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Research Article

Investigating biological characteristics of two jellyfish (*Rhopilema nomadica* and *Chrysaora hysoscella*) venoms on human fibroblast

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Abstract

This paper aims to investigate the biological and cytotoxic characteristics of venoms extracted from *Rhopilema nomadica* and *Chrysaora hysoscella* jellyfish collected from the Persian Gulf. The cytotoxic effect of the venoms is surveyed on human fibroblast skin cells (HU02) using the The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Moreover, the protein molecular weight was determined and the toxicity test (LD50) of venoms were explored on BALB/c mice. The *R. nomadica* venom possessed a higher protein concentration with the lowest molecular weight protein via SDS-PAGE (12.5 %) along with more peaks obtained by HPLC. In addition, the results of both LD50 and MTT are affected by the venom characteristics. Besides, intravenously and intraperitoneally LD50 were 1.26 and 2.35 for *C. hysesella* and 0.65 IV and 1.6 IP for *R. nomadica*, respectively, suggesting that *R. nomadica* venom was more lethal. The results of the MTT assay on Hu02 fibroblast cells for 24 h revealed that *R. nomadica* had more lethal effects on skin cells compared to *C. hysoscella*. Escherichia coli, whereas methicillin-resistant Staphylococcus aureus had no antibacterial activities in the presence of both *R. nomadica* and *C. hysoscella* venoms. Meanwhile, the venoms had antibacterial effects on Pseudomonas aeruginosa and S. aureus, which were still weak compared to the other reported species. Overall, *R. nomadica* venom was more lethal in the case of mice and human skin cells and likely with more symptoms in prey. Finally, considering the relatively high viability of different bacteria in diverse dilutions of venom, it seems that victims not only should face dermal injuries and possible scars caused by direct stings but also the presence of venomous protein on the dermal tissue may provide a cultivation medium for different kind of bacteria.

Keywords: Bacterial viability rate, Bradford assay, Human fibroblast skin cells [HU02], Jellyfish venom, MTT assay

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Introduction

Major investigations on marine organisms have been focused on bioactive compounds for various purposes, including the discovery of novel drugs, nutritional supplements, and applications for industrial biotechnology (Urbarova *et al*., 2012). In recent years, attention has been paid to natural products derived from the sea, because they are natural, more compatible with the human body and the environment, and less harmful than synthetic products (Gulhan and Selamoglu, 2016). Venomous marine organisms are potentially an important source of pharmacological tools with promising biological activities. One of the ways to use marine products is to manage resources and control pollution in order to increase the production efficiency of products (Selamoglu, 2021).

The Persian Gulf with high and extensive temperature fluctuations and naturally elevated salinity is a hostile environment for marine organisms (Sheppard *et al.,* 1992). This sea accommodates numerous venomous marine organisms including jellyfish such as *Rhopilema* sp. (Jafari *et al.*, 2019), *Chrysaora hysoscella* (Gharibi *et al.,* 2016), *Cassiopea Andromeda* (Mirshamsi *et al.,* 2016), sea anemone, *Stichodactyla haddoni* (Moghadasi *et al.,* 2018), some gastropods (Mohebbi and Nabipour, 2021); green scat, *Scatophagus argus* (Ghafari *et al.*, 2013), black spot stone fish, *Synanceia* sp. (Fathi *et al.,* 2020), and sea snake, *Hydrophis cyanocinctus* (Calvete *et al.*, 2012; Mohebi and Nabipour 2016). The Persian Gulf, represented as a semi-enclosed sea with harsh environmental conditions, provides an opportunity to form a variety of venoms compounds with diverse structures, biological activities, toxicity, clinical symptoms, and pharmacological aspects (Moghadasi *et al.,* 2018; Ballesteros *et al.,* 2022; Nabipour *et al.,* 2015; Avci *et al*., 2023). For example, the venom of the sea snake, *H. cyanocinctus,* contains a combination of organic and inorganic bioactive substances such as protease and non-enzymatic proteins that provide a wide range of medicinal activities. Note that high value of LD_{50} is comsidered in these poisons to kill mice (Calvete *et al.,* 2012; Mohebi and Nabipor, 2016). Venomous organisms can play a very important role in identifying, developing and treating various diseases (Kakoolaki *et al.,* 2013). These effects depend on the type of toxic substance or organism used and also on the water quality and exposure time (Orun *et al.,* 2005).

The venom of the sea anemone, *S. haddoni,* from the Persian Gulf has analgesic activities, proteolytic characteristics, edematogenic activity, inflammation properties, and tissue necrosis effects (Moghadasi *et al.,* 2018). For instance, the green scat, *S. argus* in the Persian Gulf produces venom with toxic activities, a protein structure with 7-250 kDa, hemolytic activity on human red blood cells, phospholipase C activity, and edematous activity (Ghafari *et al.,* 2013). The black spot stone fish, *Synanceia* sp., is the

most dangerous venomous organism in the Persian Gulf that contains venom with LD_{50} equal to 185 and a protein percentage of 2.272 mg/mL (Fathi *et al.*, 2020). The statistical reports describe that scorpionfish and jellyfish are the main marine organisms responsible for *severe injuries* in humans in Iran due to inhabiting coastal waters (Nabipour *et al.*, 2015; Dehghan Madiseh *et al.*, 2017; Zeng *et al*., 2016; Mi *et al*., 2022). Jellyfish with the largest known medusa in the Cnidaria phylum are the most abundant species in the Persian Gulf and Gulf of Oman in all seasons. Therefore, incidence with jellyfish pose more threats to human health than other venomous marine organisms (Ghafari *et al*., 2023). Nowadays, attention to social and economic well-being has been paid attention to, along with reducing environmental hazards, increasing the health of the environment, and reducing its pollution (Mesut, 2021). Also, the estimation of the risks caused by toxic organisms on non-target organisms such as fish is a significant sign of the greatest impact on the economic trade of the sea (Orun and Erdogan, 2014). Fish is a rich nutrient in the human diet and plays an important role in industry and commerce (Selamoglu, 2021). Therefore, toxic organisms and toxic environmental pollution in the seas and oceans can directly or indirectly affect the nutritional cycle of humans through the effect on fish tissues, as well as the effect on fresh water and its different ecosystems (Selamoglu, 2018).

Globally, jellyfish create environmental risks, economic crises, and serious impacts on human health with about 150 million stings annually (Purcell *et al.*, 2007; CIESM, 2011; Montgomery *et al.*, 2016; Ballesteros *et al.*, 2022). Jellyfish have a gelatinous bell and trailing tentacles equipped with stinging epithelial cells named cnidocysts (Cegolon *et al.*, 2013; Helm, 2018) which are specialized to inject venom for capturing prey and defense (Lassen *et al.*, 2011).

