Bioassay-Guided Isolation and Mass Spectrometric Characterization of Antinociceptive Components from *Naja naja oxiana* Snake Venom

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Abstract

Introduction. Snake venoms contain structurally diverse peptides and proteins with pharmacological activities relevant to nociception. Given the reported analgesic potential of some cobra venom three-finger toxins (3FTxs), this study aimed to fractionate previously selected antinociceptive material from *Naja naja oxiana* (NNO) venom using a modified chromatographic sequence and characterize the resulting fractions by electrospray ionization-ion-trap tandem mass spectrometry (ESI-Ion-Trap MS/MS).

Methods. Crude venom was fractionated by Sephadex G-50 size-exclusion chromatography (SEC), followed by semi-preparative RP-HPLC of the active fraction and subsequent CM-Sepharose cation-exchange chromatography. The resulting F3121 and F3122 fractions were evaluated for toxicity and antinociceptive activity using the formalin-induced nociception model and characterized by ESI-Ion-Trap MS/MS. Peptide assignments were obtained by Mascot searching against Swiss-Prot.

Results. The modified SEC–RP-HPLC–CM-Sepharose workflow successfully separated the selected antinociceptive material into two final fractions, F3121 and F3122, which were independently evaluated in the formalin test. Both fractions reduced nociceptive responses compared with the saline control, with F3122 showing a greater reduction in pain scores at the tested dose. Mascot analysis provided peptide-level evidence for a cytotoxin 1a-related profile in F3121 (score 92; 38% sequence coverage) and a cytotoxin 2-related profile in F3122 (score 90; 35% sequence coverage). The corresponding database entries had reported theoretical molecular masses of approximately 9.40 and 7.09 kDa, respectively. Given the limited peptide-level evidence and the sequence homology among cobra cytotoxins, the assignments remain provisional and do not establish the complete molecular identities of the active fractions. The LD_{50} values of F3, F312, final F3121, and F3122 were 2985, 2285, 1248, and 980 µg/kg, respectively.

Conclusion. The modified SEC–RP-HPLC–CM-Sepharose pathway yielded two biologically active fractions and provided peptide-level evidence for cytotoxin-related components. Further structural and pharmacological studies are required to determine their molecular identity and direct contribution to antinociception.

Keywords: antinociception; Caspian cobra; cytotoxin-related protein; ESI-MS/MS; formalin test; ion trap; Mascot; *Naja naja oxiana*; snake venom

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1. Introduction

Pain involves complex peripheral and central neural pathways, and limitations of current analgesics continue to motivate the search for alternative molecules. Venoms are rich sources of bioactive proteins and peptides, including phospholipases A2, proteases, Kunitz-type inhibitors, disintegrins, and three-finger toxins (3FTxs). Several venom-derived molecules modulate nociception through ion channels, neuronal receptors, and intracellular signaling pathways [1-4]. Peptides and proteins are of particular interest because their structural diversity enables interactions with ion channels, receptors, and other molecular targets [5-7].

Cobra venoms contain numerous three-finger toxins (3FTxs), including neurotoxins and cytotoxins/cardiotoxins. Cobrotoxin and α-cobratoxin are α-neurotoxins, whereas cytotoxins/cardiotoxins form a distinct subgroup with different pharmacological properties. Previous reports of antinociceptive cobra-venom components, including najanalgesin, which has been reported to show sequence homology to cardiotoxins, provide a rationale for investigating toxin-related components in active venom fractions [8–19]. These reports do not establish that cytotoxins generally have antinociceptive activity or that a specific toxin is responsible for the effects observed in the present study. **Table 1** summarizes selected venom-derived analgesic components and their reported molecular targets.

Table 1. Selected venom-derived analgesic components and their reported molecular targets and antinociceptive findings.

Toxin /Component	Source (venom)	Targets / Mechanism	Analgesic Findings	References
Cobrotoxin (short α-neurotoxin)	*Naja atra*	Neuronal nAChRs (central cholinergic); adenosine A1 R at low dose.	Potent analgesia in mice (hot-plate, writhing) at 33-75 µg/kg IP; central injection is analgesic; effect blocked by atropine (M receptor), not by naloxone; involves A1 adenosine signaling at low dose.	[13, 14]
α-Cobratoxin (long α-neurotoxin)	*Naja atra* (and similar in *N. kaouthia*)	Neuronal nAChRs (central cholinergic); muscarinic M4 receptor inhibits T-type Ca^{2+} channels	Dose-dependent analgesia in rodents at 30-60 µg/kg; atropine (10 mg/kg) blocks effect; inhibits DRG T-type Ca^{2+} currents via M4/PKA. (Analgesia is CNS-mediated; opioid-independent).	[15, 16]
Cobratoxin (long α-neurotoxin)	*N. kaouthia*	Spinal α7 nAChR	Intrathecal 0.56-4.5 µg/kg produces dose-dependent analgesia in neuropathic pain model; effects mimicked by α7 agonist and reversed by α7 antagonist (MLA), showing α7-R involvement.	[17]
µ-EPTX-Na1a (62-AA peptide)	*Naja atra*	Voltage-gated Nav1.8 channel	Highly selective Nav1.8 blocker (IC50 in low nM); produces potent analgesia in inflammatory and neuropathic pain (rodents) surpassing morphine; no observed toxicity at effective doses.	[10]
Najanalgesin (~6.7 kDa toxin)	*Naja atra*	Spinal MAPK (inhibits c-JNK pathway)	Reverse neuropathic pain (spinal nerve ligation) by inhibiting JNK phosphorylation in the spinal cord; analgesic effect evident in allodynia assays.	[18]
Mambalgin-1 (peptide)	*Dendroaspis polylepis*	ASIC1a/ASIC1b (acid-sensing ion channels)	Blocks ASIC1a/1b with IC50 ~ 11-300 nM; produces potent analgesia in inflammatory and neuropathic models (rodents) by inhibiting proton-mediated nociception.	[19, 20]

