Neurotoxic effects of *Alicia mirabilis* and *Aurelia aurita* venoms on *Callinectes sapidus* Rathbun, 1896: behavioural results

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Abstract

Cnidaria constitute an important phylum of venomous animals, several of which have a significant impact on human health and activities. Cnidarian venoms are included in a special capsule called nematocyst, and are known to consist of peptides, proteins, phospholipids, glycoproteins, sterols, bioactive amines and carbohydrates. Cnidarian venoms are used for hunting and defence, and have paralytic, neurotoxic, cytotoxic, dermotoxic and hemolytic effects on other living organisms. In this study, the neurological and behavioural effects of different doses of venom obtained from the nematocysts of *Alicia mirabilis* and *Aurelia aurita* were observed on blue crabs (*Callinectes sapidus*) individuals. For this purpose, various doses of venoms were injected on the linkage between merus and carpus parts of the cheliped of blue crab individuals. The most common effects of *A. mirabilis* and *A. aurita* venoms were observed to be stiffness and trembling behavior in the legs. These symptoms indicate that venom causes neural paralytic syndrome. It has been observed that the effect of venom increases with time and paralysis occurs before death.

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Introduction

Scyphozoa (Phylum Cnidaria) jellyfish make swarms by overproducing or gathering by currents and winds in case of suitable temperature and food availability.[1,2] Jellyfish can be disturbing and dangerous for bathers and sea-workers because of burning and toxicity they can cause after stinging, an event which can occur mainly in coastal areas.[3]

Cnidarian venoms are stored into nematocysts, special capsules typical and exclusive of Cnidaria phylum, which are included into special cells called cnidocytes. Jellyfish use nematocysts for protecting against predators and to catch preys for feeding. Nematocysts are produced by Golgi apparatus and include a protein-based capsule, the venom and a tightly wound thread. When a mechanical stimulus is produced on the nematocyst, the thread penetrates into the prey's tissues in 3 milliseconds, at 2 metres/second speed and with an acceleration of 40,000 g. This event is one of the fastest processes in biology.[4] Tentacles carry about 1,000 to billion counts of nematocysts which after contact with the skin will immediately paralyse or kill predators or preys within fractions of minutes. As a result of the contact with jellyfish, toxic and/or allergic reactions can be induced in humans,[3,6] which can be evidenced by dermal necrosis, oedema, neurotoxic effects, respiratory problems, cardiovascular symptoms and even fatal cases.[7]

Several studies have shown that nematocysts have different shapes and different venom content. More than 30 types of nematocysts have been described.[8,9] Nematocysts are mostly found in tentacles, mouth arms and umbrella margin. Each nematocyst is used only once, and ruptured nematocysts are lysed by adjacent cells.[10]

Cnidaria venoms contain peptides, proteins, phospholipids, glycoproteins, sterols, bioactive amines and carbohydrates.[11] It is

thought that different types of nematocysts have different functions and different venom contents for catching prey.[12] Therefore, each venom has a different effect on stung organisms. Paralytic, neurotoxic, cytotoxic, dermototoxic and hemolytic effects of Cnidaria venoms are known; their activity at cell level, with *in vitro* and *in vivo* haemolysis and cytotoxicity to a wide range of different normal and transformed (cancerous) cell types was shown and summarized in some reviews.[3,13] Recent data report the apoptosis-like cell death and potent cytotoxicity induced by *Chrysaora helvola* nematocyst venom to a human breast cancer cell line (MCF-7) and a poorly differentiated nasopharyngeal carcinoma (NPC) epithelial cell line (CNE-2),[14] the antiproliferative and antioxidant properties of crude nematocyst venom from *Acromitus flagellatus* to human lung cancer (A549) and liver cancer (HepG2) cells,[15] and the protective activity of epigallocatechin-3-gallate in inhibiting the proteases of *Nemopilema nomurai* venom using the immortalized human keratinocyte cell line (HaCaT) and human dermal fibroblasts (HDF) in culture.[16]

Given the increasing interest in the development of *in vitro* systems that aim to avoid the use of living organisms in biological research as much as possible, the use of cell cultures in the framework of applied studies and methodologies is becoming increasingly common.[17] The study by Bulati *et al.*[18] revealed that four fractions of *Anemonia viridis* venom showed different protein contents and cytotoxic effects against human Peripheral Blood Mononuclear Cells (PBMC) and three epithelial cancer cell lines.