If contact happens, death seldom happens, however, local systemic reactions such as itching, pain, rash, terrible poisoning, muscle spasm, nausea, and vomiting are common (Öztürk and İşinibilir, 2010; Gharibi *et al.*, 2016). Clinically, the main factor is the extent of the venom put into the blood. In recent decades, jellyfish venom has sparked interest among researchers around the world and also in the Persian Gulf and the Gulf of Oman, but the diversity of venom structures and their functional mechanisms remain unknown. Generally, jellyfish venom has a complex mixture of organic and inorganic compounds, including peptides and proteins with variable degrees of complexity (Currie, 2003; Fenner, 2014). In some jellyfish-like *Crambionella orsini* which have venom with a protein structure and protein bands between 11 and 250 kDa, the LD_{50} was reported to be 0.5 mL (Saki *et al.*, 2017). The anticancer properties have been identified for the venom of the jellyfish *Cassiopea andromeda*, in which the crude venom significantly decreases mitochondrial succinate dehydrogenase activity and induces

apoptosis in tumoral mitochondria (Mirshamsi *et al.,* 2016). Among different jellyfish species, *Rhopilema nomadica* and *Chrysaora hysoscella* belonging to class Scyphozoa bloom in several parts of the world, and are abundant in warm waters including the northern coastal waters of the Persian Gulf (Siokou-Frangou *et al.,* 2006; Mariottini and Pane, 2010; Gharibi *et al.,* 2016; Jafari *et al.,* 2019). The hemolytic effects of venom from the jellyfish *Rhopilema* sp. with protein bands below 100 kDa were investigated in humans, mice, and sheep cells (Jafari *et al.,* 2019). Protein bands of *C. hysoscella* were in the range of 72-250 kDa, with lower toxicity than other jellyfish species (Gharibi *et al.,* 2016). Here, it is worthwhile to mention that the venoms extracted from jellyfish are an available source of potential pharmaceuticals. Moreover, swimmers, fishermen, and researchers are always at risk of jellyfish venom, which causes some immediate and delayed immune reactions (Buecher *et al.,* 2001; Fathi *et al*., 2020). However, the causative allergens are mostly unknown. Unfortunately, although many excellent studies have recently been conducted to identify the characteristics and effects of venoms extracted from jellyfish, there has been little effort committed to exploring these issues in the Persian Gulf.

Therefore, this paper aims to compare the biological and cytotoxic characteristics of venoms extracted from the nomad jellyfish *Rhopilema nomadica* and the compass jellyfish *Chrysaora hysoscella,* collected from the Persian Gulf. In doing so, the quantity and characteristics of the protein structure of the venoms from these species were investigated. the nomad jellyfish is a jellyfish that cause injury to human (Buecher *et al.,* 2001; Benov and and Al-Ibraheem, 2002; Mariottini and Pane, 2014). It is an epipelagic-neritic and planktotrophic jellyfish, native to the West Indian Ocean, including the Red Sea, and the Persian Gulf. Generally, its umbrella-shaped body is about 30 to 50 cm in diameter (Feng *et al.,* 2010). Its envenomation almost emerges via immediate pain and redness, although its skin reactions may be postponed (Bloom *et al.,* 1988). The compass jellyfish is reported as a nearly low-risk jellyfish and its venom has adverse effects on skin cells (Del Negro *et al*., 1992).

It occurs abundantly in oceanic coastal water such as the Persian Gulf (Morandini and Marques, 2010). The compass jellyfish is 6 to 30 cm in length and is composed of a yellowish medusa associated with brown lines on the bell surface (Buecher *et al.,* 2001; Houghton *et al.,* 2007). *Rhopilema nomadica* and *Chrysaora hysoscella* species were caught with fishing nets from the coastal waters around Qeshm Island and Bushehr Province, Iran, respectively, from July to September 2019 (Fig. 1B and D). After the biometry, the tentacles of specimens were removed immediately and stored on ice until delivery to the laboratory where kept at -20°C. The laboratory analyses were conducted in the Venomous Animal Laboratory of Razi

Vaccine and Serum Institute, Karaj, Iran.

Materials and methods

Biological sampling and venom extraction

The employed methods were protein assaying by Bradford test, exploring protein bands by electrophoresis to obtain the differences in peptide structure of venoms, and confirming the results of protein assaying by HPLC and LC Mass. In this regard, the venom pH, and its antibacterial properties were determined using MIC test and growth inhibition halo. Using the MTT test, the cytotoxic effects of the venoms were investigated on human skin fibroblast cells [HU02]. The toxicity test (LD_{50}) of venom was explored on the animal model (Balb/c mouse) (Gundogdu *et al.*, 2021; Mamur *et al.*, 2020; José Palmieri *et al.*, 2021). Several methods have been suggested to extract the intracellular proteins, and one of the most common is the rapid freeze-thaw method, which induces the lysis of prokaryotic and eukaryotic cells (Benov and Al-Ibraheem, 2002). Here, the protocol proposed by Bloom *et al*. (1988) was employed with some modifications. The containers of tentacle matrices were placed in a freeze-drier. The consecutive freeze-thaw with liquid nitrogen was employed to break down the wall of nematocysts as well as to release the venom. The obtained material was centrifuged to extract the supernatant solution of the venom at 13,000 ×g at 4°C for 20 min (Feng *et al*., 2010).

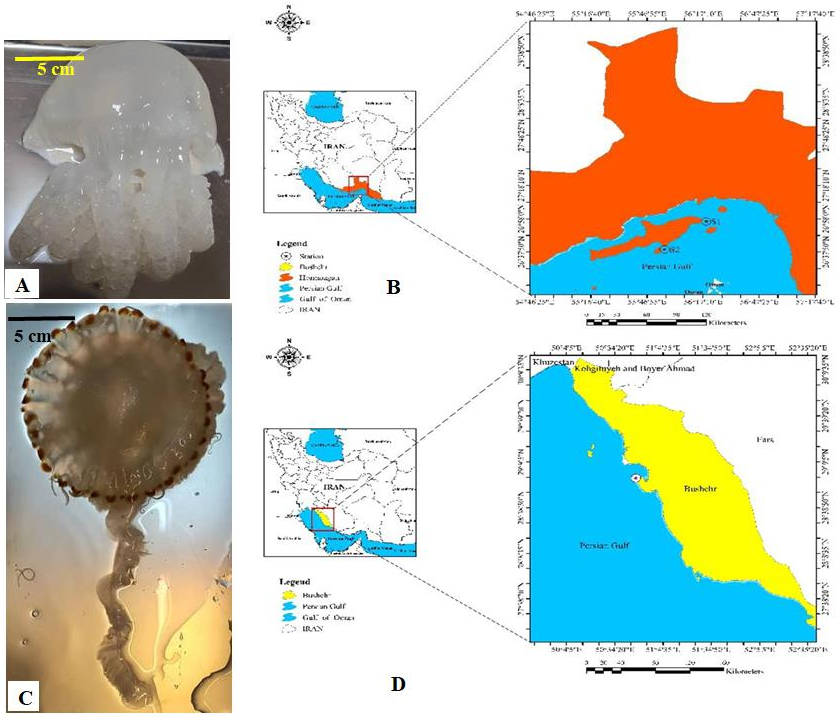

Figure 1: Jellyfish species and their distribution in the Persian Gulf. A. Rhopilema nomadica and B. its distribution in Qeshm Island near-shore water. C. Chrysaora hysoscella and D. its distribution in Bushehr province near-shore water.