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The Caspian cobra *Naja naja oxiana* (NNO) is an important venomous species distributed in the Caspian region and adjacent areas. In our previous study, an antinociceptive fraction (F381) was isolated from NNO venom using SEC followed by cation-exchange chromatography and RP-HPLC and was shown to attenuate formalin-induced nociceptive behavior in rats [21], but the active constituent was not definitively identified.

The present work was designed as a complementary purification and molecular-characterization study. The chromatographic order was changed to SEC → semi-preparative RP-HPLC → CM-Sepharose cation exchange. The final F3121 and F3122 fractions were independently evaluated for antinociceptive activity and characterized by ESI-Ion-Trap MS/MS. Unlike our previous study, the present work includes mass-spectrometric analysis of the final fractions, providing peptide-level evidence about their composition, and does not establish that the alternative sequence is superior in separation efficiency.

2. Materials and Methods

2.1. Venom and Chemicals

Lyophilized NNO venom was obtained from at the Venomous Animals and Antivenin Production Department, Razi Vaccine and Serum Research Institute, Karaj, Iran. Chromatographic reagents and general purification materials were the same as those described previously [21].

2.2. Animals and formalin test

Antinociceptive activity was evaluated in Wistar rats weighing 140-180 g according to the previously established protocol [21]. Fractions were administered intraperitoneally 30 min before intraplantar injection of 50 µL of 5% formalin into the right hind paw. The screening dose was 130 µg per rat (equivalent to approximately 750 µg/kg. Animals were observed for 60 min, and pain-related behavior was scored every 15 *s* using a four-point scale. 0, no unusual movement of the injected paw; 1, brief lifting of the injected paw; 2, repeated lifting or holding of the paw above the floor; and 3, licking, biting, or extensive movement of the injected paw. Mean scores were calculated for each 5-min interval; 0-10 min and 10-60 min represented the early and late phases, respectively. Saline was the negative control, and morphine (5 mg/kg) served as the reference analgesic. Detailed individual scores and complete statistical outputs are provided in the supporting information.

2.3. Bioassay-guided purification

Crude NNO venom (200 mg) was dissolved in 10 mL double-distilled water and centrifuged at 11,000 *rpm* for 15 min at 6°C. The supernatant was applied to a Sephadex G-50 column (150 ×5 cm) equilibrated with 0.05 M ammonium acetate buffer, pH 7.0, at 4°C. Elution was performed at 60 mL/h with 10-mL fractions and monitored at 280 nm. The active fraction F3 was subjected to semi-preparative RP-HPLC on a ZORBAX 300SB-C18 column (9.4 × 250 mm, 5 µm). Solvent A was water/0.1% TFA, and solvent B acetonitrile/0.1% TFA. After a 0-20% B step over 1 min, B was increased to 55% over 45 min at 1 mL/min. Collected peaks were desalted and concentrated using Amicon centrifugal ultrafiltration devices with a 3 kDa molecular weight cut-off (MWCO), according to the manufacturer's instructions. The active RP-HPLC fraction F312 was separated on

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CM-Sepharose column (20 × 1 cm) equilibrated with 20 mmol/L sodium acetate buffer, pH 6.7, at 4°C and eluted with a linear 0-1.0 M NaCl gradient. Two major peaks were collected and designated as F3121 and F3122, desalted, concentrated, and tested independently.

2.4. Toxicity and dose selection

Acute toxicity was evaluated as described previously [21]. Briefly, groups of NIH mice (18-20 g; n = 4/group) received intravenous administration through the tail vein in a volume of 0.5 mL/mouse, and mortality was monitored for 24 h. LD_{50} values were calculated using the Spearman-Karber method as described previously [21]. Previously reported crude-venom and F3 toxicity values were used for dose selection; the present study additionally determined LD_{50} values for F312 and the final F3121 and F3122 fractions.

2.5. ESI-Ion-Trap MS/MS and Mascot analysis

F3121 and F3122 were desalted and concentrated before ESI-Ion-Trap MS/MS. Electrospray generated peptide ions, precursor ions were isolated according m/z, and collision-induced dissociation produced product-ion spectra. The resulting MS/MS spectra were searched against Swiss-Prot database using Mascot (Matrix Science). Searches used trypsin specificity, carbamidomethylation of cysteine as a fixed modification, and deamidation of Asn/Gln as a variable modification. Mascot score, expectation value, precursor mass, peptide sequence, and protein sequence coverage were considered together. Because Naja cytotoxins are highly homologous, the highest-scoring database hit was not treated as species- or isoform-specific unless supported by discriminatory peptide evidence. The peptides were separated by reversed-phase LC using a binary acetonitrile/water gradient containing 0.1% formic acid and analyzed by an Agilent 6330 Ion Trap mass spectrometer operated in Expert mode with the AutoMS(n) acquisition strategy and Peptide Scan option.

