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Proteomic analysis of the venom of *Conus flavidus* from Red Sea reveals potential pharmacological applications

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ABSTRACT

Background: Venomous marine cone snails produce unique neurotoxins called conopeptides or conotoxins, which are valuable for research and drug discovery. Characterizing *Conus* venom is important, especially for poorly studied species, as these tiny and steady molecules have considerable potential as research tools for detecting new pharmacological applications. In this study, a worm-hunting cone snail, *Conus flavidus* inhabiting the Red Sea coast were collected, dissected and the venom gland extraction was subjected to proteomic analysis to define the venom composition, and confirm the functional structure of conopeptides.

Results: Analysis of *C. flavidus* venom identified 117 peptide fragments and assorted them to conotoxin precursors and non-conotoxin proteins. In this procedure, 65 conotoxin precursors were classified and identified to 16 conotoxin precursors and hormone superfamilies. In the venom of *C. flavidus*, the four conotoxin superfamilies T, A, O2, and M were the most abundant peptides, accounting for 75.8% of the total conotoxin diversity. Additionally, 19 non-conotoxin proteins were specified in the venom, as well as several potentially biologically active peptides with putative applications.

Conclusion: Our research displayed that the structure of the *C. flavidus*-derived proteome is similar to other *Conus* species and includes toxins, ionic channel inhibitors, insulin-like peptides, and hyaluronidase. This study provides a foundation for discovering new conopeptides from *C. flavidus* venom for pharmaceutical use.

1. Background

Cone snails are a group of 1000 species of venomous marine molluscs,[1] that are flourish in subtropical and tropical waters,[2] commonly close to coral reefs in the Indo-Pacific region.[3–4] Conidae have notable taxonomic and ecological diversity. They showed the fastest rate of diversification among gastropods.[5] All cone snails are predators and have their own unique, complex, and peptide-rich venom. They use venom mainly to help predation on small fish, other molluscs and worms and for defence.[6] Venom is the main weapon used by these carnivorous molluscs comprising small cysteine-rich peptides called conopeptides or conotoxins that show various pharmacological activities for defence, competition, capture of prey and further biological purposes.[7] These venoms are formed along a specialized venom duct and injected into the target through a hollow radula tooth.[6] Cone snails are usually categorized into three main groups according to the preferred prey: piscivore, molluscivore or vermivore.[8] Venoms of cone snails have drawn immeasurable interest as factual pharmacological resources as a result of their biological activity, astonishing diversity. Several biological studies initiated a way for biomedicine research.[9] For example, conotoxins could act as an anti-adhesion therapy for malaria. They also hamper the crucial protein of life-threatening viruses such as AIDS and COVID-19, possibly leading to their treatment.[10] Conopeptides can affect ion channels and control the nervous system of their targets, therefore, turn out to be a good candidate for developing new drugs.[11–13] Although many of the conopeptides are

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in the early phases of development, Ziconotide (promoted as Prialt; a synthetic form of ω-conotoxin MVIIA), is the only conotoxin permitted by the USA FDA for treatment of acute chronic pain through inhibiting the voltage-gated N-type calcium (Cav2.2) channel.[14] Cone snail insulins are another conopeptides draw interest as possibly new pharmacological means or therapeutic leads, which are active at the human insulin receptor,[15–17] and recently discovered somatostatin analogues in fish-hunting cone snail venoms.[18] Conopeptides are basically diverse with different cysteine frames and many posttranslational modifications (PTMs), In addition, they are stable and relatively small peptides.[19–20] Conopeptides are produced from mRNA-encoded conopeptide precursors that contains hypervariable mature peptide and a variable region following to the signal peptides.[21–22] Conotoxins are usually classified according to their endoplasmic signal sequence into different superfamilies. They are also can be classified based on cysteine framework into several set of conotoxin families acting on target receptors, channels, or transporters.[23] The extensive evolutionary history and harsh marine environment had encouraged the formation of many compounds that have distinctive structures to help the continued existence of these organisms[24] Analysis of the venom constituents of different species of cone snails indicated that each species generates an exclusive venom reflecting their heritage with some modifications to utilize different ecological habitats.[25–27]

In this work, we studied the venom from *C. flavidus*, a previously uninvestigated *Conus* snail collected from Red Sea. We used a proteomic approach including HPLC (high performance liquid chromatography) fractionation combined with LC-MS (LC/mass spectrometry) to evaluate the conopeptide content in its crude venom. The worm-hunting (mainly sedentary *Terebellidae* polychaetes) *C. flavidus* is generally spread over the Red Sea coast of Egypt, whilst no previous studies have been done on its venom. This analysis presents an incipient overview of *C. flavidus* venom components and reports data about promising bioactive peptides which may have pharmacological significance.

2. Materials and methods

2.1. Venom collection

Ten *C. flavidus* specimens were collected from different locations on the Red Sea coast of Egypt (Fig. 1). Venom apparatus were carefully dissected, and the venom ducts were cut into small fragments and suspended in 2% acetic acid twice. The venom was freeze-dried and kept at −80°C until use. Protein quantification was performed using bicinchoninic acid assay following the manufacturer's instructions.