Protein assay

The Bradford protein assay with bovine serum albumin (BSA) solution at 595 nm was employed to measure the protein concentration of venom after preparing 0.25 mg/µL of BSA solution (Bradford,

1976). Different concentrations of the solution were prepared with double-distilled water to achieve the final volume of 800 µL. Then, the obtained solution was vortexed for a few seconds, and its optical density (OD) was read under a spectrophotometer after 5 min. The protein concentration diagram was plotted in Excel.

Electrophoresis

The molecular weight of proteins obtained from crude venom was measured by SDS-PAGE following Laemmli (1970). According to the silver nitrate method, 12% polyacrylamide gel was used to isolate protein bands based on their molecular weight (Chevallet *et al*., 2006).

Toxicity test (LD_{50})

To determine the toxicity (LD_{50}), the prepared venom was injected into 10 BALB/c mice 20 g, both intravenously (IV) and intra-peritoneally (IP) for 24 h. This experiment was repeated on three groups, and the average data was investigated. For the control group, 20 g saline solvent was injected into BALB/c mice. LD_{50} was measured by the Spearman-Karber method (Hamilton *et al.*, 1977) using Eq. (1):

$$\mathrm{LD}_{50} = 100\%\ \text{lethal log} \pm \frac{\text{Logarithm coefficient}}{\text{Number of samples per dose}}[\Sigma\ \text{Number of dead} - \mathrm{n}/2] = \text{A \& B} \quad (1)$$

In this method, a double answer was calculated by the antilogarithm in Eq. (1) of which the received number in the dose range was chosen as the correct answer.

High-performance liquid chromatography (HPLC)

HPLC was employed to isolate and identify the proteins of venom in an advanced manner (Gharibi *et al.,* 2016). For this purpose, 100 µg of the crude venom was dissolved in 5 µL of distilled water. Then, the insoluble residue was centrifuged at 13,000 ×g for 15 min at 4°C to become removable. Meanwhile, to separate different parts of the venom obtained by HPLC and UV detector, 0.12 µL of crude venom of *R. nomadica* as well as 0.11 µL of crude venom of *C. hysoscella* were passed through a 0.22-µm membrane filter and were injected into vials along with 988 and 989 µL of HPLC water for the previous and latter ones, respectively. Afterward, 20 µL of the extract was analyzed by HPLC. The flow rate in the C18 column was 1 mL per minute [5 µm, 250 mm × 4.6 mm]. The HPLC and detector specifications were Water2695 separations Module and Waters996 Photodiode Array Detector. In the next step, 20 µL of filtered venom solution was injected into the HPLC WATERS2695 to analyze in the analytical column C18 by the gradient method, in which different percentages of Solution A containing 0.1% TFA, HPLC water, Solution B containing 0.1% TFA, and acetonitrile were combined. This process was performed at 280 nm via a flow rate of 1

mL/m for 60 min. Because the column used in HPLC is column 18 which is a polar column, if the constituents of the sample are polar, they will be removed later due to greater interaction with the stationary phase. So the compounds that are later in the graph, have changed and are more polar.

Cytotoxicity and MTT assay

Human skin fibroblast cells (HU02) were provided by the Iranian Biological Resources Center. Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), and trypsin were purchased from Gibco-BRL (US) while gentamicin, phosphate-buffered saline, and MTT powder [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide] were prepared from Sigma-Aldrich (St Louis, MO, US). To prepare 10 mL of complete culture medium, a mixture of 8.9 mL of DMEM powder, 1 mL of FBS, 0.1 mL of L-glutamine (100X), and 2 µL of gentamicin were cultured in a 25-mL flask and then incubated under 5% CO2 at 37°C. After the second passage by checking the viability and proliferation of cells, the subsequent experiments were implemented. Cells were cultured on a 96-chamber plate at a concentration of 5000 cells per chamber and further incubated for 24 hours to allow the cells to adhere to the plate.

Then, normal skin cells (Hu02) were exposed to the venom of both jellyfish under graduated concentrations for 24 h. Then an MTT test was performed. The crude venom was also aliquoted for 1/2 and 1/4 solutions. Then they were treated with 1, 3, 7, and 10 µg per µL of crude venom at concentrations of complete, 1/2, and 1/4. The cytotoxicity and viability of normal human skin fibroblast cells were measured by MTT assay, (Freimoser *et al.*, 1999), which was later performed within 24 hours. The culture medium was discharged into the microplates and then rinsed with PBS buffer, and added to the serum-free culture medium (100 µL). In the next step, 10 µL of MTT solution was added to each chamber and then incubated for 2 hours. The supernatant solution was rinsed with a PBS buffer. Next, the values were read by an ELISA microplate reader at 570 nm after adding 10 µL of DMSO solution to each chamber. The viability rate was calculated using Eq. (2):

$$Viability\ rate = \frac{Mean\ OD\ of\ Test\ Group}{Mean\ OD\ of\ Control\ Group} \times 100 \qquad (2)$$

The pH of crude venom was also measured by a pH meter (WPA CD510) at room temperature.

Antibacterial properties and growth inhibition halo of venom

To determine the antibacterial properties of venoms, a 0.5 McFarland suspension was prepared at 600 nm. The chamber wells were classified into a series of

dilutions under three replicates apiece. The MIC (Minimum Inhibitory Concentration) tests of the venoms were performed (Kowalska-Krochmal *et al.*, 2021) on four bacteria, including *Pseudomonas aeruginosa* (gram-negative), *Staphylococcus aureus* (gram-positive), methicillin-resistant *Staphylococcus aureus* (MRSA**,** gram-positive), and *Escherichia coli* (*E. coli***,** gram-negative). All bacteria belonged to American Type Culture Collection (ATCC). Then, dilutions of 1.2, 1.4, 1.8, 1.16, 1.32, 1.64, and 1.128 were prepared under three replicates to be used for MIC testing of laboratory bacteria at 37°C.