2.6. Statistical analysis

Pain scores were expressed as mean ± SEM and analyzed using GraphPad Prism. Time-course data were evaluated by two-way ANOVA followed by Tukey's multiple-comparisons test, with $p < 0.05$ considered significant. Full ANOVA and post hoc outputs are provided in the Supporting Information **Tables S7A** and **S7B**.

3. Results

3.1. Bioassay-guided purification

SEC of crude NNO venom produced several major fractions (**Figure 1**), and F3 was selected on the basis of the antinociceptive screening results. Semi-preparative RP-HPLC of F3 yielded 13 fractions (**Figure 2**), and F12 that designated as F312 was selected for further purification. CM-Sepharose cation-exchange chromatography of F312 produced two major peaks, designated F3121 and F3122 (**Figure 3**). Importantly, F3121 and F3122 were each tested independently in the formalin model before MS/MS characterization. Thus, the biological activity of the final fractions was experimentally demonstrated rather than inferred from their chromatographic origin.

3.2. Acute toxicity and antinociceptive activity

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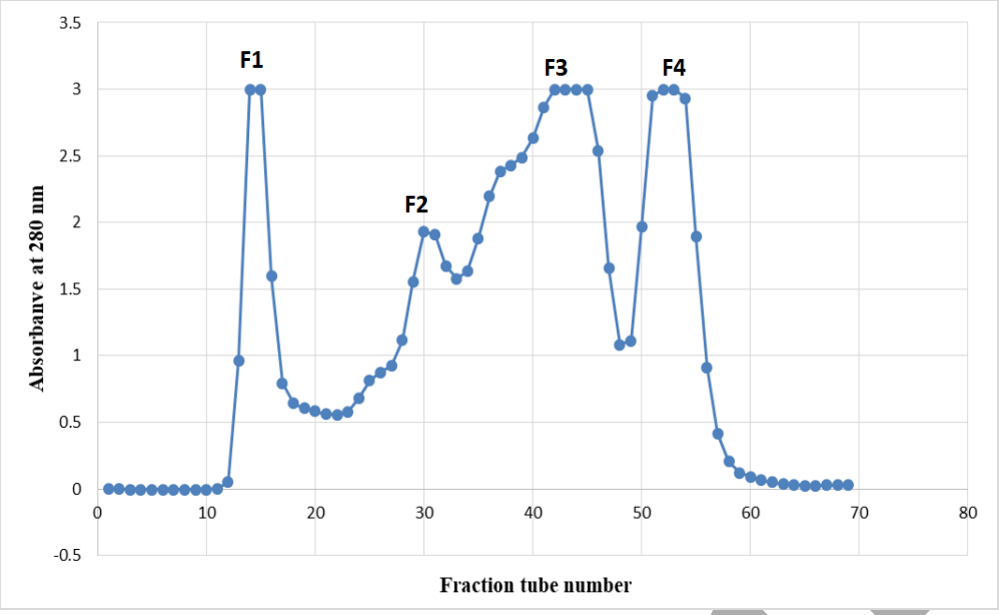

Figure 1. Size-exclusion chromatographic profile of crude NNO venom on Sephadex G-50. Absorbance was monitored at 280 nm. F3 showed antinociceptive activity and was selected for further purification.

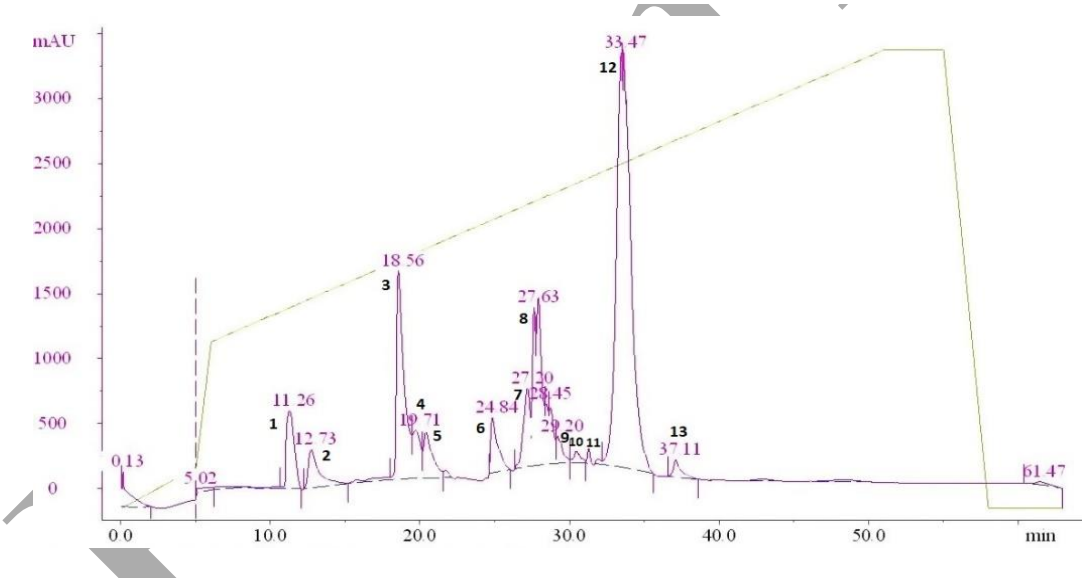

Figure 2 Semi-preparative RP-HPLC profile of the selected SEC antinociceptive fraction (F3), the collected fractions are indicated, and fraction F12 (designated as F312) was selected for further cation-exchange separation on the basis of the antinociceptive screening results.

