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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 purify 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-MVIIA (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

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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).

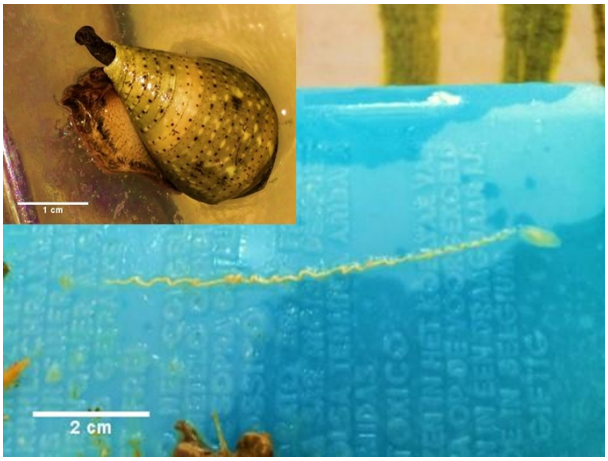

Fig. 1: Venom duct of *C. coronatus*.

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 anti-nociceptive 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).

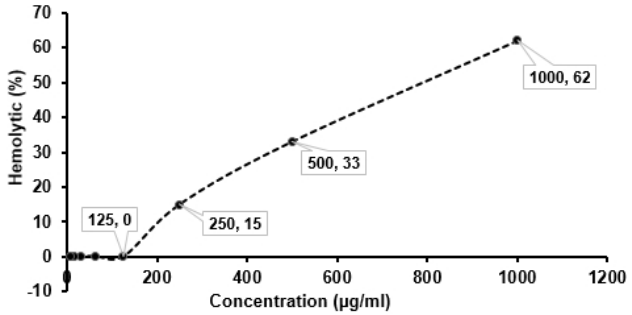

Fig. 2: Different concentrations of the crude venom of *C. coronatus* and their hemolytic activity on human erythrocytes.

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).

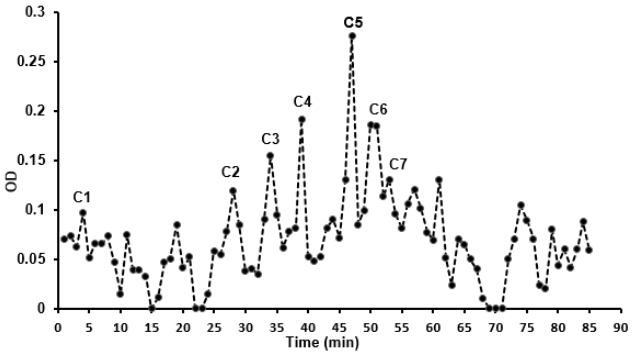

Fig. 3: Purification of the crude extract of *C. coronatus* by Sephadex G-25.

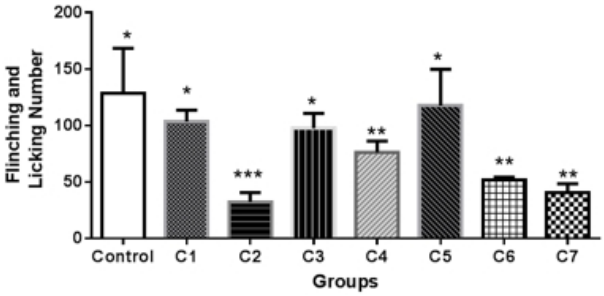

Fig. 4: Effects of different venom fractions on formalin-induced flinching and licking responses in the first phase. (First phase was defined as the flinching and licking response 0–5 min after formalin injection, P<0.05, n=7).

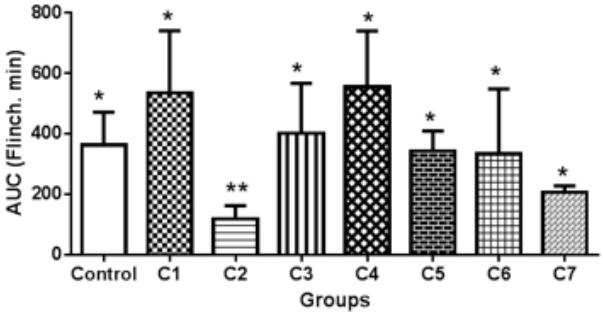

Fig. 5: Effects of different venom fractions on formalin-induced flinching and licking responses in the second phase. (Second phase was defined as the flinching and licking response 20–60 min after formalin injection and AUC was calculated to comparison with the different groups, P<0.05).

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.

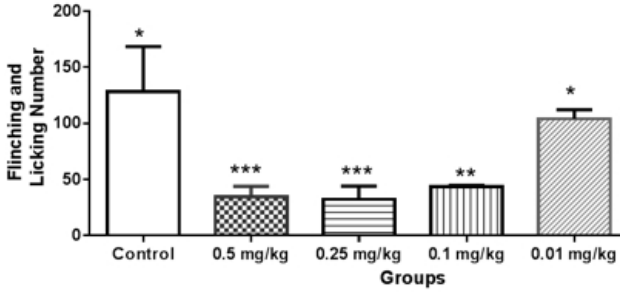

Fig. 6: Effects of different doses of the C2 fraction on formalin-induced flinching and licking. responses in the first phase.

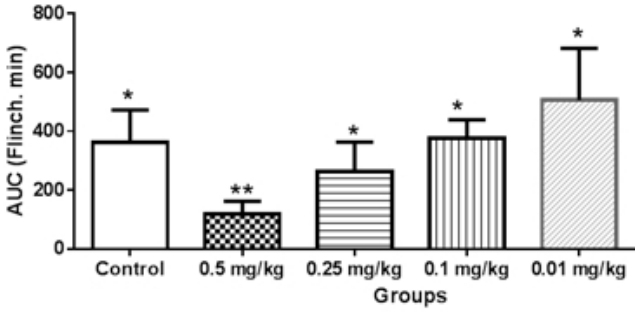

Fig. 7: Effects of different doses of the C2 fraction on formalin-induced flinching and licking responses in the second phase.

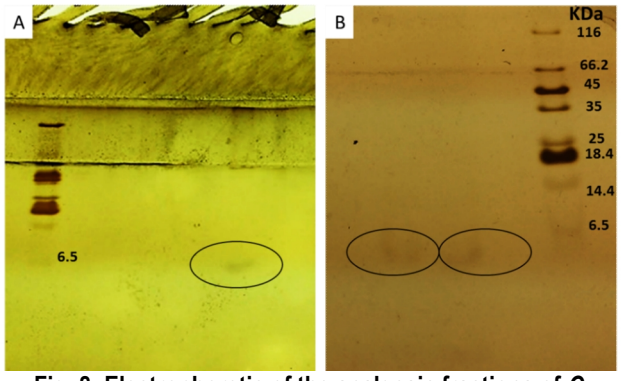

Fig. 8: Electrophoretic of the analgesic fractions of *C. coronatus* venom with silver staining. (Molecular weight marker was obtained from Sitomatin gene Company, Isfahan, Iran).

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 (Woolf, 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.

Acknowledgment

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