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چکیده فارسی پایان‌نامه یا رساله (شامل: خلاصه، اهداف، روش‌های اجرا و نتایج به دست آمده – حداکثر در 10 سطر):

اسکات خالدار *Scatophagus argus argus* یک ماهی آکواریومی متعلق به خانواده Scatophagidae است که می تواند زخم های دردناک و فلج موضعی در اندام آسیب دیده دراثر تماس با خارهایش ایجاد کند. خارهای سخت پشتی و شکمی این ماهی شامل سلول هایی هستند که زهر تولید می کنند و فعالیت های سمی دارند.

بر طبق اطلاعات منتشر نشده جمع آوری شده از بیمارستان های محلی در مناطق ساحلی جنوب ایران، اسکات خالدار بعنوان یک ماهی سمی گزارش شده است . مسمومیت عوارض کلینیکی مانند درد موضعی ، فلج نسبی ، بی نظمی ضربان قلب و خارش ایجاد می کند. در مطالعه حاضر82 عدد اسکات سبز (خالدار) از آبهای ساحلی خلیج فارس جمع آوری شد. SDS_PAGE از زهرخام 12 باند مختلف در اندازه 10-

250 کیلودالتون تشخیص داد.

1/7 میکروگرم از زهر خام در خون 1٪ (گلبول قرمز) انسان ٪100 همولیز ایجاد نمود(LC). زهر خام 813 میکرو گرم پروتئین از کازئین ٪ 0/5 جدا کرد. فعالیت فسفولیپاز C در غلظت 3/125 میکروگرم زهر خام ثبت گردید. یافته های ما نشان داد که فعالیت ادم بیشتر از 48 ساعت بعد از تزریق باقی ماند. خالص سازی زهر خام با HPLC انجام شد و 30 پیک در طی 80 دقیقه بدست آمد ولی تنها پیک جدا شده در دقیقه 68 فعالیت همولیتیک نشان دادکه در استونیتریل ٪ 90 جدا شد. پروتئین همولیتیک شامل٪ 32 از مجموع پروتئین های زهرخام بود و وزن مولکولی آن 72 کیلودالتون نشان داده شد. نتایج آشکار ساخت که زهر خام استخراج شده از اسکات سبز ایرانی دارای خواص آنزیمی و سمی متفاوت است.

واژه های کلیدی:

***Scatophagus argus argus*،همولیز ، HPLC ، فعالیت همولیتیک، LC ، فسفولیپاز C.**

In the name of

Allah

The beneficent the

merciful

Islamic Azad University

Sciences and research branch

"Ph.D." Thesis

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Venomics and biological analysis of *Scatophagus argus argus*(Spotted scat) venom isolated in Persian Gulf, extraction and purification of hemolytic protein

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*To Imam **al-Mahdi***

Whom all of the world are looking forward to seeing his emersion

And

*To my **parents** whom I love most*

Table of Figures

Index of tables

Abstract

Green scat namely as *Scatophagus argus argus* is a venomous aquarium fish belonging to Scatophagidae family. It can induce painful wounds in injured hand with partial paralysis to whom that touch the spines. Dorsal and ventral rough spines contain cells that produce venom with toxic activities. According to unpublished data collected from local hospitals in southern coastal region of Iran, *S. argus* is reported as a venomous fish. Envenomation induces clinical symptoms such as local pain, partial paralysis, erythema and itching. In the present study green scat (spotted scat) was collected from Persian Gulf coastal waters. SDS-PAGE indicated 12 distinct bands in the venom ranged between 10-250 KDa. The crude venom had hemolytic activity on human erythrocytes (1%) with an LC100 (Lytic Concentration) of about 1.7 µg. The crude venom can release 813 µg proteins from 0.5% casein. Phospholipase C activity was recorded at 3.125 µg of total venom. Our findings showed that the edematic activity remained over 48 h after injection.

The purification of the venom was done by HPLC and 30 peaks were obtained within 80 min but only one peak in 68 min retention time showed hemolytic activity at 90% acetonitril was isolated. The area percentage of the hemolytic protein showed that this hemolytic protein consist of 32 percent of total proteins and its molecular weight was 72 KDa in SDS_PAGE.

The results demonstrated that crude venom extracted from Iranian coastal border has different toxic and enzymatic activities.

Key words: *Scatophagus argus argus*; Persian Gulf; Venomous fish; Hemolytic activity; Phospholipase C (PLC); HPLC.

Preface

During last decade, the toxins that are used for defense by venomous animals have been isolated and found useful tools for physiological studies besides giving valuable lead to drug development. Toxins with remarkable characteristics have been isolated from the venoms of snakes, scorpions, spiders, snails, lizards, frogs and fishes.

The venomous property of certain fishes has been recognized for thousands of years; however, scientific investigations have only begun recently on these fishes. The study of fish venomology encompasses many fields such as anatomy, physiology, pharmacology, and biochemistry.