The LD_{50} values determined in the present purification pathway were 2985 µg/kg for F3, 2285 µg/kg for F312, 1248 µg/kg for final F3121, and 980 µg/kg for F3122. The previously reported crude-venom LD_{50} was 414.9 µg/kg [21]. A sublethal screening dose of 130 µg/rat was used for the formalin experiments, corresponding to approximately 750 µg/kg. Because the study was not designed as a dose-response experiment, these results should be interpreted as evidence of antinociceptive activity at the tested dose rather than as measures of potency or therapeutic index.

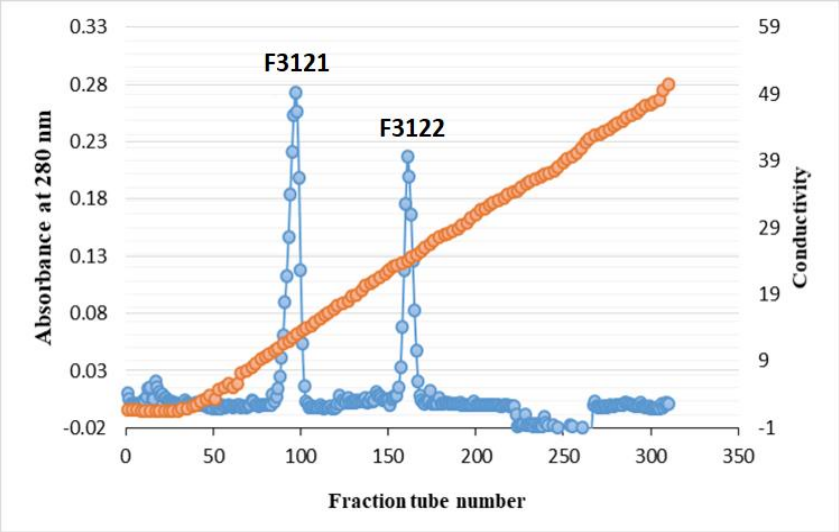

Figure 3. CM-Sepharose cation-exchange chromatogram of fraction F312. The two major peaks collected for separate characterization are designated F3121 and F3122.

3.3. Antinociceptive activity

The formalin assay showed the expected biphasic nociceptive response. Upstream bioassay-guided screening identified F3 and F312 for further purification. **Figure 4** illustrates the time-dependent changes in pain intensity following administration of F3 and F312 in the formalin test. Similarly, both final fractions, F3121 and F3122, reduced nociceptive behavior compared with the saline-treated control and Morphine groups (**Figure 5**). F3122 produced the greater overall reduction in pain scores at the tested dose. These findings indicate that both F3121 and F3122 contain components associated with antinociceptive activity. Pain-scores data for NNO venom fractions and morphine (5 mg/kg) are presented in **Table 2**. The complete time-course data, individual observations, ANOVA results, and Tukey's comparisons are supplied as Supporting Information.

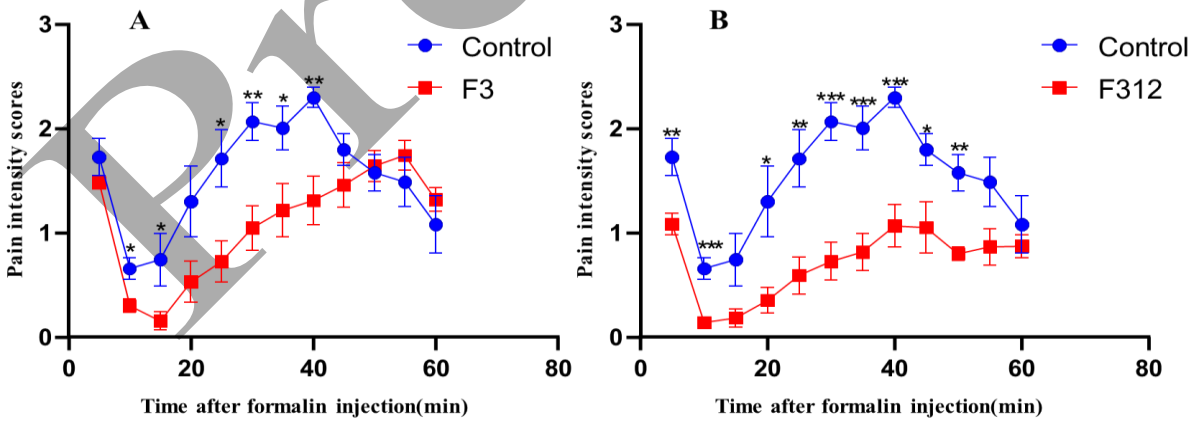

Figure 4. Pain intensity scores recorded during the formalin-induced nociception assay (data are expressed as mean ± SEM (n = 6–10 rats per group). Intraplantar administration of the F3 fraction from SEC (graph A) and F312 from semi-preparative RP-HPLC (graph B) into the right hind paw significantly attenuated nociceptive responses compared with the saline-treated Control. Statistical significance is denoted by *p ≤ 0.05, **p < 0.01, and ***p < 0.001.