After importing the data into Excel, the MIC was determined based on the dilution series of each bacteria. To evaluate the growth inhibitory halo, the chambers on the agar plate were filled with the venom of the studied jellyfish. A 0.5 McFarland suspension was prepared from the tested bacteria and further cultured on the surface of the plate, where the compartments were placed on the agar plate. In addition, pure and dilute extracts of venom compounds were added to the chambers. Since bacteria under the antimicrobial properties cannot grow on the edges of the chambers containing this compound, a clear area is formed on the edges of the chambers, called the growth inhibitory halo. It should be mentioned that for the fidelity of results, each assay was undertaken in triple replicates.

Results

Protein assay and electrophoresis

According to the concentration of bovine serum albumin protein as well as the obtained formula, the concentration of toxin protein measured by UV spectrophotometer in the range of 595 nm 0.898 mg/mL, and 0.948 mg/mL was estimated by BSA for the species of *C.hysoscella* and *R. nomadica* respectively (Fig. 2). Moreover, the molecular weight of proteins was determined by electrophoresis and SDS-page gel at 13, 21, 25, 33, 53, 98, 120, 170, and 190 kDa for *R. nomadica* as well as 21, 25, 35, 38, 40, 53, 100, 120, and 190 kDa for *C. hysoscella* (Fig. 3).

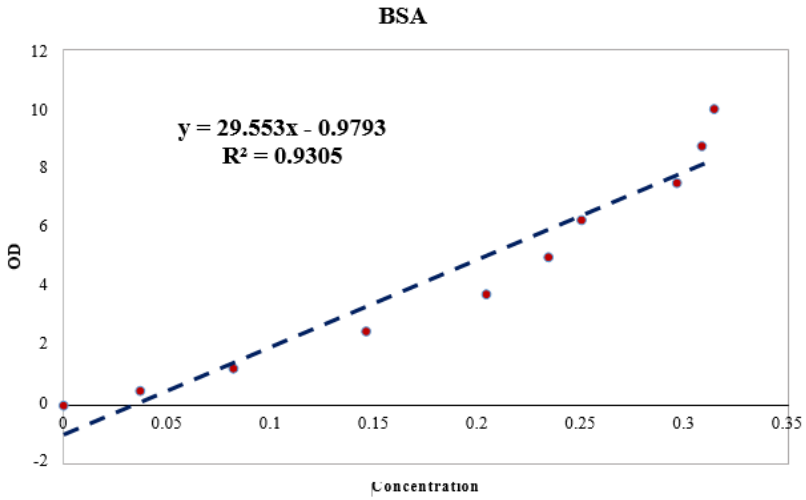

Figure 2: Protein profile of toxin concentration measured concerning the bovine serum albumin protein concentration.

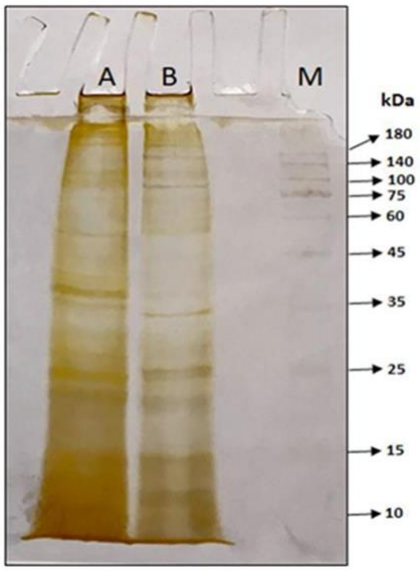

Figure 3: Electrophoresis with silver nitrate staining. A. Rhopilema nomadica, B. Chrysaora hysoscella, and M. Protein marker.

A large number of protein bands indicate the presence of a variety of proteins with different molecular weights. Note that as the number of protein bands with a molecular weight of fewer than 100 kDa is considerable, it seems that venom is mostly made up of lighter protein compounds. In a previous study, some proteins associated with molecular weights of 72 and 250 kDa were reported for *C. hysoscella*, caught from the Persian Gulf (Gharibi *et al*., 2016). Jafari *et al*. (2019) reported the molecular weights of 45, 65, and 95 kDa for venom proteins in *R. nomadica* caught from the Persian Gulf. Likewise, Gusmani *et al*. (1997) reported proteins with molecular weights of 16, 50, 91, 148, and 186 kDa in *R. nomadica*. The variation in recognized protein bands could be related to some differences in the number of nematocytes, venom component variants based on the seasonal or life cycle changes, differences in the methods of extracting venom, and even the peptides diversity (Gharibi *et al.*, 2016).

Toxicity test (LD_{50})

By IV injection of *C. hysoscella* venom, the LD_{50} value was 25.1 µgr/mouse and 1.26 mg/kg mouse body weight. Moreover, intraperitoneally (IP) injection of 52 µg/mouse and 2.35 mg/kg body weight was observed. In the case of *R. nomadica* intravenously (IV) injection, the LD_{50} value was 12.9

µgr/mouse and 0.65 mg/kg body weight. However, IP injection was reported as 32.5 µgr/mouse and 1.6 mg/kg body weight. These results confirmed that by decreasing the LD_{50}, the lethality of the venom is increased (as reported in Table 1). More importantly, in the case of both species, injecting a lower volume of crude venom using the IV method, i.e. injecting intravenously was more lethal.

Table 1: LD_{50} was determined by Intravenously (IV) and intraperitoneally (IP) venom injection during 24 hours and calculated after the Sperman-Karber method.

No	Species	µg/mouse		mg/kg body weight	
		IV	IP	IV	IP
1	*Chrysaora hysoscella*	25.1	52	1.26	2.35
2	*Rhopilema nomadica*	12.9	32.5	0.65	1.6

In the case of both IV and IP injection methods, the result revealed that the LD_{50} for *R. nomadica* was lower than that for *C. hysoscella*. This suggests that *R. nomadica* venom is more lethal and even brief contact with human skin can be harmful. In the study by Jafari and Zargan (2019), IP injection of *R. nomadica* venom in the mice yielded LD_{50} of about 0.45 µg/kg which was lower than the results of the present study. Compared to the other jellyfish, like *Carybdea alata*, LD_{50} was in the range of 5 - 25 µg/kg (Lee *et al.,* 2015), the LD_{50} of both examined species was nearly low whereas they might cause more injuries than death terms of contact. The difference in calculated LD_{50} could be related to the differences in the size of the jellyfish, the sampling location of the studied species, the method by which the venoms were stored before injection, the method of injection, and venom variations (Gharibi *et al.,* 2016; Jafari *et al.,* 2019).

The crud venom of *R. nomadica* had a pH of 6.45, whereas *C. hysoscella* had a pH of 6.6 at the laboratory temperature indicating that the venom of *R. nomadica* was slightly more acidic than the venom of *C. hysoscella*. As such, the venom of *R. nomadica* could leave a more lethal effect on human skin cells.