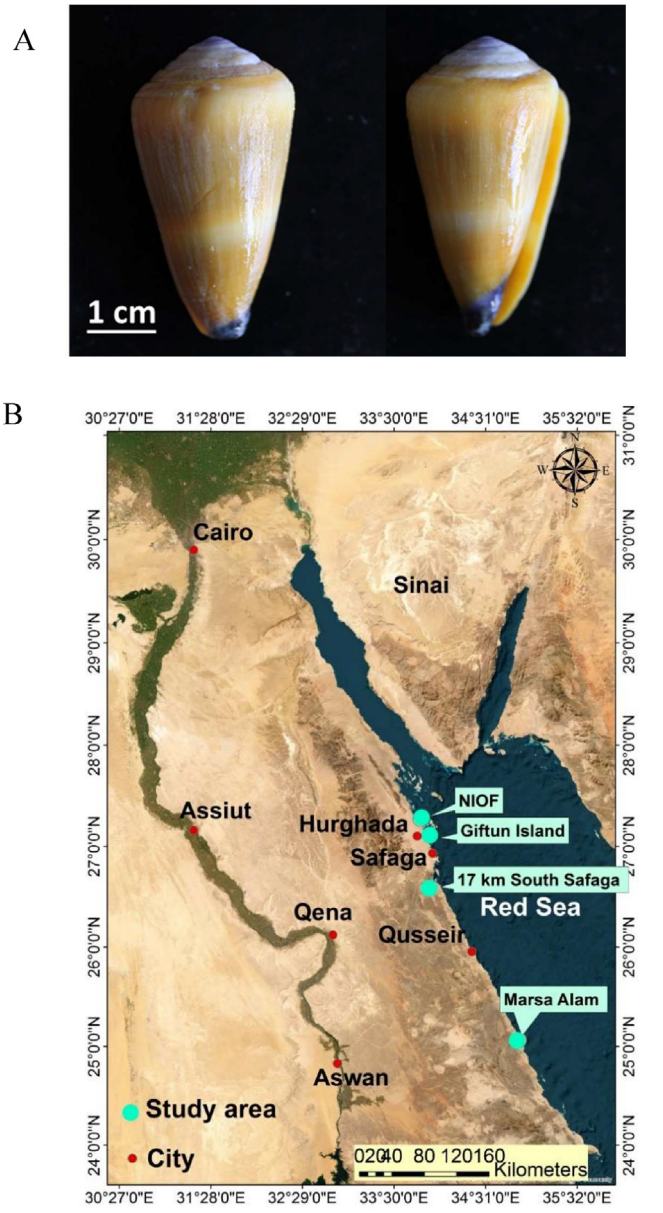

Fig. 1. (A) Morphology of *C. flavidus* shell (scale bar = 1 cm), (B) Map showing the collection sites of *C. flavidus* from the Red Sea coast in Egypt.

2.2. Reduction, alkylation, and trypsin digestion of extracted venoms

Crude venom (30 μg) was incubated at RT for 45 min in a solution; 0.13 M NaHCO3 (pH 8.5) and 2.7 M urea, reduced with 35 mM dithiothreitol (DTT). For alkylation, the mixture was then incubated with 125 mM iodoacetamide (IAA) for 45 min in dark at RT. For digestion of the peptides, Sigma proteomic sequencing-grade trypsin was used as described previously.[28]

2.3. LC/MS analysis

NanoLC system consisting of Eksigent nanoLC 400 autosampler and Ekspert nanlLC425 pump was used. A reversed-phase C18 HPLC (RP-HPLC) column (CHROMXP-C18-CL, 120A (150x0.3 mm) was used for separation. At a flow rate of 10 μl/min for 55 min a 1.0 μg of crude venom was injected and the column was eluted with 0.1% formic acid (FA) in water (dissolvent A) and 0.1% FA in CH3CN (dissolvent B). Mass spectrometry analysis was done using Sciex TripleTOF™ 5600 + (AB Sciex, Canada). The venom sample was desalted before the measurement by using reversed-phase C18 columns (GlSciences, Cat. No. 5010–21701). Using a matrix solution consists of α-cyano-4-hydroxy-cinnamic acid (HCCA, 2.5 mg, Bruker Daltonics) that dissolved in CH3CN (50%, 0.1% FA, Sigma-Aldrich) the venom was dissolved. 1.0 μl of the matrix solution was speck onto a target plate (Bruker Daltonics) then kept drying at RT. In positive ion mode the measurements were conducted, and the MS/MS and MS ranges were 170-1500 and 400-1250 *m/z*, respectively. Mass spectra raw data (the TripleTOF™ 5600+ files) were changed to Mascot generic format (mgf) files by use of the script provided by AB Sciex and ProteoWizard.

2.4. Proteomic data analysis

Using X! Tandem in a Peptide-shaker (v1.16.38) spectra of the MS/MS were searched against the UniProt *Conus* organism (Swiss-Prot and TrEMBL containing 10,684 proteins) with reversed sequences. With initial *m/z* tolerances of 10.0 and 20.0 ppm, fragment masses and the precursor were established, respectively. Post-translational

modifications (PTM) used in the search included the carbamidomethylation of cysteine, the oxidation at methionine, acetylation of the protein N-terminus, deamidation of asparagine, and deamidation of glutamine. Consequently, to decide the gene superfamilies found in the crude venom of *C. flavidus* the Entrez PubMed database and the UniProtKB database[29–30] were used.

3. Results

In the venom of *C. flavidus*, a total of 117 peptide fragments were detected (Table 1). A protein sequence similarity search in the database revealed 75 fragment peptides attributed to 65 conotoxin proteins across 16 conopeptide superfamilies: A, B1, B3, I1, J, M, N, O1, O2, O3, P, S, T, Conkunitzin, Cerm, and 17, as presented in Table 2. Notably, the T, A, O2, and M superfamilies comprised the highest percentages (33.9%, 14.5%, 14.5%, and 12.9%, respectively) of the identified superfamilies. Furthermore, rare superfamilies of conotoxins were also found in the venom of *C. flavidus* (Fig. 2). Within each of the following conotoxin superfamilies—B3, I1, J, N, P, Conkunitzin, Cerm, and I7—only one peptide fragment sequence was detected. Additionally, 42 fragment peptides belonging to 19 non-conotoxin proteins were identified, including Insulin, Hyaluronidase conohyal-P1, protein disulfide isomerase, arginine kinase, and vitamin K-dependent gamma carboxylase (Table 3).

Table 1
Showing peptide sequences revealed by LC/MS analysis of *C. flavidus* venom.