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Table 2 Analgesic effects, expressed as pain scores, of active *Naja naja oxiana* venom fractions and morphine (5 mg/kg) following 5% formalin injection. Data are presented as mean ± SEM. Statistical differences are indicated as *p<0.05, **p<0.01, and ***p<0.001. ANOVA and Tukey's multiple-comparisons results are provided in Supplementary information Tables S7A and S7B.

Intervals (min)	0-5	5-10	10-15	15-20	20-25	25-30	30-35	35-40	40-45	45-50	50-55	55-60
	Mean ± SEM											
Control (saline)	1.72 ± 0.56	0.66 ± 0.33	0.74 ± 0.80	1.3 ± 1.08	1.71 ± 0.87	2.06 ± 0.58	2.005 ± 0.67	2.29 ± 0.31	1.79 ± 0.48	1.57 ± 0.55	1.48 ± 0.75	1.08 ± 0.87
F3	1.48 ± 0.21	0.30 ± 0.21*	0.15 ± 0.27*	0.53 ± 0.62	0.72 ± 0.63*	1.04 ± 0.67**	1.21 ± 0.81*	1.31 ± 0.74**	1.46 ± 0.68	1.64 ± 0.47	1.75 ± 0.45	1.32 ± 0.36
F312	1.08 ± 0.11**	0.14 ± 0.06***	0.18 ± 0.09	0.35 ± 0.12*	0.59 ± 0.18**	0.72 ± 0.18***	0.82 ± 0.18***	1.07 ± 0.20***	1.05 ± 0.24*	0.80 ± 0.07**	0.87 ± 0.18	0.87 ± 0.11
F3121	1.43 ± 0.04	0.33 ± 0.04*	0.07 ± 0.02*	0.20 ± 0.02*	0.40 ± 0.03**	0.79 ± 0.03***	0.84 ± 0.02***	0.77 ± 0.02***	0.73 ± 0.02***	0.93 ± 0.03**	0.82 ± 0.04*	0.71 ± 0.03
F3122	1.00 ± 0.07**	0.10 ± 0.04***	0.00 ± 0.00*	0.03 ± 0.02*	0.12 ± 0.05***	0.38 ± 0.05***	0.60 ± 0.02***	0.45 ± 0.02***	0.56 ± 0.03***	0.50 ± 0.02***	0.53 ± 0.04***	0.50 ± 0.02
Morphine	1.34 ± 0.18**	0.14 ± 0.13	0.09 ± 0.06*	0.06 ± 0.07**	0 ± 0***	0.05 ± 0.04***	0.24 ± 0.16***	0.22 ± 0.10***	0.44 ± 0.29***	0.57 ± 0.19***	0.67 ± 0.38*	0.69 ± 0.19