Adhikari *et al.*[19] observed haemolytic activity of sea anemone *Paracondylactis indicus* venom in rat erythrocytes. Kalina *et al.*[20] reported 159 peptides including neurotoxins, proteinase, inhibitors and Acid-Sensing Ion Channels (ASICs) in *Heteractis crispa*. Palytoxin which was isolated from sea anemones is one of the most potent known toxins, with *in vivo* studies demonstrating its severe effects across multiple mammalian species, including humans. Its toxic effects include neurotoxicity, rhabdomyolysis, and cardiovascular collapse.[21] Two polypeptides from the sea tropical anemone *Heteractis crispa* were shown to have analgesic effect on mice.[22] Also, it was seen that sea anemones have been known as an important source of peptides and other molecules having biomedical potentials.[23]

Although the potency of Cnidaria venoms depends mainly on the nature of the toxin, also the amount of injected venom, the size of the contact area, the duration of the contact, and the general health and age of the stung person are known to play a fundamental role.[24] Radwan *et al.*[25] noted that *Aurelia aurita* venom causes erythrocyte destruction and loss of skin tissue in humans. It has been determined that *Chrysaora hysoscella* nematocysts cause inflammatory lesions on the skin.[26] *Pelagia noctiluca* venom has been reported to cause bronchial constriction and respiratory distress.[27,28] In addition, *Rhopilema nomadica* and *Cassiopea andromeda* species have been found to cause erythrocyte and leukocyte destruction in humans.[25]

Cnidarians represent an important potential source of new biologically active compounds with different utilizations and therapeutic activity.[29] Neuroactive peptides found in jellyfish venom are thought to have the potential to be used to develop new therapies for ion channel dysfunctions and combating age-related neurological diseases.[30,31] In this connection, this study was aimed to behaviourally observe the neurological effects of different doses of venom obtained from the nematocysts of *Alicia mirabilis* and *Aurelia aurita* on *Callinectes sapidus* individuals. These are the study's preliminary results and the research at the cellular level continues.

Materials and Methods

According to the "Regulation on Working Rules and Principles of Animal Experimentation Ethical Committees" published in the Official Gazette of the Republic of Turkey on 15.02.2014, there is no need for ethics committee permission in animal experiments conducted with invertebrates. Despite this, although invertebrates were not under official legal protection during the study, considering that behavioral research was conducted and live animals were required, possible harms were tried to be minimized as much as possible and the guidelines regarding animal use in the said regulation were carefully followed.

Sampling

This study was conducted between September 2020 and April 2022, but the sampling started in November 2021 because of the pandemic restrictions due to coronavirus disease (COVID-19) in Turkey. Sea anemones *Alicia mirabilis* and medusae *Aurelia aurita* were sampled by trammel net and hand net from a boat in Gökova Bay. The specimens were transported to Muğla Sıtkı Koçman University, Faculty of Fisheries, Department of Basic Sciences, Marine Biology Laboratory. The tentacles of *A. mirabilis* and oral arms of *A. aurita* were separated and stored in containers and subsequently frozen at -20°C.