N	Sequence	N	Sequence
1	LSLEQQQK	60	KRDECLPGGK
2	LCLPVFIILLLLVSPAATLLVKSK	61	EFQRILLR
3	DLPCGNKR	62	KLSVTFLLILMILPSVTGEK
4	YKEYNRPVK	63	RCLPVVVILLLLIASTPNVDARPKTK
5	TDFASGIK	64	ILQVIESK
6	EKLTVLILVATVLLTIQVLAQSDR	65	EKNNTQRVNK
7	QLMLRNNLQK	66	ELKEKDDVK
8	NAENKQDHVPDK	67	QSEEGGSNATK
9	DFLEGNYLQEQVR	68	MEFILHALGQR
10	AAASLEEEKSNIK	69	QTLQILSNKR
11	TASDIVQWAMEK	70	SGKLLQLL
12	EKLTILVLVAAVLLSTQVLVQGDGEKPQKK	71	LSGADPNSIWSK
13	DQESAGALALK	72	KSGMLLFVLLLVLPLAFPKLVPVQR
14	NIEKVGSQNGKTSAK	73	KYQDESIK
15	VTPGSPGTAQLSGHR	74	GGTNDGGKK
16	SIPNKLGGVIGLAGSVLVLFILPLAHQAK	75	TLQILSNNR
17	LGILLANFLILVIFPLAGK	76	SAIKEFLSQECLGMCGIRTMSNAGDFR
18	NLTKGVFEQLK	77	QLQCQRLQEHIR
19	MLFLPVFVILLLLIASAPSVDVRPKAK	78	ASDGGNAVAKK
20	RCIPVFVILLLLIASAPSVDVRPKAK	79	MSKLGAMFFLLLLFTLASSQEK
21	AATALEEQNLNVKLGKVDATVEDSLAAK	80	DDMSLASFQDNAKR
22	MTKRCTPAGK	81	RCLPVVIILLLLIPSALSVHAQPKTK
23	KIEASETDERDKPK	82	GILLANFLILVILPLISKKWSWYLK
24	KAHHEMKNPEASK	83	NSKLSVHFDLQR
25	EKLTVLILVATVLLAIQVLVQSDREKPLK	84	GEKQATQR
26	QDISPNERKR	85	MVPARPYWR
27	TLQTLSNK	86	GGVEKRQEAK
28	RCLPVFIILLLLIPSALSLIAKPK	87	QAVDIVNWLK
29	DMALPIQEMLVKQEK	88	ASDGRNAEAK
30	SALMRGPR	89	GQGLTDHYRNLR
31	GLPVFVILLLLIASAPSVDARPKTK	90	QASDIVQWLK
32	RCLPVFVILLLLIASPPSVDVRPKAK	91	ADERGQGLTEQYR
33	ELGDYITNL	92	EELMNYVR
34	NDVHRAILHDVAK	93	GKLNLPPYLTNEAR
35	NAAAKASNR	94	LPTEDHPLYD
36	MLGILLANFLILVIFPLTGKKWSWYLK	95	DGLGNLFSKTQHEMKNPETSK
37	LGFGNLDPEGKMIK	96	ANVIQEMNNTQAK
38	LSKEEIER	97	LCLPVFIILLLLVSPAATLPVK
39	EGTFQGYHSIK	98	EVVMEKQAMMEK
40	DRDRLTYHAGCPVLMGNK	99	EEEKPLPQNEFQR
41	TKDDMSLASFHENAKR	100	EKLTILVLVATVLLAIQVLVQSDGEKPLKR
42	NSKVSRDCQK	101	KLAAMQQQQR
43	MLCLPVFIILLLLVSPAATLLVKSK	102	MKVAVVLLVSLLAVTYALPEKR
44	CLPVFVILLLLIASISSVDALLKTK	103	QTSDIINWLNKK
45	QVASDRATSIAR	104	NDVILDSLR
46	EDNLSITEDWLQGMCLEKR	105	DNAKRILQVLESK
47	CLPVFVILLLLTASVPSVDARPMTK	106	TLQTLMNK
48	IGLSVSPVK	107	SEPVPENNDQPVK
49	KLTVLILVATVLLMIQVLAQSGGDKHLK	108	DITKDNRAVQK
50	RCLPIFVILLLLIASAPSVDVRPKAK	109	GGSLSMLKARAK
51	SSEEEREHAEK	110	CLEKSGAQPNK
52	ELKEKDEAK	111	EKLTFLILVATVLLTIHVLVQSVGDKHLK
53	NANKQGLKPDER	112	LGGVIGLAGSVLVLFILPISHQAK
54	ASDGASAAADLVAR	113	DEGSPLQR
55	LEKNAVAEK	114	ATLQLDAEQR
56	RAADRGMWGK	115	CSTKKCDTLCCQR
57	AATTLEEEKLDIK	116	STNDNGKDTQMK
58	ASDLENVAANRK	117	SSLPSCPRHIVR
59	ETLQEKQE		

Table 2
Venom of *C. flavidus* identified conotoxin proteins and their congruent gene superfamilies.

