Pharmacological studies (Analgesic and Hemolytic) on the cone snail venom
*Conus coronatus* Gmelin, 1791

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**Abstract:** The venom of the cone snails has a rich source of novel peptides with pharmaceutical activity. The aim of this study was to investigate the hemolytic, and analgesic effects of *Conus coronatus* venom. Samples were collected from Qeshm Island, Persian Gulf. The venom ducts were isolated and kept on ice then homogenized. The mixture was centrifuged and the supernatant was considered as a crude venom. The hemolytic activity was performed on human red blood cell and purification was carried out by using gel filtration chromatography on Sephadex G-25. The analgesic effect was evaluated via intraperitoneally (IP) injection in mice. Finally, the molecular weight of the analgesic fractions was determined by using Tricine-SDS-PAGE. Results showed that the crude venom exhibited no hemolytic activity on human erythrocytes and the purified fraction number C2 with dose 0.5 mg/kg showed the best analgesic activity in both acute and inflammatory pain and exhibited a dose-dependent analgesic effect (*P*<0.05) containing peptides with the molecular weight less than 6.5 kDa. The venom of the *C. coronatus* from the Persian Gulf contains an analgesic component for relieving acute and inflammatory pain with a small size and no toxicity which can lead to finding a new analgesic drug.

**Keywords:** Analgesic, Hemolytic, Venom, Conotoxin

**Introduction**

Conotoxins and several neurotoxins have been isolated from venoms, which have analgesic activity in animal models (Han et al., 2008; Lee et al., 2010; Shi et al., 2011). Conotoxins are small peptides that divided into two classes: disulfide-rich conotoxins and peptides without multiple disulfide bonds. Both of them are found in cone snail venoms (Biass et al., 2015).

Cone snails are a type of sea snails belong to the *Conus* genus, to the phylum Mollusca which is a widespread genus of the sea snails (Favreau et al., 2012). This genus include the predatory gastropods comprising about 800 species that are found in tropical marine habits (Puillandre et al., 2014). Each *Conus* species generating a distinctive supply of 100-200 venom peptides (Bingham et al., 2012). All of them have different toxicity (Haddad et al., 2006). These unique marine organisms deliver their venoms peptides through a specialized radular tooth (Salisbury et al., 2010; Rajabi et al., 2016).

Conotoxins are very diverse and more than 100 have purified from the cone snail venoms and grouped into pharmacological families according to their molecular targets (Chen et al., 2008; Bingham et al., 2012). Conotoxins are interesting molecules with a diverse human therapeutic potential, such as analgesic, antiepileptic, cardio- and neuro-protective activity (Chen et al., 2008; Bernaldez et al., 2013; Kumar et al., 2014). Also, different conotoxins are under processing to produce drugs. For example, ω-MVI1A (ziconotide) is FDA approved and used to treat acute pain on incurable diseases. The other conopeptides like Conantokin-G, A-Vc1.1, and CGX-1204 are candidates as pharmaceutical drugs, too (Rodreguez et al., 2015).

In this study, analgesic effect was investigated in acute and inflammatory pain in a mouse model of pain induced by formalin. Injection of formalin into mice paws induces a biphasic nociceptive response showed by flinching, licking or biting of the affected paw. An early phase starting immediately after injection and lasting for 0–5 min and a late phase from 20 to 60 min after injection. It is now known that the first phase is due to the direct action of formalin on nociceptors, while the second phase involved the combination of peripheral input and spinal cord sensitization (Dubuisson and Dennis, 1977; Lee et al., 2010; Shi et al., 2011).

*Conus coronatus* is the most common cone snail found in the Persian Gulf coasts. There is no study on
the medicinal potential of the *C. coronatus* venom. Conotoxin diversity as well as their medicinal potential, and eventually the dominant presence of this specie in the Persian Gulf, were the importance and the reasons for this study. The results of this study showed a significant pain relief of conotoxin extracted from the *C. coronatus* of the Persian Gulf in animal models on formalin test without toxicity on blood cells that have not been reported before.

**Materials and Methods**

**Materials**

Acetonitrile, Formalin, Tris base, Acrylamide, and Bis-Acrylamide were purchased from Merck Chemical Company. Also, BSA were obtained from Bio-Rad, Sephadex G-25 and Tricin from Sigma-Aldrich. *Animals, specimens and venom extraction*

Male albino mice weighing 22 to 25 g were chosen after an acclimatization period at least 7 days, in the laboratory environment. Standard food pellets and water was provided for them.