Callinectes sapidus individuals were obtained alive from Dalyan Fishermen's Cooperative (DALKO) in Köyceğiz, Turkey. Crab samples were brought to Muğla Sıtkı Koçman University, Faculty of Fisheries, Marine Biology Laboratory in a cool and humid environment. The length and weight of the individuals were measured and recorded. The crabs were placed in aquaria of 50×60×100 cm, which were prepared according to the characteristics of their natural environment (ventilation was installed and sediment sand was placed). Aquarium water was prepared from artificial sea aquarium salt, considering the temperature and salinity values of the natural environment of crabs. The crabs were maintained in aquaria for 7 days to adapt before starting the experiments. During the adaptation and trials, the crabs were fed with fish food.

Venom isolation and nematocyst identification

The venom isolation procedure was arranged according to Mariottini *et al.*[32] and Frazão and Antunes.[33] Frozen samples were thawed at +4°C for 2 hours and stirred by shaker overnight for the separation of nematocysts from the tissues. Samples were filtered through 100 µm plankton net to remove tissue residues. Then, the samples were centrifuged at +4°C and 3,000 rpm for 5 minutes. The supernatant was removed and nematocyst suspension was counted under an Olympus CX21 light microscope by the Sedgewick Rafter counting chamber. Nematocyst isolation was continued by repeating centrifugations until the amount of 200,000 nematocysts/mL was reached. Then, nematocyst suspension was sonicated in ice 90 times at 30-second intervals. Sonicated samples were centrifuged at +4°C, 4,000 rpm for 3 minutes to precipitate nematocysts debris. The supernatant (crude venom) was taken and stored at -20°C. The protein content of crude venom was determined by the Bradford method.[34]

The nematocysts of *A. mirabilis and A. aurita* were measured and photographed under a light microscope Olympus CX21 and described according to Godknecht and Tardent[35] and Östman.[9]

Neurotoxic activity test

Juveniles, males and females *C. sapidus* were treated with crude venom of *A. mirabilis* and *A. aurita* and the effects were observed. *A. aurita* venom was injected to 5 females, 3 males and 4 juvenile individuals, and *A. mirabilis* venom to 4 females, 4 males and 4 juvenile individuals. Four doses of the stock solution were applied: i) 150,000 nem/mL; ii) 100,000 nem/mL; iii) 50,000 nem/mL; iv) 30,000 nem/mL, all suspended in isotonic saline.

Crude venom of *A. mirabilis* was applied on *C. sapidus* in two different times; the first experiment was done shortly after the venom isolation and the second experiment was applied about 5 weeks later for considering the possible differences between a fresh sample and a sample maintained in freezing, because it is known that freezing could change the response of cells to venoms. Until the second experiment the venom was stored at -20°C. Venom was injected at the linkage between the merus and carpus parts of the cheliped. The experiments were made in two replicates. In addition, a control group was injected with the same doses of isotonic saline for each application. After venom application, crabs were observed and recorded by video. During these observation periods, crabs were allowed to feed, the nutrition activity was observed to see if they took food or not, and abnormalities in swimming behavior, slowdown in movements, involuntary muscle movements, paralysis and death were recorded.

Results

The crude venom protein amount of *A. mirabilis* and *A. aurita* was determined as 7.926 mg/mL and 2.103 mg/mL, respectively. Gender, length and weight values of *C. sapidus* individuals used in the experiments, application doses and the effects of *A. aurita* and *A. mirabilis* crude venoms are given in Table 1, Figure 1 and 2.

No behavioral or physiological changes were observed in male and female individuals treated with *A. aurita* venom. Only two juveniles (females) were paralyzed after treatment with dose 1 and 2, and these individuals died after 30 minutes. The effect of venom was observed in 2 females, 1 male and 1 juvenile individual injected with *A. mirabilis* venom at doses 3, 2 and 1 respectively. As to the temporal variation of the effects of venom, these individuals released their chelipeds as soon as the venom was injected and tremors were observed in the walking legs in the first minute of the application after putting back them to the aquarium. In the same experiment, this behaviour was not observed in the control group.