HPLC and Liquid Chromatography Mass (LC Mass)

Chromatography analysis of *C. hysoscella* venom divided the C_{18} column into seven sections. Adsorption was recorded at 280 nm and main peaks were recorded at 3, 6, 7, 9, 15.3, 27, and 48 min (Figs. 4A, B). Chromatography analysis of *R. nomadica* venom divided the C_{18} column into eight sections and the main peaks were recorded at 3.5, 5, 6.1, 7, 9, 15, 48.8, and 54 min. (Figs. 4C and D).

The results of HPLC and LC Mass indicate seven and eight peaks per 60 min for *C. hysoscella* venom and *R. nomadica* venom, respectively. Besides, the venom for *R. nomadica* with more peaks indicates more components than that of *C. hysoscella* venom. Gharibi *et al*. (2016) showed 15 sections in HPLC analysis and the main peaks of 8.62 and 11.23 min for *C. hysoscella* venom.

Furthermore, Ras *et al*. (2020) found five peaks of *C. hysoscella* proteins identified by FPLC and HLPC from the column determined, which were related to hemolytic activity, proteolytic activity, and phospholipase A. The same results for *R. nomadica* were expressed by Gusmani *et al*. (1997) who found 5 main peaks related to hemolytic activities, proteolytic activity, and phospholipase A.

MTT test

In the MTT test, venom toxicity on Hu02 fibroblast cells for 24 h in crude form and 1/2 and 1/4 diluted concentrations indicated that the *R. nomadica* venom (10 and 7 µg/µL of crude venom) had more capability to destroy the skin cells. On the other hand, in the case of *C. hysoscella*, venom toxicity on Hu02 fibroblast cells was recorded in 10 µg/µL of crude venom. The highest percentage of cell viability was recorded in 1/4 diluted concentration of the venoms in both species, with a higher rate in *R. nomadica.* The results revealed that although the highest volume of crude *R. nomadica* venom (10 µg/µL) had the highest mortality, mortality was significantly reduced at 1/4 dilution (Fig. 4; Tables 2 and 3).

Therefore, washing the damaged skin with fresh water can be an effective way to reduce cell damage. Moreover, an adverse relationship was found between the volume of the venom and the viability of the cells. This can be related to the different protein structures or different kinds of venoms associated with various types of nematocytes. In this regard, three types of nematocytes have been reported in *C. hysoscella* . However, there is a gap in knowledge about their toxicity. While robust skin cell destruction has been previously reported for *R. nomadica* venom (Mariottini and Pane, 2010), little information is available on *C. hysoscella* toxicity (Del Negro *et al.,* 1992; Parodi, 2009). Parodi (2009) reported 55% mortality for the *C. hysoscella* venom.

Here, it is worthwhile to mention that the obtained results are in accordance with those obtained by Mariotini *et al*. (2014) in which they argued that *C. hysocella* was almost harmless while *R. nomadica* was relatively toxic. Likewise, Lee *et al*. (2015) investigated the effect of *R. nomadica* venom on various types of cancer cells and using an MTT assay demonstrated that jellyfish venom could strongly inhibit the growth of HepG2 cancer cells.

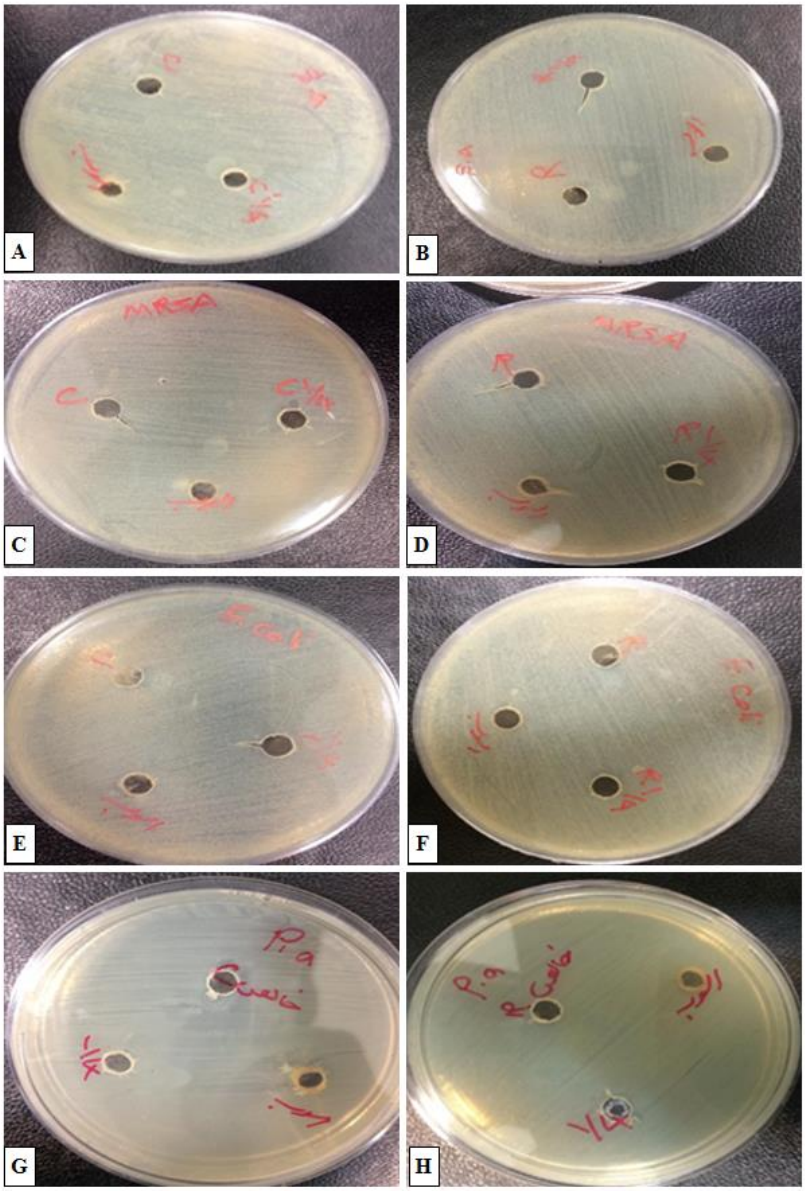

Figure 4: Absence of growth inhibition halo of different bacteria in the presence of venom. A. Interaction of *Staphylococcus aureus* (SA) and *Chrysaora hysoscella,* venom, B. SA and *Rhopilema nomadica* venom; C. *C. hysoscella venom* and methicillin-resistant *Staphylococcus aureus* (MRSA), D. *R. nomadica* venom and MRSA; E. *Escherichia coli* (E. coli) and C. *hysoscella venom and* F. *E. coli* and *R. nomadica* venom; G. *C. hysoscella* venom and *Pseudomonas aeruginosa* (Pa) H. *R. nomadica* venom and Pa.

Table 2: Viability of skin fibroblast cells with the effect of *Chrysaora hysoscella* venom.