Protein superfamily	Sequence	Identified protein	Accession numbers	Number
A	DLPCGNKR	Conotoxin [*C. praecellens*]	ATF27581	11
	TDFASGIK	Conotoxin [*C. betulinus*]	ALM87488	
	NAENKQDHVPDK	Conotoxin, partial [*C. betulinus*]	AMP44769	
	NSKVSRDCQK	Conotoxin [*C. praecellens*]	ATF27696	
	ASDGGNAVAKK	RHO conotoxin A-superfamily protein, partial [*C. tulipa*]	ADN79119	
	NSKLSVHFDLQR	Conotoxin [*C. betulinus*]	AMP44603	
	ASDGRNAEAK	Alpha conotoxin lp1.3 [*C. leopardus*]	AAS93426	
	GQGLTDHYRNLR	Conotoxin [*C. andremenezi*]	ATF27411	
	NAAAKASNR	Alpha-conopeptide precursor Fi1.1 [*C. figulinus*]	AIF30337	
		Alpha-conopeptide precursor Bt1.5 [*C. betulinus*]	AIF30336	
		Conotoxin [*C. betulinus*]	AMP44635	
B1	STNDNGKDTQMK	ConRl-B; Precursor [*C. rolani*]	P0DKZ0	2
		Conotoxin precursor B1 [*C. judaeus*]	UMA83357	
B3	CLEKSGAQPNK	AlphaB-conotoxin VxXXIVA; precursor [*C. vexillum*]	J7JU64	1
I1	KLSVTFLLILMILPSVTGEK	Conotoxin Ep11.1; precursor [*C. episcopatus*]	P0C253	1
J	VTPGSPGTAQLSGHR	J-superfamily conotoxin Vt14.7 precursor [*C. planorbis*]	ADZ74179	1
M	ATLQLDAEQR	M superfamily MLKM group conopeptide Ec2C03 [*C. emaciatus*]	AEX60186	12
		Conotoxin precursor M [*C. judaeus*]	DAZ86453	
	QSEEGGSNATK	M superfamily MLKM group conopeptide Bt3-I04 [*C. betulinus*]	AEX60050	
		Conotoxin precursor M, partial [*C. judaeus*]	DAZ86728	
	MEFILHALGQR	M superfamily MLKM group conopeptide Ca3-Y01 [*C. caracteristicus*]	AEX60069	
	LPTEDHPLYD	M superfamily MMSK group conopeptide Tx3-WP04 [*C. textile*]	AEX60315	
	NANKQGLKPDER	M superfamily MLKM group conopeptide Bt3-D05 [*C. betulinus*]	AEX60096	
		Conotoxin Pl168; alpha-conotoxin Vt1.24; precursor [*C. planorbis*]	D9IWN7	
	QDISPNERKR	M superfamily MLKM group conopeptide Vr3-DPP03 [*C. varius*]	AEX60203	
		Conotoxin precursor M, partial [*C. judaeus*]	DAZ86748	
	NAENKQDHVPDK	M superfamily MLKM group conopeptide Bt3-F02 [*C. betulinus*]	AEX60160	
	ADERGQGLTEQYR	Conotoxin precursor superfamily M, partial [*C. ammiralis*]	UBT01827	
N	MVPARPYWR	Conotoxin Mr15.3; Mr095; precursor [*C. marmoreus*]	P0DM20	1
O1	MTKRCTPAGK	Four-loop conotoxin, partial [*C. coronatus*]	ABO31223	4
	DGLGNLFSKTQHEMKNPETSK	Conotoxin superfamily O1 [*C. episcopatus*]	BAS22531	
	ASDLENVAANRK	Conotoxin precursor superfamily O1, partial [*C. ermineus*]	AXL95517	
	KAHHEMKNPEASK	Omega-conotoxin TxVII; Flags: precursor [*C. textile*]	P56714	
O2	EKLTVLILVATVLLTIQVLAQSDR	Conotoxin Ml15a; precursor [*C. miles*]	C8CK74	12
		Conopeptide Mi037 [*C. miles*]	AKB91375	
	EKLTILVLVAAVLLSTQVLVQGDGEKPQKK	Conotoxin superfamily O2, partial [*C. magus*]	QFQ61085	
	GGVEKRQEAK	Conotoxin VnMEKL-024; precursor [*C. ventricosus*]	Q9BPC5	
	EKLTILVLVATVLLAIQVLVQSDGEKPLKR	XV conotoxin Tx15a precursor [*C. textile*]	AGK23206	
	KLTVLILVATVLLMIQVLAQSGGDKHLK	Conotoxin Lv15a; precursor [*C. lividus*]	C8CK76	
		Conopeptide Mi037 [*C. miles*]	AKB91375	
	RAADRGMWGK	Conotoxin LeD51; flags: precursor [*C. litteratus*]	Q3YEF7	
	EKLTFLILVATVLLTIHVLVQSVGDKHLK	Conotoxin Vx15a; precursor [*C. vexillum*]	C8CK79	
		Conopeptide Mi037 [*C. miles*]	AKB91375	
	EKLTVLILVATVLLAIQVLVQSDREKPLK	XV conotoxin Rt15c precursor [*C. rattus*]	AGK23201	
	SALMRGPR	Conotoxin Bt15a; flags: precursor [*C. betulinus*]	B0KZ78	
O3	QLMLRNNLQK	Conotoxin Eb6.6 [*C. eburneus*]	ADZ99332	2
	GEKQATQR	Conotoxin ArMSGL-0122; precursor [*C. arenatus*]	Q9BP67	
P	CSTKKCDTLCCQR	Alpha-conotoxin PiXXA; precursor [*C. princeps*]	P0DQX2	1
S	EFQRILLR	AlphaS-conotoxin RVIIIA; precursor [*C. radiatus*]	P0C1W3	2
	MSKLGAMFFLLLLFTLASSQEK	Conotoxin superfamily S [*C. episcopatus*]	BAS22718	
T	LCLPVFIILLLLVSPAATLLVKSK	T superfamily conotoxin Bt5.2 precursor [*C. betulinus*]	AGK23247	23
		T superfamily conotoxin Lv5.7 precursor [*C. lividus*]	AGK23257	
	MLFLPVFVILLLLIASAPSVDVRPKAK	Conotoxin superfamily T [*C. episcopatus*]	BAS23011	
	RCIPVFVILLLLIASAPSVDVRPKAK	Conotoxin superfamily T [*C. episcopatus*]	BAS23049	
	TLQTLSNK	Conotoxin superfamily T [*C. episcopatus*]	BAS24914	
	RCLPVFIILLLLIPSALSLIAKPK	Conotoxin leo-T1; precursor [*C. leopardus*]	P0C906	
	NDVHRAILHDVAK	T superfamily conotoxin Lv5.4 precursor [*C. lividus*]	AGK23254	
	TKDDMSLASFHENAKR	Conotoxin superfamily T [*C. episcopatus*]	BAS25030	
	RCLPVVVILLLLIASTPNVDARPKTK	Conotoxin superfamily T [*C. episcopatus*]	BAS25357	
	ILQVIESK	Conotoxin superfamily T [*C. episcopatus*]	BAS23613	
	QTLQILSNKR	Conotoxin superfamily T [*C. episcopatus*]	BAS23504	
	TLQILSNNR	Conotoxin Pn-B01121; Flags: precursor [*C. pennaceus*]	Q9BPF3	
	DDMSLASFQDNAKR	Conotoxin pu5.3; flags: precursor [*C. pulicarius*]	P0C638	
	RCLPVVIILLLLIPSALSVHAQPKTK	Conotoxin superfamily T [*C. magus*]	QFQ61110	
	LCLPVFIILLLLVSPAATLPVK	T superfamily conotoxin Bt5.4 precursor [*C. betulinus*]	AGK23249	