After 2 minutes from injection, tremors were observed in walking and swimming legs. Within 6 minutes, involuntary contractions and tremors increased in the legs. At the 7th minute, tremors continued in the legs and chelipeds were motionless. Then, extreme tremors were observed in the swimming legs, the body was constricted and no response to stimuli occurred. After 9

Table 1. Mean, standard deviation (SD), minimum and maximum values of length (mm) and weight (g) of *Callinectes sapidus* individuals.

		Mean	SD	Min	Max
♀	L	159.18	11.38	142	177
	W	185	16.64	156.7	221.2
♂	L	158.56	20.56	133	185
	W	228.5	41.72	139.2	322.1
J♀♂	L	96.78	8.56	85	110
	W	94.69	18.87	65.3	117.56

J, juvenile; L, length; W, weight.

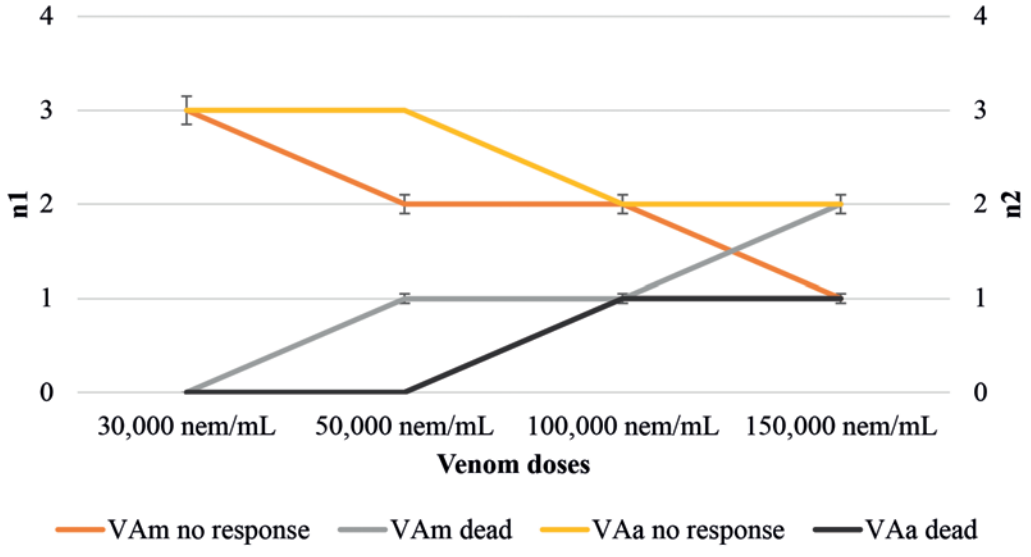

Figure 1. Number of crabs affected and unaffected by venom according to application doses. VAm, *Alicia mirabilis* venom; VAa, *Aurelia aurita* venom; n1, number of crabs treated with *Alicia mirabilis* venom; n2, number of crabs treated with *Aurelia aurita* venom.

minutes, sudden contractions and tremors in the chelipeds and legs were observed. Within 12 minutes, abnormal movement, upside down posture of the body, contractions and tremors in the legs were sighted. After 19 minutes, the abdomens were opened and the swimming legs were contracted, no movement was observed. From the 12th minute onwards, the crabs became motionless and lost their reflexes. Cramp-like movements of the swimming legs were observed and then they showed paralysis. Crabs died at 30 minutes after the injection. The control individuals showed normal activity and gave normal responses to stimuli during the experiment.

In this study, nematocyst types of *A. aurita* and *A. mirabilis* were identified. Microbasic mastigophores and spirocysts were observed in *A. mirabilis* while a-isorhiza, A-isorhiza, eurytele and polyspiras were shown to be the nematocysts of *A. aurita* (Figure 3). Lengths of spirocysts and mastigophores were between 20-45 µm and 50-100 µm, respectively. A-isorhiza, a-isorhiza, eurytele and polyspiras lengths were measured as 6-7 µm, 2-5 µm, 6-14 µm and 8-14 µm, respectively.

