 3D structure will provide more insight in understanding the structure and function of the protein. The present study gives us information about biological properties and *in silico* approach for *Plotosus canius* . Moreover, detailed studies could then be made on purification and characterization of the venom into several active components that may lead to the discovery of new potent antimicrobial drugs and its structure can be used for drug designing or understanding the interactions between proteins in near future.

Conflict of interest statement

We declare that we have no conflict of interest.

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