Concentration µg/µL	C1	C3	C7	C10	C1/2 1	C1/2 3	C1/2 7	C1/2 10	C1/4 1	C1/4 3	C1/4 7	C1/4 10
Viability%	61	45	20	11	55	44	39	35	61	55	47	42

Table 3: Viability of skin fibroblast cells with the effect of *Rhopilema nomadica* venom.

Concentration µg/µL	R1	R3	R7	R10	R1/2 1	R1/2 3	R1/2 7	R1/2 10	R1/4 1	R1/4 3	R1/4 7	R1/4 10
Viability%	64	39	10	6	61	50	42	36	86	75	64	61

Antibacterial properties and growth inhibition halo of venom

Comparing the venoms of two jellyfish species with the same concentrations, showed that when a more lethal venom was added to the bacterial suspension, the percentage of bacterial survival decreased. As a result, the OD of the bacterial suspension was further reduced when exposed to the lethal toxin. The lack of visible inhibitory growth halo around the discs indicates the absence of antimicrobial activity. In the control group, the bacteria survived and there were no harmful substances. Their viability was optimal and their OD was measured as the constant OD of the control groups. In the experimental group, when the venom triggered the growth of bacteria, the survival of the bacteria was higher than that of the control group (100%) (Eq2). Regarding the effect of *C. hysoscella* venom, the toxin had a variable but stronger effect on Pa. The most antibacterial effect was observed on the 1/2 dilution of the venom on Pa, which killed about 48% of the bacteria. No constant adverse trend was observed and the antibacterial effects were noticeable at concentrations of 1/64 and 1/128, more than 1.4 to 1/32 of the venom concentration. The effects of the toxin on *E. coli* were neutral as in the control group in concentrations of 1/64 and 1/128, and in other dilutions that caused more growth of the bacteria. The highest effect of *C. hysocella* toxin on Sa was observed at 1/4 concentration. As for other concentrations, the effect of the venom was almost neutral. In the case of MRSA, similar to *E. coli*, at lower concentrations, the effect was almost neutral, and at higher concentrations, it promoted bacterial growth (Fig. 5A). Pa had different behaviors in the presence of *R. nomadica* venom. The venom caused bacterial growth in the concentrations of 1/2 to 1/8 while a weak antibacterial effect was observed at 1/16 to 1/128 concentrations (~ 20 %). The venom raised E. coli growth in 1/2 to 1/16 concentrations and was almost neutral in the rest of the concentrations. The venom of *R. nomadica* almost neutrally affected Sa bacteria in 1/2 to 1/16 and caused negligible growth. At the concentrations of 1/32- 1/128, a slight antibacterial activity was observed against Sa (~ 13 %). MRSA bacterial growths were increased in the presence of 1/2 to 1/32 venom concentrations and their growth was slightly dropped at 1/64 to 1/128, which was about the neutral rate. Compared to the control group, OD is a very accurate method for any bacteria that has an optical absorbance given by Eq. (2) (Fig. 5B).

The antibacterial effects of the venoms were investigated by the viability of 2 Gram-positive bacteria (MRSA and Sa) and 2 Gram-negative bacteria (*P. aeruginosa* and *E. coli*) against jellyfish venom. The results varied depending on the bacteria and the dilutions applied. The viability of MRSA bacteria in dilutions of 1/2, 1/4, 1/8, 1/16, and 1/32 against *C. hysoscella* toxin was similar to *E. coli* and was higher compared to the control samples. While in 1/64 and 1/128 dilutions, the survival rate was almost equal to the

control group. The highest survival rate was for MRSA at 1.8 dilution and for *S. aureus* at 1/16 dilution. Instead of antibacterial properties, it seems that the protein substances of this venom cause more growth of MRSA and *E. coli* bacteria. In general, almost no antibacterial properties were observed for these venoms. Bacteria grew around and on the edge of the wells and there was no clear halo. Therefore, their venom had almost no antimicrobial activity (Fig. 4).

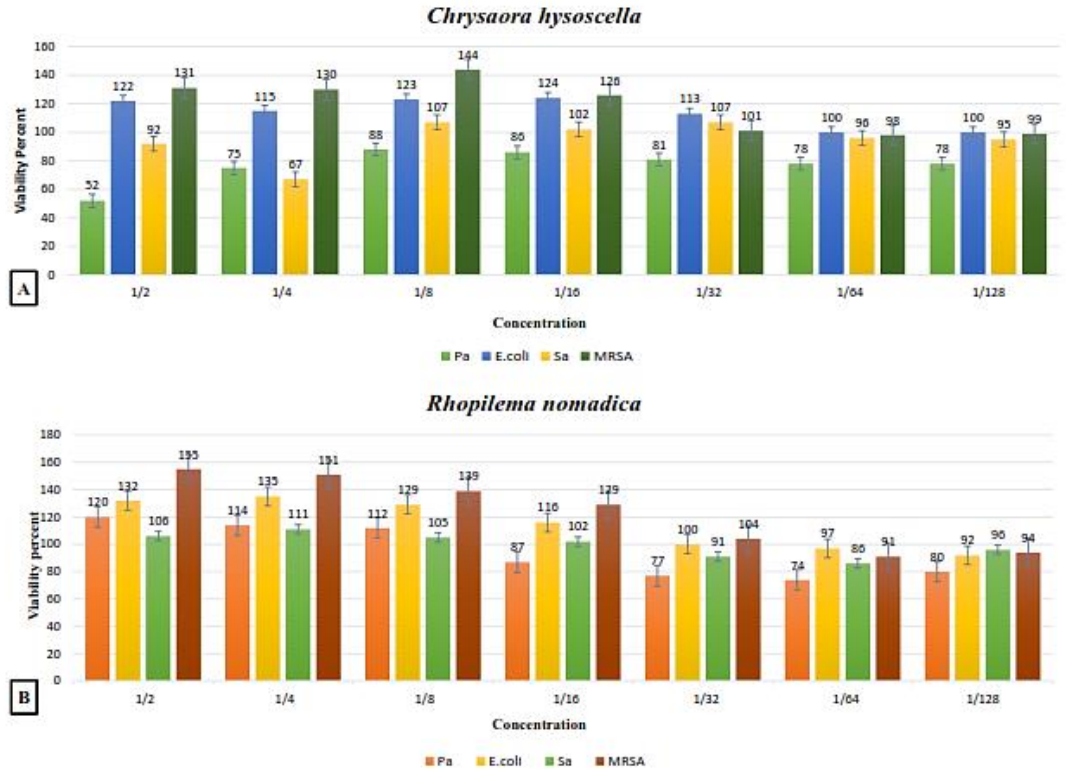

Figure 5: Effect of *Chrysaora hysoscella* (A) and *Rhopilema nomadica* (B) venoms on four studied bacterial species, Pseudomonas aeruginosa: Pa, Escherichia coli: E. coli, Staphylococcus aureus : Sa, methicillin-resistant Staphylococcus aureus: MRSA.