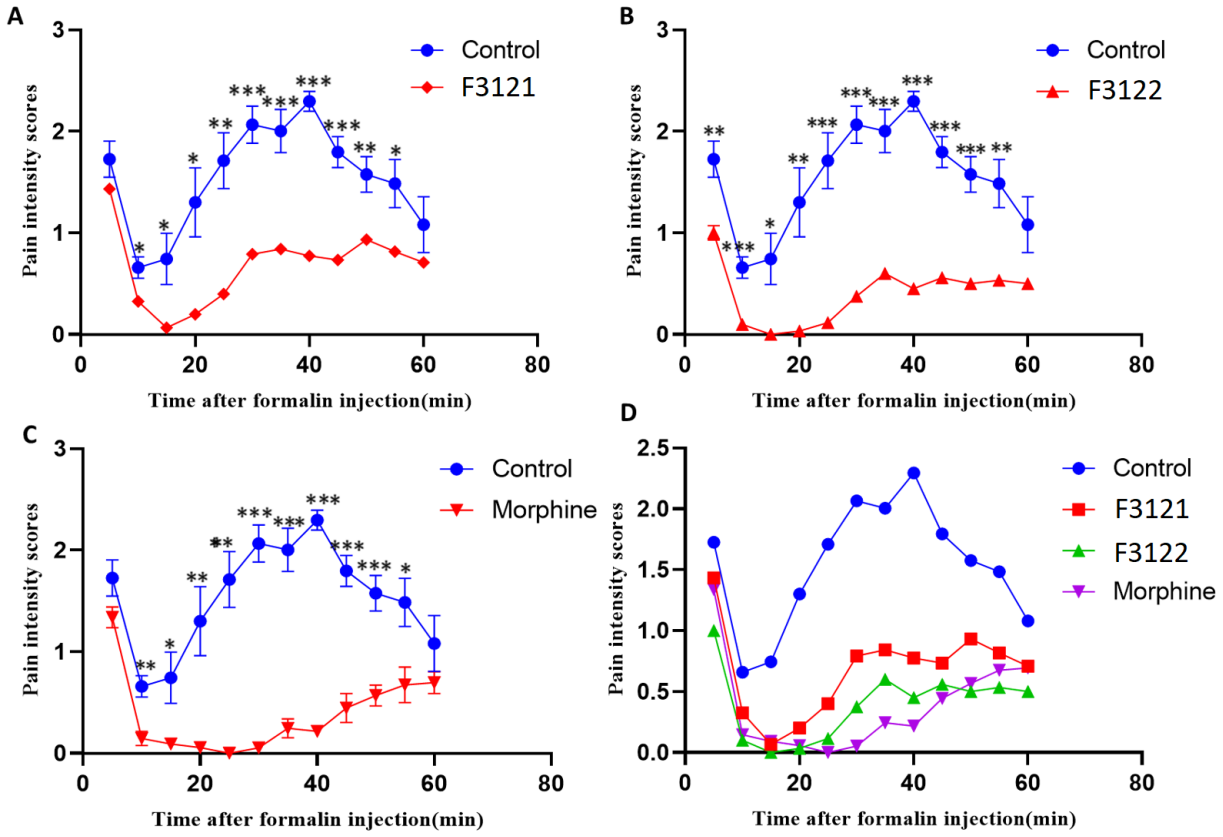

Figure 5. Pain intensity scores following formalin-induced nociceptive stimulation in rats treated with the final CM-Sepharose fractions F3121 and F3122. Data are presented as mean ± SEM (n = 6–10 rats per group). Significant attenuation of nociceptive responses during the evaluated Phases was observed for both F3121 and F3122 compared with the saline-treated control (graph A and B respectively). Panels also show the comparative responses of the control, F3121, F3122, and morphine (5mg/kg) groups (C and D). Statistical significance is indicated by *p ≤ 0.05, **p < 0.01, and ***p < 0.001. Detailed pain-score data and complete ANOVA/Tukey outputs are provided in Supplementary information Tables S7A, S7B)

3.4. Mascot/MS/MS Characterization

The Mascot search of F3121 returned a top-scoring database hit corresponding to cytotoxin 1a, from *Naja atra* (3SA1_NAJAT), with score 92 and 38% sequence coverage, corresponding to a predicted molecular mass of approximately 9.40 kDa. Four non-duplicate peptide-spectrum matches (PSMs) were reported: MFMMSDLTIPVKR (score 76; expectation 1.7×10^{-6}), GCIDVCPK (40; 0.037), YVCCNTDR (38; 0.015), and YVCCNTDRCN (32; 0.016). The strongest peptide-level score, MFMMSDLTIPVKR, showed a clear annotated MS/MS spectrum and was selected as the primary representative F3121 spectrum (**Figure 6A**); a second representative F3121 spectrum for GCIDVCPK is shown in **Figure 6B**.

The database assignment to *Naja atra* was interpreted cautiously. The observed peptide sequences occur within highly homologous cobra cytotoxins, and the experimentally analyzed venom was NNO. Thus, the result supports a cytotoxin-related 3FTx component with a CT1-like peptide profile but does not establish a definitive *Naja atra* species assignment. A separate Mascot search of the smaller F3121 dataset produced a different top cytotoxin entry from *Naja sputatrix* (score 47; 19% coverage) based mainly on shared peptides, illustrating the dependence of database ranking on the available spectra and the non-specific nature of several cytotoxin peptides.

F3122 yielded a more consistent assignment. The Mascot search of F3122 returned 3SA2_NAJOX (cytotoxin 2, *Naja oxiana*) with score of 90 and 35% sequence coverage, corresponding to a predicted molecular mass of approximately 7.09 kDa. Four PSMs representing three peptide sequences were observed: MFMVAAPHVPVK (score 53; expectation 0.0049), the same peptide in a second charge state (34; 0.015), MFMVAAPHVPVKR (70; 0.0001), and YVCCNTDK (30; 0.085). Representative spectra for the two principal F3122 peptides are shown in **Figure 6C-D**. An independent search of the F3122 dataset gave the same protein assignment (score 91; 21% coverage), with MFMVAAPHVPVK (53; 0.0046) and MFMVAAPHVPVKR (70; 0.00026), providing reproducible support for a CT2-related component. Representative Mascot peptide-spectrum matches are summarized in **Table 3**.

Table 3. Representative Mascot peptide-spectrum matches from the Mascot searches. Protein-level interpretations are intentionally phrased as related/CT-like because of sequence homology among *Naja* cytotoxins.