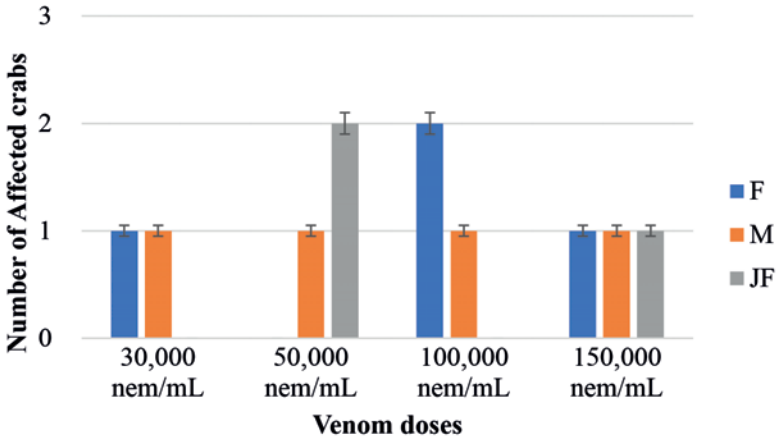

Figure 2. Number of affected crab groups by anemone venom. F, females; M, males; JF, juvenile females.

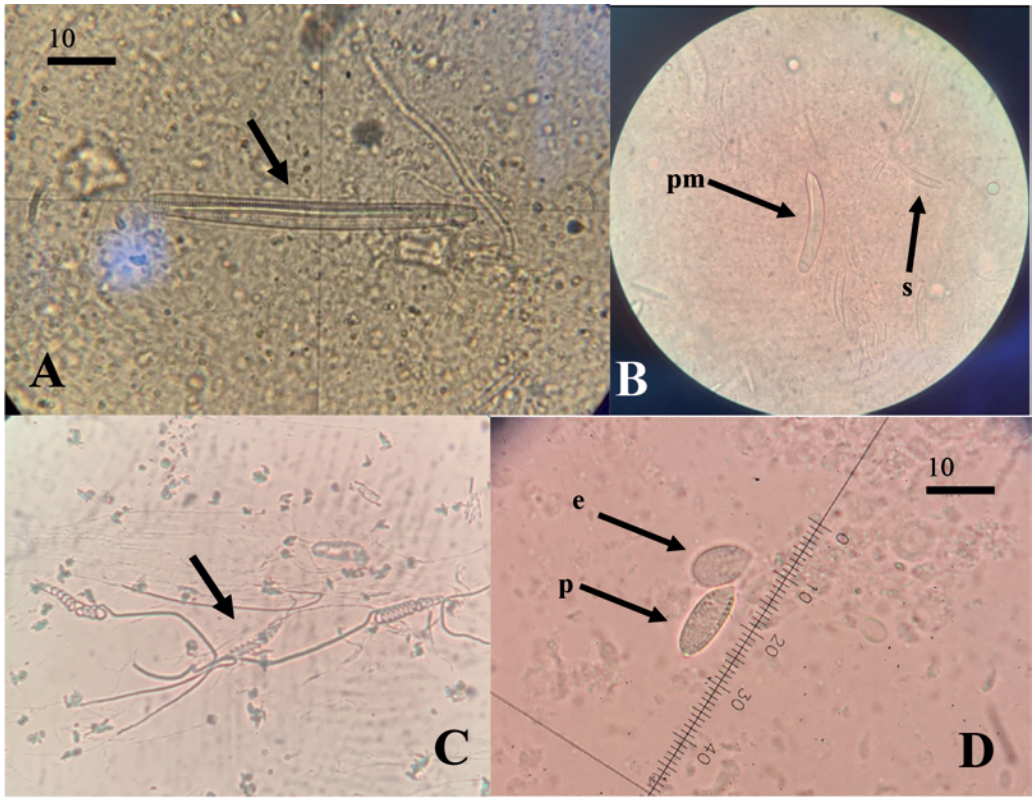

Figure 3. Nematocyst types of *A. mirabilis* and *A. aurita*. A, microbasic b-mastigophore in *A. mirabilis*; B, microbasic p-mastigophore (pm) and spirocysts (s) in *A. mirabilis*; C, discharged spirocysts in *A. mirabilis*, D, polyspiras (p) and eurytele (e) in *A. aurita*.