Table 2 (*continued*)

Protein superfamily	Sequence	Identified protein	Accession numbers	Number
	CLPVFVILLLLIASISSVDALLKTK	Conotoxin superfamily T [*C. episcopatus*]	BAS24261	
	CLPVFVILLLLTASVPSVDARPMTK	Conotoxin superfamily T [*C. episcopatus*]	BAS25235	
	RCLPIFVILLLLIASAPSVDVRPKAK	Conotoxin superfamily T [*C. episcopatus*]	BAS23127	
	GLPVFVILLLLIASAPSVDARPKTK	T superfamily conotoxin Eb5.4 precursor [*C. ebraeus*]	AGK23262	
	NDVILDSLR	Conotoxin im5.5 [*C. imperialis*]	ADZ99324	
	DNAKRILQVLESK	Conotoxin superfamily T [*C. episcopatus*]	BAS25452	
	TLQTLMNK	Conotoxin superfamily T [*C. episcopatus*]	BAS24911	
Conkunitzin	EVVMEKQAMMEK	Conotoxin superfamily conkunitzin 10, partial [*C. magus*]	DAC80558	1
Cerm	MKVAVVLLVSLLAVTYALPEKR	Conotoxin precursor Cerm06 [*C. ebraeus*]	UMA82331	1
17	SSLPSCPRHIVR	Conotoxin-like precursor unassigned superfamily 17 [*C. ermineus*]	AXL95660	1
Total	**65**			**76**

3.1. Potential pharmacological applications C. flavidus conopeptides

The potential pharmacological activities of conopeptides from *C. flavidus* were investigated, revealing a diverse array of bioactive peptides within the venom. Notably, seven peptide fragments belonging to protein superfamily A were identified (Table 2). Members of superfamily A are known to target voltage-gated ion channels, presenting therapeutic potential in the management of various neurological disorders such as epilepsy, schizophrenia, and neurodegenerative diseases like Alzheimer's and Parkinson's disease. Moreover, two peptides from protein superfamily B1 exhibited specificity towards the N-methyl-D-aspartate receptor (NMDA), suggesting potential applications in pain management and epilepsy treatment. Twelve peptides from superfamily M, known to interact with K+ channels, Na+ channels, and nicotinic acetylcholine receptors (nAChR), suggest potential roles in pain management, stroke treatment, epilepsy, and neurological disorders, as well as potential implications in cancer therapeutics. Within the O1 superfamily, four peptides were identified, recognized for their interactions with Ca+ channels, K+ channels, Na+ channels, and nAChR, indicating therapeutic avenues for pain relief, stroke mitigation, hypertension management, and treatment of various neurological disorders including epilepsy and cancer. Similarly, the twelve peptides from the O2 superfamily, previously known to modulate neuronal pacemaker and Ca+ channels, show potential for pain, hypertension, arrhythmias, and epilepsy. Proteins from the S superfamily (two identified) is known to exhibit affinity towards serotonin receptors and nAChR, highlighting their potential in managing neuropathic pain and related conditions. The identification of 23 peptides from the T superfamily, known to target the noradrenaline transporter, somatostatin-3 receptor, and possibly Ca2+ and Na+ channels, suggests therapeutic implications in pain management, stroke treatment, hypertension, and epilepsy. Furthermore, a peptide from the I1 superfamily was found to target Na+ channels activator, indicating potential applications in the treatment of heart failure and pain. Lastly, Conkunitzin superfamily proteins, which target K+ channels, show promise in the management of neurological disorders and cancer. In conclusion, the diverse range of conotoxin peptides derived from *C. flavidus* presents a significant opportunity for drug discovery. These findings underscore the potential of these peptides as promising candidates for the development of novel therapeutics across a spectrum of medical conditions, ranging from neurological disorders to cancer.

3.2. Potential pharmacological activities of non-conopeptides of C. flavidus

Our analysis of *C. flavidus* venom revealed a complex bioactive profile. We detected 42 peptide fragments belonging to 19 non-conotoxin proteins, including insulin, hyaluronidase conohyal-P1, protein disulfide isomerase, arginine kinase, and vitamin K-dependent gamma carboxylase (Table 3). Of particular interest, the presence of three insulin peptide fragments suggests potential hypoglycemic activity of *C. flavidus* venom. These peptides could potentially reduce blood glucose levels by activating the human insulin receptor (hIR). This discovery highlights the possibility of cone snail insulin peptides contributing to the future development of fast-acting insulin analogs with improved hIR affinity.

4. Discussion

Marine neglected creatures represent promising sources for drug discovery. Eight drugs from ignored marine organisms were approved, most of them are used in treatments of cancer.[31] These approved drugs are from sponge and tunicate.[32] Conopeptides from piscivorous and molluscivorous cone snail venoms have attracted biomedical attention[33] However, the peptide constituents of worm-hunting species are still poorly understood, even though they have potential as sources of pharmacological compounds.[34–36] Therefore, vermivore snails could also be valuable for pharmacology.[37–38] Marine cone snails endowed with venoms rich in bioactive peptides to target different biological activities for defence and quickly immobilize their prey. Conopeptides have been well studied especially from mollusc and fish-hunting cone snail venoms.[33] On the other hand, conopeptides profile of worm-hunting species is poorly understood, although they have capacity as promising sources of pharmacological compounds.[35] In view of that vermivore snails could also be important for pharmacology field.[38–39] In our study we used proteomic techniques based on MS which have become popular for studying conotoxin sequences on a large scale. For example, different parts of the venom duct of *C. textile* were analyzed by proteomics to understand how the venom is processed.[40] Conopeptide profile of vermivores cone snails; *C. taeniatus* was studied from Red Sea,[41] *C. flavidus* and *C. frigidus* from Queensland.[42] Combining the data from proteomics and transcriptomics would help to make sense of the MS results.[43–44] In the present study, we detected more than one hundred different components in the venom of *C. flavidus*. This variability may enable *C. flavidus* to modify the composition of the injected venom according to the predatory or defensive stimuli. Variations in number of peptide sequences between *C. flavidus* and other *Conus* peptides was observed. For example, a total of 276 peptides were identified in *C. imperialis* venom, 298 in *C. fulgetrum* venom and 488 different molecular masses in *C. crotchii* venom and 290 in *C. taeniatus*.[19,41,45] Substantial differences in peptide numbers in the proteomic analysis of *Conus* species may be due to different conditions used for peptide authentication or difference in methods of venom collection or total number of collected specimens and pooled data.[46–47] Conopeptides in the venom of *C. flavidus* was analyzed the resulted data revealed that T, O2, A and M were the main groups of conotoxins in *C. flavidus*, suggesting important role in the venom. Previous studies