Fraction	Interpretation	Peptide sequence	Mascot score	Expect
F3121	Cytotoxin 1a-related/CT1-like	MFMMSDLTIPVKR	76	1.7×10^{-6}
F3121	Cytotoxin-related	GCIDVCPK	40	0.037
F3121	Cytotoxin-related	YVCCNTDR	38	0.015
F3121	Cytotoxin-related	YVCCNTDRCN	32	0.016
F3122	Cytotoxin 2-related	MFMVAAPHVPVK	53	0.0049
F3122	Cytotoxin 2-related	MFMVAAPHVPVK	34	0.015
F3122	Cytotoxin 2-related	MFMVAAPHVPVKR	70	0.0001
F3122	Cytotoxin 2-related	YVCCNTDK	30	0.085

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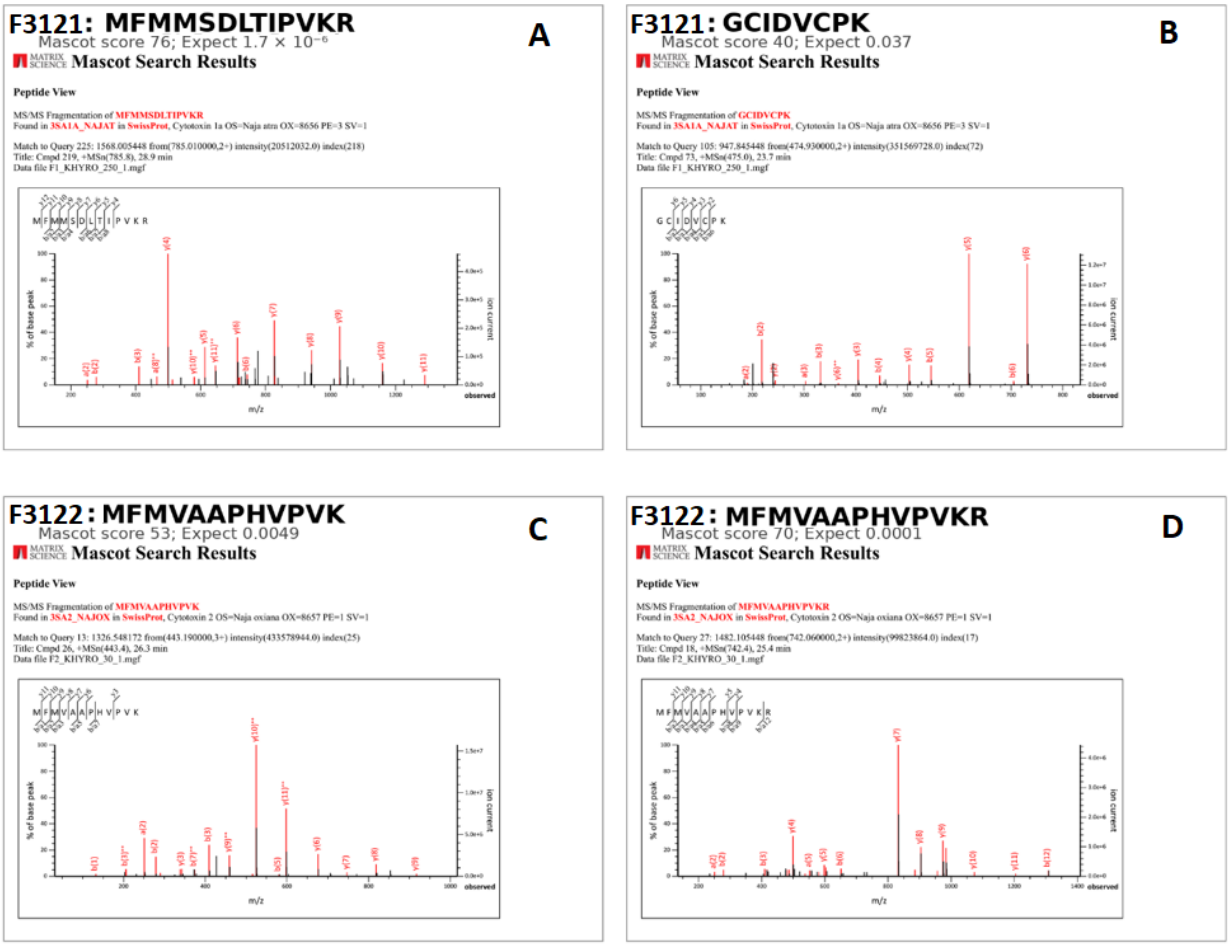

Figure 6. Representative Mascot MS/MS spectra from the final purified active fractions. (A) F3121 peptide MFMMSDLTIPVKR (score 76; expectation 1.7 × 10-6); (B) F3121 peptide GCIDVCPK (score 40; expectation 0.037); (C) F3122 peptide MFMVAAPHVPVK (score 53; expectation .0049); and (D) F3122 peptide MFMVAAPHVPVKR (score 70; expectation 0.0001). Peptide scores and expectation values are summarized in Table 3.

4. Discussion

The principal contribution of this study is the use of an alternative bioassay-guided purification sequence to further resolve antinociceptive material previously obtained from NNO venom. The earlier work used SEC → cation exchange → RP-HPLC and identified F381 as the final antinociceptive fraction [21]. Here reversing the order of the last two chromatographic steps produced F3→ F312→ F3121/F3122 and allowed the final two fractions to be tested separately. In contrast to the previous study, the present work also includes MS/MS characterization of the final fractions. This provides complementary information on their peptide profiles; however, because no direct head-to-head comparison was performed, improved chromatographic efficiency, recovery, purity, or potency cannot be claimed.