Discussion

From ancient times it is well known that plants and animals can be an excellent source of compounds having interesting and useful therapeutic properties.[36] The *Alhagi maurorum* (Camel thorn) plant has been used by the Ayurvedic people, and the *Agaricus compestris* species of manta rose has been used in the treatment of diseases in the Caribbean.[36] Among the natural compounds, biotoxins when used at sublethal doses have been viewed as an interesting tool due to their different pharmacological properties, and their study have had an impressive advance during the last decades.[32] In this connection, the venoms and the associated biotoxins coming from aquatic organisms, such as cnidarians, seem to be a potential promising source of useful compounds for biotechnological and pharmacological/therapeutical purposes to be used, in perspective, as antimicrobial, antiviral, antiinflammatory and antitumoral agents.[37] Notably, some substances have been judged useful for the treatment of neurological diseases, thanks to their muscle relaxant, analgesic, and anesthetic properties, and for the study of nerve impulse transmission in neurophysiological research.

Really, the neurotoxins utilized by cnidarians to paralyze and catch preys in their ecological relationships seem to be of special relevance in breaking nervous transmission.[38] This activity of cnidarian venoms seems to be specifically addressed to ionic conductance and neuromuscular junctions, affecting action potentials and voltage-gated ion channels, which are essential for the neuromuscular and neuronal transmission.[30,35]

In the experiments here reported it was seen that the freezing reduced the effect of venom. Fresh venom experiment caused the effects showed in Figure 1, while in the second experiment the crude venom did not affect the crabs.

Previous research was carried out about the activity of cnidarian venoms to crabs showing that a concentration of 10 mg/mL crude extract from sea anemones *Lebrunia danae* caused convulsions, paralysis and death within one minute after treatment in ghost crabs *Ocypode quadrata*; furthermore, the purified toxin injected at lowest concentration (1 mg/mL) induced immediate death.[30] In the same paper, the authors stated that sodium channels in voltage-sensitive nerve and muscular cells of crabs were affected by toxin from another sea anemone too, *Bartholomea annulata*. In another research, Thangaraj and Bragadeeswaran observed similar neurotoxic effects to crabs *Ocypode macrocera* caused by *Stichodactyla mertensii* and *Stichodactyla gigantea* extracts.[39] According to Türeli,[40] in *C. sapidus*, the chelipeds and legs can be deliberately lost through autotomy in case of danger. Venom of *A. mirabilis* had an effect on two females, one male and one juvenile *C. sapidus* at the doses 3, 2 and 1. These crabs released their chelipeds as soon as the venom was injected. Such an effect of the injection was not observed in the control group, and it is thought that the crabs released their chelipeds due to the effect of the venom, not due to the injection process. Other data showed that in fiddler crabs *Uca pugilator* the action potentials of ventral nerve cord giant fibers were affected by two soluble protein fractions of *Aiptasia pallida* nematocyst venom injected into the hemocoele, causing death after convulsive motion.[41]