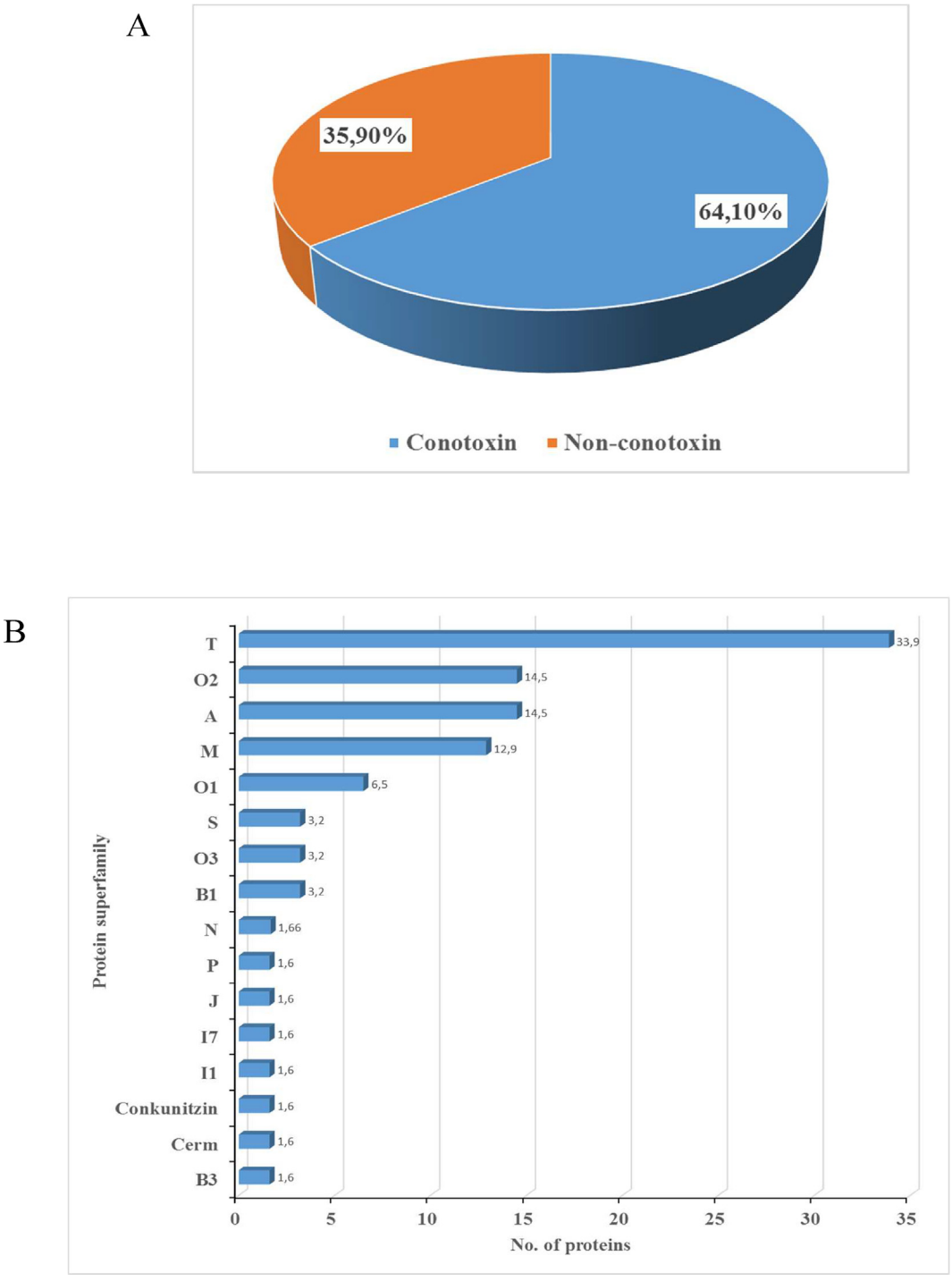

Fig. 2. (A) *C. flavidus* venom proteome; percentage composition of non-conotoxin proteins and conotoxin superfamilies. (B) *C. flavidus* venom relative abundance of conopeptide superfamilies. (The percent relative abundance of total identified conotoxin proteins by LC-MS/MS).

showed that O1, M, and T conotoxins are common among cones and may give a fundamental set of conotoxins necessary for the venom to work well.[25,48] It was also found that I 2, O1, O2, M, and T superfamilies are dominant among worm hunters.[42] The T-superfamily peptides in *Conus* venom can affect various types of neurotransmitters or ion channels.[49–50] The T-superfamily was found to be dominant in another cone snails; *C. taeniatus*,[37] and *C. victoriae* venom.[51] However, this group of conotoxins is poorly understood, despite being abundant in *C. flavidus* and other *Conus* species. Conotoxins have various targets that make them valuable for treating a variety of diseases, such as cancers, depression and pain.[52–53] For instance, peptides of M − superfamily, that are common in *Conus* venom,[54] able to block nicotinic acetylcholine receptors or voltage-gated potassium and sodium channels. O-superfamily conopeptides can block potassium channels and voltage-gated calcium.[55–56] A member of O1 superfamily; ziconotide, is commercially available as potent analgesic which relief pain by specifically blocking the N-type voltage-gated Ca^{++} channel,.[57–58] M- and O-superfamilies are the main superfamilies in *C. bullatus*, *C. marmoreus*, and *C. pulicarius*, *C. tribblei*,.[51] Moreover, conopeptides of A-superfamily are the dominant in *C. bullatus C. consors* and

Table 3
Venom of *C. flavidus* identified non-conotoxin proteins list and their congruent gene superfamilies.