In the case of *S. aureus*, a relatively reliable antibacterial effect, and the only lethal dose, were observed in 1/4 of the diluted *C. hysoscella* venom (67% bacterial survival). In the remaining dilutions, survival was almost identical to the control group. Likewise, in all dilutions, the viability of *P. aeruginosa* decreased and signs of the antibacterial effect of *C. hysoscella* venom were observed. The highest antibacterial effects were observed in 1/2 dilution with 52% survival of bacteria.

In the case of *R. nomadica*, the growth of MRSA and *E. coli* was observed in dilutions of 1/2, 1/4, 1/8, 1/16, and 1/32. At 1/32 dilution of *E. coli* and 1/64 and 1/128 dilutions of both bacteria, viability was almost equal to the control group. The result that at 1/32 dilution of *E. coli* and 1/64 and 1/128 dilutions of both bacteria, viability is almost equal to the control group indicates that *R. nomadica* venom contains proteins that provide a suitable environment for the growth of bacteria. The highest growth rates in

MRSA and *E. coli* were at 1/2 and 1/4 dilutions, respectively. The reaction of *S. aureus* against *R. nomadica* venom was nearly neutral with no explicit antibacterial effect. The only dilution which showed a slight antibacterial effect was 1/64 [86% bacterial survival].

For *P. aeruginosa*, significant bacterial growth was observed in dilutions about 1/2, 1/4, and 1/8, while further dropped in dilutions about 1/16, 1/32, 1/64, and 1/128 where there were some antibacterial effects. The most effective antibacterial dilution was 1/64 (viability: 74%). Given no clear halo around the wells, the results confirmed that the venom had no antimicrobial properties. Considering the relatively high viability of different bacteria in diverse dilutions of venom, it seems that victims not only should face dermal injuries and possible scars caused by direct stings but also the presence of venomous protein on the dermal tissue may provide a cultivation medium for different kind of bacteria. Despite our observations, Bhosale *et al*. (2002) demonstrated that jellyfish Cassiopeia sp. exhibited significant activity against *Bacillus pumilus* and Pseudomonas vesicularis. Likewise, Liu *et al*. (2011) found inhibitory activity against pathogenic bacteria including MRSA 3089 in the venom of jellyfish *Nemopilema nomurai*. They further argued that the venom of different species of jellyfish may have diverse reactions against variants of bacteria.

Research on venomix and their poisoning mechanism can facilitate the possibility of obtaining some suitable, cheap, and low-risk medicines of marine origin in the future, and in addition, it can contribute to therapeutic methods and lead to the improvement of food and cosmetic industries (Mariottini and Pane, 2014; Riccio *et al.*, 2022). Moreover, cytotoxicological studies are the basis for developing an antivenom to treat or reduce the effects of marine organisms' stings. In this way, there are some problems in studying the effect of jellyfish stings such as catching the animal, determining the sensitivity of the victim to the venom due to temperature and the properties of the venom protein, and the problem of separating and purification of the toxin from the unnecessary parts of nematocytes (Lassen *et al*., 2011; Ghaffari *et al*., 2013). Accordingly, this paper investigates the characteristics of the venom of *R. nomadica* and *C. hysoscella* from the Persian Gulf by employing conventional methods.

The large number of found protein bands in the venoms indicates the presence of a variety of proteins with different molecular weights. Meanwhile, a significant number of protein bands with molecular weights less than 100 kDa indicate that the venoms are mainly composed of lighter protein compounds. The reported molecular weights were in the range of venom of *C. hysoscella*, caught from the Persian Gulf, in which there were some proteins associated with molecular weights of 72 and 250 kDa (Gharibi *et al*., 2016). Jafari *et al*. (2019) also studied the venom of *R. nomadica* and further reported the molecular weights for the obtained proteins about

45, 65, and 95 kDa. Gusmani *et al*. (1997) reported proteins with molecular weights of 16, 50, 91, 148, and 186 kDa in *R. nomadica.* The differences in identified protein bands probably reflect differences in some issues such as the number of nematocytes, variations in the venom components based on the seasonal or life cycle changes, methods of extracting venoms, and even the peptides diversity (Gharibi *et al.,* 2016).

LD_{50} for *C. hysoscella* and *R. nomadica* crude venoms were obtained at 2.35 mg/kg and 1.6 mg/kg of mice body weight, respectively. In the case of both species, injecting a lower volume of crude venom using the IV method (i.e., intravenously injecting) was more lethal. LD_{50} for *R. nomadica* in both IV and IP methods was lower than that for *C. hysoscella.* This suggests that *R. nomadica* venom is more lethal, and even brief contact with human skin can be harmful. In the study by Jafari and Zargan (2019), LD_{50} for *R. nomadica* through the IP method being injected into mice was about 0.45 µg/kg which was lower than in the present study. In contrast to the other jellyfish, such as *Carybdea alata,* which its LD_{50} was in the range of 5 - 25 µg/kg (Lee *et al.,* 2015), the LD_{50} of both examined species in the present study was nearly low, and in case of contact, and they could cause more injuries than death. The differences in calculated LD_{50} could be related to the size of the jellyfish, the difference in sampling location of the same species, the way the venoms were handled and stored before injection, the method of injecting, and the diverse characteristics of the venoms from different species (Gharibi *et al.,* 2016; Jafari *et al.,* 2019)

The number of peaks recorded for the venoms of *C. hysoscella* and *R. nomadica* were seven and eight peaks per 60 min, respectively. Since the number of HPLC columns was 18 which is a polar column, they would be removed more slowly due to greater interaction with the stationary phase if the constituents of the sample were polar. Therefore, the compounds that were later illustrated in the diagram could be changed and became more polar. The venom of *R. nomadica* had more peaks and was composed of more compounds than that of *C. hysoscella*. Gharibi *et al*. (2016) found 15 fractions in HPLC analysis in which the main peaks were 8.62 and 11.23 min for *C. hysoscella* venom. Ross *et al*. (2020) also found five peaks in *C. hysoscella* venom proteins and attributed them to hemolytic activity, proteolytic activity, and phospholipase A.

The same results were reported for *R. nomadica* by Gusmani *et al*. (1997), where there were five main peaks related to hemolytic activity, proteolytic activity, and phospholipase A. The results of the MTT test, exploring the venom toxicity (crude venom and 1/2 and 1/4 diluted concentrations) on Hu02 fibroblast cells for 24 h indicated that *R. nomadica* venom (10 and 7 µg/µL of crude venom) had more ability to destruct the skin cells compare to *C. hysoscella* (10 µg/µL of crude venom). As was expected 1/4 diluted concentration of the venoms from both

species had the highest percentage of cell viability which was higher in *R. nomadica*. However, the results showed that in the case of the highest volume of crude venom (10 µg/µL), *R. nomadica* resulted in more mortality, and the mortality decreased dramatically in 1/4 dilution. Therefore washing the injured skin with freshwater could be an effective method to decrease cell damage. There was an inverse relationship between the volume of the venom and the viability of the cells might be related to the differences in protein structures, and different types of venoms belonging to various types of nematocytes.