In a study by Morales-Landa *et al.*,[42] *Carybdea marsupialis, Bartholomea annulata*, and *Stichodactyla helianthus* venoms were injected into adult tilapia fish, and cramp-like movements of the fins and mouth were observed in the fish. Subsequently, the fish began to lose reflexes and coordination, turn sideways and swim in a semicircle, weakening of tremors or loss of reflexes, paralysis, arrhythmia and respiratory obstruction, and the fish died within the first 24 hours after the injection because of *Stichodactyla helianthus* venom. These reactions, except arrhythmia and respiratory obstruction which were not surveyed here, were also seen in our study. In a study of Gülşahin,[43] the venom of *Cassiopea andromeda* was isolated and injected intramuscularly to *Cyprinus carpio* juveniles. Signs of partial paralysis, raking, and immobilized fins were observed in the juveniles consequently. Death was observed for the fishes which were 3-4 g in the weight range. This study observed that *A. aurita* and *A. mirabilis* venoms were effective at doses 2 and 1 in juveniles and low-weight adult individuals. Granulitoxin (GRX) obtained from the anemone *Bunodosoma granulifera* is a neurotoxic peptide and, when applied to mice, it was found to cause circular movements, aggressive behavior, convulsions and death.[44] With the injection of GRX into rats, head tremor, salivation, erectile dysfunction, jumping, wet dog shakes, clonic movements of the forelimbs and death were observed.[45] Because sea anemone neurotoxins affect both neuronal and myocardial sodium channels, further research with purified venom components is needed to determine which effects are due to a specific toxin.[46]

This study is the only *in vivo* study conducted with *Alicia mirabilis* venom. It is seen that *A. mirabilis* venom has similar neurotoxic effects to other venoms from sea anemones. It has also been observed that females and juveniles of *C. sapidus* are more affected by the venom. The smaller body size of juveniles may explain why they are more affected by the venom, but there is no significant size difference between male and female adults. Therefore, it can be said that females are more sensitive to neurotoxic substances than males. This is thought to be due to hormonal, biological and metabolic differences.

PhcrTx2, isolated from the sea anemone *Phymanthus crucifer*, is an Acid-Sensing Ion Channel (ASIC) inhibitor. PhcrTx2 was injected into crabs at 7 different doses and the results were observed. It was reported that the toxin was not lethal but caused tetanic paralysis, upward turning and uncontrolled movements of the legs in crabs starting from the ED50 dose of 707 µg/kg.[47] In our study, venom purification process was not performed. However, it can be said that the behavioral effects of anemone venoms on crabs are the same as in other studies and the peptides obtained from the venom of *A. mirabilis* species have paralyzing-activity.

Rodríguez *et al.*[48] distinguished macrobasic p-amastigophores from the column vesicles of *Alicia mirabilis*. Cengiz[49] defined 6 nematocyst types (A-isorhiza, a-isorhiza, O-isorhiza, eurytele, birhopaloid and polyspiras) from *A. aurita*. The length of these types showed similarity with what was observed in the present study. Spirocysts are typically more abundant than nematocysts in the tentacles of sea anemones.[50] As far as we observed, 64 out of every 100 cnidocysts in the tentacles of *A. mirabilis* were spirocysts.

Neurotoxins interfere with the signal communication process by affecting the transmission of neurotransmitters in synaptic sites.[51] In this study, it was also demonstrated behaviorally that anemone venom affects neurotransmission. Although this study was conducted as a student project and with a very low budget, it revealed important findings regarding the neurotoxic effects of Cnidaria species. However, further studies are needed to physiologically demonstrate the neurotoxic effects of Cnidaria venoms.

Conclusions

In this study we observed that the neurotoxic effect of jellyfish venoms began immediately after application on *C. sapidus* individuals. The most common effect of *A. mirabilis* and *A. aurita* venoms is stiffness and trembling behavior in the legs. These symptoms indicate that venom causes neural paralytic syndrome. It has been

observed that the effect of venom increased with time and paralysis occurred before death.

It has been determined that the venom isolated from *Aurelia aurita* affects juvenile individuals at high doses only. The protein amount of *A. aurita* venom was found to be lower than *A. mirabilis* venom, and therefore it is thought to not affect adult individuals. The high amount of protein increases the effect of venom. Also, it has been determined that the effects of venoms of 2 different species with different nematocyst types are different.

This study revealed that jellyfish crude venoms are neurotoxic and induce behavioral effects to blue crabs. The damage caused by these effects on nerve cells should be investigated in further studies.

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