Protein superfamily	Sequence	Identified protein	Accession numbers	Number
Arginine kinase	NLTKGVFEQLK	Arginine kinase, partial [*C. araneosus*]	AQM52449.1	3
	LGFGNLDPEGKMIK	Arginine kinase, partial [*C. frigidus*]	ARU12142	
	KLAAMQQQQR	Arginine kinase [*C. litteratus*]	ARS01451	
Conopeptide class: Cono-NPY	EELMNYVRELNL	Neuropeptide Y2-like conopeptide; NPY2-like conopeptide [*C. betulinus*]	P0CJ23	2
	EELMNYVR	Neuropeptide Y1-like conopeptide; NPY1-like conopeptide [*C. betulinus*]	P0CJ22	
Cytochrome *b*	SIPNKLGGVIGLAGSVLVLFILPLAHQAK	Cytochrome *b* [*C. isabelarum*]	ATZ70070	2
	LGGVIGLAGSVLVLFILPISHQAK	Cytochrome *b* [*C. irotchii*]	ATZ70161	
Cytochrome *c* oxidase subunit	EGTFQGYHSIK	Cytochrome *c* oxidase subunit 3 [*C. infinitus*]	ATZ69735	1
Endoplasmic reticulum oxidoreductin isoform X2	EDNLSITEDWLQGMCLEKR	Endoplasmic reticulum oxidoreductin isoform X2, partial [*C. geographus*]	AYU65461	
Ferritin	DFLEGNYLQEQVR	Venom-related protein ferritin [*C. judaeus*]	UMA83642	3
	ELGDYITNL	Venom-related protein ferritin [*C. judaeus*]	UMA83642	
	ETLQEKQE	Venom-related protein ferritin [*C. ebraeus*]	UMA82671	
Glutaredoxin	SGKLLQLL	Glutaredoxin, partial [*C. ebraeus*]	ATY36129	1
Glucose-regulated protein	DITKDNRAVQK	78 kDa glucose-regulated protein, partial [*C. amadis*]	AKZ17802	1
Hyaluronidase conohyal-P1	QLQCQRLQEHIR	Conohyaluronidase, partial [*C. magus*]	DAC80621	2
	KYQDESIK	Hyaluronoglucosaminidase; precursor [*C. purpurascens*]	C0HKM3.1	
Hypothetical protein	SSLPSCPRHIVR	Hypothetical protein, partial [*C. magus*]	QFQ61209	1
Insulin	DEGSPLQR	Hormone insulin-related peptide [*C. judaeus*]	UMA83320	3
	GGSLSMLKARAK	Venom gland insulin precursor Mo2 [*C. mucronatus*]	UNO36808	
	GGTNDGGKK	Con-Ins G2b A chain; Precursor [*C. geographus*]	A0A0B5ADT3	
NADH dehydrogenase subunit	LGILLANFLILVIFPLAGK	NADH dehydrogenase subunit 4 [*C. venulatus*]	APH08616	3
	MLGILLANFLILVIFPLTGKKWSWYLK	NADH dehydrogenase subunit 4 [*C. venulatus*]	ATZ69978	
	GILLANFLILVILPLISKKWSWYLK	NADH dehydrogenase subunit 4 [*C. trochulus*]	ATZ69913	
Prolyl 4-hydroxylase	DRDRLTYHAGCPVLMGNK	Prolyl 4-hydroxylase [*C. miles*]	AXL97329	2
	IGLSVSPVK	Prolyl 4-hydroxylase, partial [*C. amadis*]	AXL97325	
Protein disulfide isomerase (PDI)	QTSDIINWLNKK	Venom-related protein PDI [*C. ebraeus*]	DAZ85958	12
	ELKEKDEAK	Protein disulfide isomerase [*C. eburneus*]	ADZ76591	
	SEPVPENNDQPVK	Venom-related protein disulfide isomerase [*C. ebraeus*]	UMA82691	
	QTSDIINWLNKK	Conotoxin-specific protein disulfide isomerase variant 2 [*C. textile*]	AMM62657	
	ELKEKDEAK	Protein disulfide isomerase [*C. eburneus*]	ADZ76591	
	QAVDIVNWLK	Protein disulfide isomerase [*C. tessulatus*]	AOZ19957	
	QASDIVQWLK	Protein disulfide isomerase [*C. virgo*]	ADZ76590	
	ELKEKDDVK	Venom-related protein PDI [*C. judaeus*]	DAZ87008	
	AAASLEEEKSNIK	Protein disulfide isomerase [*C. magus*]	QFQ61179	
	TASDIVQWAMEK	Venom-related protein disulfide isomerase [*C. judaeus*]	UMA83674	
	DQESAGALALK	Protein disulfide isomerase [*C. tessulatus*]	AOZ19957	
	AATALEEQNLNVKLGKVDATVEDSLAAK	Protein disulfide isomerase [*C. textile*]	AMM62648	
Peptidyl prolyl cis–trans isomerase (PPI)	NIEKVGSQNGKTSAK	Peptidyl prolyl cis–trans isomerase A, partial [*C. frigidus*]	ARU12144	2
	KIEASETDERDKPK	Peptidyl prolyl cis–trans isomerase B, partial [*C. frigidus*]	ARU12147	
Potassium voltage-gated channel Kv1.1-like protein	EEEKPLPQNEFQR	Potassium voltage-gated channel Kv1.1-like protein [*C. betulinus*]	ASK12219	1
Rimp-03	KSGMLLFVLLLVLPLAFPKLVPVQR	Superfamily Rimp-03, partial [*C. magus*]	QFQ61145	1
Ribosomal protein S6 kinase beta-1 protein	GKLNLPPYLTNEAR	Ribosomal protein S6 kinase beta-1 protein, partial [*C. ebraeus*]	ASF90537	1
Vitamin K-dependent gamma carboxylase	ANVIQEMNNTQAK	Vitamin K-dependent gamma carboxylase, partial [C. imperialis]	AAL78318	2
	EKNNTQRVNK	Vitamin k-dependent gamma carboxylase, partial [C. monile]	ANC48003	
Total	**45**			**43**