The *C. coronatus* specimens were collected from Qeshm Island, Zeyton Park (south part of Iran) in September 2014. Coordinates of sampling place was: N 26 55' 631", E 56 15' 209". The specimens were kept alive in salt water till dissection and venom duct isolation (Fig. 1). Specimens were dissected on a petri dish on ice and the venom ducts were removed. Conotoxins were extracted from freshly venom ducts. The venom ducts were homogenized at 16000 × rpm for 5 min with cold sterile water and acetonitrile. Then, the mixture was centrifuged at 10000 × g for 20 min at 4°C. Finally, the supernatant was lyophilized and stored at -20°C (Tayo et al., 2010), and bovine serum albumin (BSA) was used as a standard to estimate the protein concentration (Bradford, 1976).

**Hemolytic Study**

Different concentrations of the crude extract from 8 to 1000 µg/ml were subjected to analysis the hemolytic activity by using fresh human red blood cells in the microtiter assay. After incubation for 1 hour at 37 °C, centrifugation was performed at 4 °C for 10 min. The degree of hemolysis was measured in OD540 nm by using an Elisa plate reader (Kumar et al., 2014).

**Purification**

The lyophilized powder was resuspended in distilled water. Gel filtration chromatography on Sephadex G-25 was performed to the purification of the crude extract. Purification protocol was performed at room temperature; the elution was monitored by absorbance at 280 nm, and Tris base 50mM was used as elution (Lee et al., 2010).

**Analgesic activity**

The study was ethically approved by the Ethics Committee of Abadan School of Medical Sciences, Ref: IRABADAN.UMS.REC 1395. 80. Throughout this experiment, the mice were placed in a transparent observation chamber. The analgesic effects of the crude venom and purified fractions were evaluated by formalin test in mice. Normal saline as a negative control and different fractions of conotoxin in the volume of 200 µl, separately injected IP, 45 min before formalin injection. Thereafter, formalin 2/5% at volume 20 µl in saline, were subcutaneously injected into the plantar surface of the left hind paw, and mice behaviors were evaluated. Licking and flinching numbers were counted to a comparative analysis between the groups. The time period for the first phase (acute pain) was from 0-5 min, while the second phase (inflammatory pain) was from 20-40 min after formalin injection. The best venom fraction was selected and used to dose determination (0/5, 0/25, 0/10, 0/01 mg/kg doses). Then, the formalin test was performed as described above (Dubuisson and Dennis, 1977; Lee et al., 2010; Shi et al., 2011).

**Tricine-SDS-PAGE Analysis**

Tricine–SDS-PAGE, based on Tricine-Tris buffer systems, are the commonly used for separating less molecular weight proteins. In this study was utilized 5% stacking gel, 10% spacer and 16% resolving polyacrylamide gels to estimate the molecular weight of the analgesic peptide (Jiang et al., 2016).

**Statistical analysis**
All the data were expressed as mean ± SE. The antinociceptive effect of the fractions was statistically compared with the controls by one-way ANOVA, where p < 0.05 was considered as significant. Statistical analyses were performed by using the Graph Pad Prism software v. 6.

Results

Protein Estimation
The protein concentration in the crude extract of *C. coronatus* venom was about 10.8 mg/ml. protein content in the purified fractions was different, between 1.3-4.2 μg/ml.

Hemolytic Assay
The hemolytic assay was conducted on human erythrocytes revealed that the crude venom of *C. coronatus* induced spontaneous hemolysis of red blood cell just when the protein content was 250 μg/ml, and in the less concentration of the protein, there was no hemolytic activity (Fig. 2).

Protein Purification
The soluble venom was separated by means of gel filtration chromatography using Sephadex G-25 (Fig. 3). Major protein fractions were selected and used for the analgesic activity.

Analgesic activity
Nociceptive behaviors induced by crude venom, fractions, and control was determined among the groups in the first and second phase. The result showed no significant differences in fraction number C1, C3, and C5 with control, and the others (C2, C4, C5, and C7) had the analgesic activity (P<0.05), (Figs 4 and 5).

Comparison between the control and different doses of C2 (0/5, 0/25, 0/10 and 0/01 mg/kg), showed significant differences between dose 0/5 mg/kg and the other doses that could relieve the pain in the second phase, too. Just dose 0/01 mg/kg was similar to the control (P<0.05), (Figs 6 and 7).

Tricine-SDS-PAGE
The analgesic fractions of *C. coronatus* venom were
containing peptide with molecular weights less than 6/5 kDa on the acrylamide gel that was shown in Figure 8.

Fig. 6: Effects of different doses of the C2 fraction on formalin-induced flinching and licking responses in the first phase.