Both F3121 and F3122 independently showed antinociceptive properties in the formalin assay. The biological result should therefore be distinguished from the molecular assignment: the formalin experiment demonstrates activity of the fractions themselves, whereas MS/MS identifies

peptides present in those active fractions. The data do not demonstrate that one identified cytotoxin sequence is the sole determinant of the antinociceptive effect, because F3121 and F3122 may contain additional co-purified molecules. In addition, although acute toxicity was assessed, this assessment alone cannot exclude non-specific behavioral effects, such as impaired movement or altered responsiveness that could influence formalin-scored behavior. Dedicated behavioral and motor function assessments, together with testing of individually purified components, will be needed to distinguish antinociception from potential behavioral impairment and to establish the contribution of specific peptide sequences.

The F3121 Mascot result illustrates the difficulty of species– and isoform-level identification in cobra venom proteomics. The strongest search assigned four peptides to cytotoxin 1a from *N. atra*, but the peptide set is compatible with closely related *Naja* cytotoxins. In particular, MFMMSDLTIPVKR is informative for a CT1-like sequence profile, whereas GCIDVCPK and the YVCCNTDR-containing peptides are less discriminatory. Accordingly, the most defensible interpretation is a cytotoxin-related 3FTx component with a CT1-like peptide profile.

F3122 provided stronger and more reproducible molecular evidence. Two independent Mascot searches assigned the spectra to cytotoxin 2 from *N. oxiana*, and the principal peptides MFMVAAPHVPVK and MFMVAAPHVPVKR were repeatedly observed. A previous proteomic study of Iranian *N. oxiana* venom also reported overlapping 3FTx peptides, including MFMVAAPHVPVK, MFMVAAPHVPVKR, and YVCCNTDR, supporting the occurrence of closely related cytotoxin/3FTx sequences in this venom [22]. Nevertheless, the evidence in the present study remains peptide-level rather than complete protein sequencing. The assignment therefore supports a putative CT2-related component, but intact-mass confirmation, broader sequence coverage, and disulfide mapping would be required for definitive structural characterization.

Previous studies have reported antinociceptive effects of several cobra-venom components, including the α-neurotoxins cobrotoxin and α-cobratoxin [9, 13–18]. Najanalgesin, isolated from *Naja atra* venom, has also been reported to produce antinociceptive effects and to show sequence homology to cardiotoxins (cytotoxins) [8, 19]. These reports indicate that antinociceptive activity has been observed among distinct cobra-toxin families, but they do not establish that cytotoxins generally have antinociceptive activity or that the peptide sequences detected in F3121 or F3122 are responsible for the effects reported here. The reported venom-derived analgesic molecules act through different mechanisms, including cholinergic receptors, Nav1.8, MAPK-related signaling, and acid-sensing ion channels. The present study did not determine the receptor or molecular mechanism responsible for F3121- or F3122-mediated antinociception and therefore does not support assignment of a specific mechanism.

Further studies should include intact-mass measurement, high-resolution MS/MS to expand sequence coverage, peptide mapping and disulfide-bond characterization, and comparison with curated *N. oxiana* cytotoxin sequences. Dose-response formalin testing of independently purified components, together with dedicated motor-function or behavioral-control assays, would help determine whether a specific peptide contributes directly to the observed antinociceptive phenotype and whether non-specific behavioral effects can be excluded.

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5. Conclusion

An alternative bioassay-guided chromatographic strategy further fractionated antinociceptive material from NNO venom into two final fractions, F3121 and F3122. Both fractions showed antinociceptive activity in the formalin model at the tested dose, with F3122 producing the greater reduction in pain scores. Unlike the previous study, the present work provides MS/MS-derived peptide-level evidence for a CT1-like cytotoxin-related profile in F3121 and a reproducible CT2-related profile in F3122. Because cobra cytotoxins are highly homologous and sequence coverage was incomplete, these assignments remain provisional at the intact-protein level. The current data do not establish that an individual cytotoxin-related peptide is responsible for the observed activity, and they do not demonstrate quantitative superiority of the chromatographic workflow. Further structural and pharmacological studies are required.

Acknowledgments

This project was a collaboration between the Chemistry & Chemical Engineering Research Center of Iran (CCERCI) and the Department of Human Vaccine and Serum at the Razi Vaccine and Serum Research Institute (RVSRI), Iran.

Authors' Contributions

Mehdi Kheirollahpour: investigation, formal analysis, software, and writing – original draft. Nader Shokoufi: supervision, project administration, validation and review. Nasser Mohammadpour Dounighi: conceptualization, methodology, resources, and review.

Ethics

All animal experiments were conducted in accordance with the relevant institutional guidelines and were approved by the Research Ethics Committee of the Razi Vaccine and Serum Research Institute (approval No. IR.RVSRI.REC.1401.018; 9 December 2022).

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

Underlying pain-score datasets and statistical outputs, are provided in the Supporting Information or are available from the corresponding author upon reasonable request. Mascot search reports and representative MS/MS spectra are retained with the study data and can be supplied as Supporting Information.

Supporting Information

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Detailed pain-score datasets and statistical outputs for the formalin assay (Tables S1-S6), including the ANOVA and Tukey's multiple-comparisons results (Tables S7A and S7B), are provided in the Supporting Information. Full Mascot search reports and additional MS/MS spectra for F3121 and F3122 are also provided.

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