C. geographus,[51] and together with the O-superfamilies, they can affect nicotinic acetylcholine receptors and potassium channels.[45] Therefore, conopeptides in *C. flavidus* seems to have potential for drug development and biomedical applications as they can target different receptors and ion channels. Venom peptides of *C. flavidus* have been studied from different geographical regions; Red Sea (this study), South China Sea near Hainan, China[59] and Queensland, Australia.[42] In these studies, marked variation of peptide profile is observed. Such variation is

the expected, because cone snail species in different marine habitats have different biotic interactions leads to a consistent difference in *Conus* venoms.

Several non-conopeptide proteins in addition to conopeptides, were also detected in *C. flavidus* venom. Hyaluronidase (Hyals), an endoglycosidase that breaks down the glycosaminoglycan hyaluronic acid, several peptide fragments were found in *C. flavidus* venom. In fact, many small predators use strong and fast acting venoms for immobilizing and killing their prey. Hyals have been discovered in venoms from different animal groups; in invertebrates such as spiders, honeybees and scorpions and in vertebrates such as stonefish, snakes and reptiles.[60–62] Basically, the venom Hyals is mainly function to degrade polysaccharides of extracellular matrix in the connective tissue of the prey, thus disrupt the integrity of its structure.[63] Therefore, venom Hyals work as spreading factors to facilitate other venom components distribution through different tissues,[64] which is essential for quick immobilisation of prey as well as protection against different predators.

Different peptide fragments of insulin were also detected, suggesting the possible hypoglycemic activity of *C. flavidus* venom. Specific insulin-like protein can be released by some cone snails in water to arrest fish and capture their prey. In this regard, *C. geographus* significantly reduces the blood glucose level and triggers hypoglycemic shock in its fish prey by releasing insulin like peptide (Con-Ins-G1).[17] It is reported that Con-Ins-G1 as the smallest naturally effective activator of the human insulin receptor (hIR).[16] Other species of cone snails such as *C. tulipa* and *C. kinoshitai* can also release specific insulins for fish hunting.[15,17] Thus, cone snail insulin peptide discovery may potentially contribute to future design of fast-acting insulin analogues with improved affinity for hIR. The enzyme family protein disulfide-isomerase (PDI), which can ensure the proper folding of proteins was detected in the venom of *C. flavidus*. PDI gives stability to proteins through linking cysteine residues.[65–66] Venom glands of several classes of insects were found to contain PDI such as *Aphidius ervi*[67] and *Psytallia* species,[68] and in *Pteromalus puparum* crude venom extract,[69] *Diversinervus elegans*[70] and *Cotesia chilonis*.[71]

Interestingly, venomous peptides and samples from COVID-19 patients have shown an intriguing correlation. patient samples were found to contain conotoxin-like peptides from various snail species including *C. flavidus*.[72] Furthermore, the SARS-CoV-2 genome has been reported to contain regions encoding oligopeptides identical to animal venom neurotoxins.[73] These findings suggest several potential mechanisms for the presence of these peptides, including direct viral replication, SARS-CoV-2 genome directly read by bacteria after bacteriophage-like activity,[74] or a kind of bacterial response to the virus.[75–77] The interaction of SARS-CoV-2 with nicotinic acetylcholine receptors (nAChRs) is well-established, with the virus potentially mimicking the actions of neurotoxins (Nadwa 2023). This interaction could lead to neuroinflammation, cytokine storm, and other COVID-19 complications.[78–80] It is well known that some conotoxins can target nicotinic acetylcholine receptors and ion channels. They also can alter acetylcholine levels and cause dysfunction of the receptors.[81–83] This could explain some of the symptoms related to the nerves that some COVID 19 patients experience, such as reduced smell, taste, and signs of the Guillain–Barre syndrome.[72] Therefore, specific conotoxin peptides of *C. flavidus* may be able to inhibit the essential protein of life-threatening viruses COVID-19, potentially leading to their treatment or cure. Therefore, further research is warranted to explore this potential connection. Key avenues of investigation include determining if there's a direct sequence homology between conotoxin-like peptides in COVID-19 patient samples and those definitively identified in *C. flavidus* venom. Additionally, computational modeling could investigate potential interactions between *C. flavidus* conopeptides and nAChRs affected by SARS-CoV-2, or with the SARS-CoV-2 spike protein itself. These investigations could offer novel insights into COVID-19 pathophysiology and potentially uncover new therapeutic strategies. While the potential applications of *C. flavidus* conopeptides in combating COVID-19 remain speculative, the connection between these peptides and the virus presents an exciting and much-needed area of research.

CRediT authorship contribution statement

Mousa O. Germoush: Writing – review & editing, Supervision, Funding acquisition. **Maged Fouda:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis. **Hamdy Aly:** Writing – review & editing, Writing – original draft, Resources, Methodology, Formal analysis. **Islam Saber:** Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis. **Barakat M. ALRashdi:** Writing – review & editing, Writing – original draft. **Diaa Massoud:** Writing – review & editing, Writing – original draft. **Sarah Alzwain:** Writing – review & editing, Writing – original draft. **Ahmed E. Altyar:** Writing – review & editing. **Mohamed M. Abdel-Daim:** Writing – review & editing. **Moustafa Sarhan:** Writing – review & editing, Writing – original draft, Resources, Data curation, Conceptualization.

Declaration of competing interest

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

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