Fig. 7: Effects of different doses of the C2 fraction on formalin-induced flinching and licking responses in the second phase.

Fig. 8: Electrophoretic of the analgesic fractions of C. coronatus venom with silver staining. (Molecular weight marker was obtained from Sitomatin gene Company, Isfahan, Iran).

Discussion
The cone snail (genus Conus), a marine gastropod lives mainly in the tropical habitat of shallow waters near the coral reefs (Puillandre et al., 2014). All species are venomous with different toxicity because they have different types of conotoxin (Haddad et al., 2006; Bingham et al., 2012; Kumar et al., 2014).

Despite the potential of conotoxin as the therapeutic agents, small numbers of them have been defined in detail, and contrary to the different species of Conus species are in the Persian Gulf coasts, no one examined the bioactivity of them.

In the present study, the hemolytic activity observed just on 250 µg/ml protein concentration, which is 25 times higher than the highest dose for formalin test (0.5 mg/kg or 10 µg/ml protein concentration). This result indicated that the Persian Gulf C. coronatus venom has a potential for new analgesic drug design without a hemolytic agent.

The hemolytic activity has been reported in a lot of venomous animals and Conus species (Nallathambi, 1993; Sakthivel, 1999; Nayak, 2011). The venom of C. coronatus has no hemolytic and cytotoxic agents. The hemolytic activity is suggestive of cytolytic activity with anticancer and antiviral agents (Nayak, 2011). Kumar et al (2014), suggested that the venom of the vermivorous cone snail, such as C. lentiginosus does not contain any hemolytic peptide, which is similar to the C. coronatus venom in this study with vermivorous diet.

In the present study, the analgesic activity was measured by using purified venom. Many pharmacologically active components such as CTx-MVIIA, SO-3, ACV1, CVID, and GVIA have been identified from the venom of Conus species (Baby et al., 2011; Elliger et al., 2011). The effect of all analgesic fractions (C2, C4, C6 and C7) were more marked during the acute phase than the chronic pain similar to the results previously reported (McIntosh et al., 2000; Zhang et al., 2007; Han et al., 2008; Favreau et al., 2012). Mechanism of analgesic effect of Conus venom shows that when the electrical impulse generated along an axon, sodium ions rush in and potassium ions rush out. Sodium ions accumulation cause to open calcium ion channels. Then the influx of calcium causes acetylcholine to be inserted to synaptic junction. Acetylcholine bindings with receptor proteins alter the shape of the ion channel. This opens the sodium ion channel to let the sodium in. Sodium ions set off an electrical impulse along the next nerve. Finally, the pain signal will work. Blocking channels by conotoxins lead to inhibit pain signals so that the peptides relieve the sensation of pain (Woo1f, 2004; Tabaraki et al., 2014).

On the basis of the results, the fraction C2 of the Persian Gulf Conus venom showed the best analgesic activity in both phases comparing to the others. This finding is similar to the previously reported results by
Lee et al. (2010) and Favreau et al. (2012). The less dose induced balance between the number of ligands and receptors, and cause the analgesic effect in a mouse model was 0/1 mg/kg. In drug delivery systems, the best dose is that it is more effective for longer time and drug can bind to receptors at the most. So, drug dosage should consume properly because of the defined receptors. When the dose of the drug is optimum, negative competition does not happen between drug and receptors (Tiwari et al., 2012).

The results of this study indicated that the Persian Gulf C. coronatus venom is effective in acute pain reduction, without toxicity and is an excellent candidate for acute pain treatment. Electrophoretic of the analgesic fractions of C. coronatus venom on Tricine-SDS-PAGE demonstrated that these conotoxins are less than 6/5 kDa band of the marker, and are about 3-4 kDa. The peptides, which have a weight range between 500 Da to 5 kDa, have recommended as a drug. Small peptides (<500 Da) have low target specificity but they synthetize easily and have high oral bioavailability and membrane permeability. But, the larger peptides (>5 kDa) generally are metabolized more rapidly than small peptides and requires intravenous administration, are expensive to produce (Craik et al., 2013). So, conotoxins have both the selectivity of the larger peptides and the stability and the ease of synthesis of the small peptides that was found in the venom of C. coronatus from the Persian Gulf, so it is suitable for drug design.

Conclusion
This study has demonstrated the bioactivity of the purified fractions of C. coronatus venom. It is supposed that the C. coronatus venom contains a rapid analgesic conopeptide, which would be applicable for treatment of acute and chronic pain comparing to the chemical drug such as morphine with side effects such as addiction. Actually, further purification and structural elucidation of compounds are required to confirm the designation of these compound for a drug.

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References