Fish is a rich nutrient in the human diet and plays an important role in industry and commerce (Selamoglu, 2021). Therefore, toxic organisms and toxic environmental pollution in the seas and oceans can directly or indirectly affect the nutritional cycle of humans through the effect on fish tissues, as well as the effect on fresh water and its different ecosystems (Selamoglu, 2018).

Furthermore, three different types of nematocytes have been identified in *C. hysoscella*, while there is no definite knowledge of their toxicity. Besides, the strong skin cell destructs has been reported earlier by the venom of *R. nomadica* (Mariottini and Pane, 2010), little data are available about the toxicity of *C. hysoscella* (Del Negro *et al.*, 1992; Parodi, 2009). Parodi (2009) explored the toxicity of *C. hysoscella* and further demonstrated that its crude venom could cause 55% mortality. Similar to our findings, Mariottini and Pane (2014) reported that *C. hysoscella* was nearly harmless while *R. nomadica* was relatively toxic. Likewise, Lee *et al*. (2015) treated different types of cancer cells using *R. nomadica* venom and performed an MTT test to indicate that jellyfish venom could strongly inhibit the growth of HepG2 cancer cells. The results of the present study revealed that the crud venom of *R. nomadica* had a pH of 6.45 whereas *C.hysoscella* had a pH of 6.6 at laboratory temperature. This suggests that *R. nomadica* venom is slightly more acidic than that of *C. hysoscella*. This could be one of the reasons underlying the highly lethal effects of *R. nomadica* venom on human skin cells.

The results of exploring the antibacterial effects of the venoms were diverse depending on the bacteria and the applied dilutions. MRSA bacterial viability was similar to *E. coli* in dilutions 1/2, 1/4, 1/8,1/16, and 1/32 against venom *C. hysoscella* and was more compared to the control samples. In contrast, in the dilutions of 1/64 and 1/128, the viability was nearly equal to those of the control groups. Survival was the highest for MRSA in dilution of 1/8 and for *S. aureus* in dilution of 1/16 suggesting that the protein substances of *C. hysoscella* venom could cause more growth of MRSA and *E. coli* rather than possessing antibacterial properties.

In the case of *S. aureus,* a relatively reliable antibacterial effect, the only lethal dose, was observed in 1/4 of the venom of *C. hysoscella* dilution (67% survival of bacteria). In the remaining dilutions, the viability was

approximately similar to the control groups. In all series of dilutions, the viability of *P. aeruginosa* was reduced and the signs of antibacterial effect were observed against *C. hysoscella*. The highest level of antibacterial effects was observed in 1/2 dilution with 52% survival of bacteria.

Discussion

Regarding *R. nomadica*, MRSA and *E. coli* growth were observed in dilutions of 1/2, 1/4, 1/8, 1/16, and 1/32. In 1/32 dilution of *E. coli* and 1/1 64 and 1/128 of both bacteria, viability was nearly equal to the control groups. This suggests that *R. nomadica* venom possesses proteins that provide a suitable environment for bacterial growth. The highest rate of growth in MRSA and *E. coli* was related to the dilutions of 1/2 and 1/4, respectively.

The reaction of *S. aureus* against *R. nomadica* venom was nearly neutral meaning that *R. nomadica* venom had no clear antibacterial effect. The only dilution that showed a slight antibacterial effect was 1/64 [86% bacterial survival]. Regarding *P. aeruginosa*, bacterial growth was significantly observed in the dilutions of 1/2, 1/4, and 1/8, while dropped in the dilutions of 1/16, 1/32, 1/64, and 1/128 where some antibacterial effects were recorded. The most effective antibacterial dilution was 1/64 (viability: 74%). Since there was no clear halo around the wells, the results confirmed that the venom had no antimicrobial properties. Considering the relatively high viability of different bacteria in diverse dilutions of venom, it seems that victims not only should face dermal injuries and possible scars caused by direct stings but also the presence of venomous protein on the dermal tissue may provide a cultivation medium for different kind of bacteria.

Despite our observations, Bhosale *et al*. (2002) showed that jellyfish *Cassiopeia* sp. exhibited significant activity against *Bacillus pumilus* and *Pseudomonas vesicularis*. Likewise, Liu *et al.* (2011) argued that compounds of jellyfish *Nemopilema nomurai* had inhibitory activity against pathogenic bacteria including *MRSA* 3089. It was assumed that the venom of different species of jellyfish may have diverse reactions against variants of bacteria. Tinta *et al*. (2012) explored the impact of jellyfish substrate on bacterial community phylotype selection and further argued that jellyfish biomass was bioavailable to associated and free–living bacteria, and could serve as an important microbial microenvironment.

In this paper, the characteristics of the venoms in two jellyfish species were investigated including *R. nomadica* and *C.hysoscella* in the Persian Gulf. The results revealed significant differences in the characteristics of the venoms. Overall, the measured toxin protein concentration by UV spectrophotometer was in the range of 595 nm which was more in *R. nomadica* than *C. hysoscella*. In electrophoresis, the number of identified protein bands was the same in both species. The molecular weight of most of the bands was more or less the

same but in two cases, the difference was meaningful.

The first identified protein for *R. nomadica* had a high molecular weight. The difference in the protein concentration, protein molecular weight, and pH affected the LD_{50} and MTT results. In IV and IP injection methods, the IV method was more lethal due to direct release into the bloodstream followed by a faster spread through the entire body. In both injection methods, *R. nomadica* venom possessed more lethality. A logical and expected MTT trend was observed such that increasing venom dilution resulted in greater lethality.

As it was expected, the lethality of *R. nomadica* venom on skin cells was more significant. The bactericidal effects of venoms were controversial and unfounded. The venom of both species not only was weak in killing bacteria but also in some cases raised their growth. This could increase the risk of infection after being bitten by the jellyfish, therefore even though the venom is not mortal, the possible post-infection could be dangerous or even fatal for the prey. However, the victim is challenged by the effects of the venom including hemolysis and phospholipase A, the immune system is under pressure due to the growth of bacteria on the wounds.

In conclusion, our results revealed some unique aspects of the venom of jellyfish species. It is hoped that jellyfish, as abundant animals in marine ecosystems, can be exploited as a research resource in the field of advanced pharmaceutical research. In this regard, more accurate data collection by HPLC and LC Mass profiles is suggested for further research.

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