As reported in “FishBase” scats are used in Chinese medicine and marketed as live fish in Hong Kong(Parenti, 2004).This work represents a study of the venom of a common tropical marine teleost fish which, in defense, is capable of inflicting extremely painful and occasionally serious wounds in humans. The emphasis of this project was at first developing an effective method of venom extracting from a natural resource and extracting hemolytic protein. This is based on the premise here being that the effects of the fish venom may be less pronounced, the animals against which the venom is presumably used in defense.

The techniques developed and described in this work, especially which of the biochemical characteristics of the venom with can hopefully be used in the further studiesfor other fish venoms.

1. History

1.1. Marine venomous creatures

1.1.1. Planktons

1.1.1. a. Red tide

Red tide is a common name for a phenomenon also known as an algal bloom (large concentrations of aquatic microorganisms), an event in which estuarine, marine, or fresh water algae accumulate rapidly in the water column and results in discoloration of the surface water. It is usually found in coastal areas. Certain species of phytoplankton, dinoflagellates such as *Gymnodinium spp*, *Pyrodinium spp*, *Alexandriumspp* and diatoms contain photosynthetic pigments that vary in color from green to brown to red.

When the algae are present in high concentrations, the water appears to be discolored or murky, varying in color from purple to almost pink, normally being red or green. Not all algal blooms are dense enough to cause water discoloration, and not all discolored waters associated with algal blooms are red. Additionally, red tides are not typically associated with tidal movement of water, hence the preference among scientists to use the term algal bloom. Some red tides are associated with the production of natural toxins, depletion of dissolved oxygen or other harmful effects, and are generally described as harmful algal blooms. The most conspicuous effects of these kinds of red tides are the associated wildlife mortalities of marine and coastal species of fish, birds, marine mammals, and other organisms (Pierce& Henry, 2008).

Fig.1.1. *Alexandrium spp.*

Fig.1.2. Aureoumbra

1.1.1. b. Brown tide

Brown tide is caused by different microalgaes: Chrysophyta (golden-brown algae) *Aureococcus spp* and *Aureoumbra spp*. Their toxins are known as Saxitoxins and Brevetoxins (Twiner, *et al*. 2012).

1.1. 2. Animals:

Fish and coelenterate stings are the most common causes of marine envenomations in humans. Most hazardous marine organisms are found in temperate or tropical oceans, particularly the Indo-Pacific region.

1.1.2. a. Invertebrates

1.1.2. a. 1.Coelenterates

Fig.1.3. *Physalia physalia* (Portuguese man of war)

Coelenterates include the corals, sea anemones, jellyfishes, and hydroids. Of the over 11,000 species, approximately 200 species are hazardous to humans. Coelenterates contain thousands of specialized stinging cells (nematocysts) capable of penetrating the skin with harpoon-like threads and injecting venom upon contact. The potency of venom varies from species to species. The venom is composed of many protein fractions, some of which have been shown to be cardiotoxic, neurotoxic, and dermonecrotic. The venom may also contain histamine, prostaglandins and serotonin. Reactions are often instantaneous, but can be delayed. They usually consist of burning, itching. Various types of skin lesions may develop after a sting, depending on the type of organism and the extent of the contact. Systemic manifestations are rare, except for the most toxic organisms. Anaphylaxis is becoming more common possibly due to the increasing number of people with previous contact, and sensitization to coelenterate venom. The Portuguese man-of-war, sea nettle, and the box jellyfish (sea wasp) can inflict serious and potentially life-threatening stings.

Portuguese man-of-war and sea nettles have caused deaths in U.S. and Caribbean waters. Fire corals, feather hydroids, and cabbage-head jellyfish are common causes of minor stings. Untreated stings resolve in 3-7 days. Most sea anemones sold for aquariums have little or no venom.

Therapy for most coelenterate stings is the same. First the area is rinsed with seawater (freshwater may cause additional firing of nematocysts). The area should be soaked for 15-30 minutes in 5% acetic acid (vinegar) to inactivate any unfired nematocysts. The vinegar will generally not relieve pain. Any tentacles should be removed using forceps or a gloved hand. Removal of the remaining nematocysts can be accomplished by applying a paste of baking soda, flour, or shaving cream, and scraping the area with a sharp knife or shaving the area with a razor. When dry, a topical corticosteroid may be applied for several days to treat the dermatitis. Chironex antivenin (produced and distributed in the Indo-Pacific regions) may be used to treat box jellyfish envenomations (Pontin& Cruickshank, 2012).

1.1.2. a. 2. Cone shells

Conusis a large genus of small to large predatory sea snails, marine gastropod molluscs, with the common names of cone snails, cone shells or cones. This genus is placed in the subfamily Coninae within the family Conidae. Geologically speaking, the genus is known from the Eocene to the Recent (Holocene) periods. Conus species have shells that are shaped more or less like geometric cones. Many species have colorful patterning on the shell surface. Conus snails are mostly tropical in distribution. They are all venomous. Live ones should be handled with care or not handled at all because they are all capable of stinging humans with unpleasant results. The species that are most dangerous to humans are the larger ones that prey on small bottom-dwelling fish. The other species hunt and eat marine worms or mollusks. Cone snails use a hypodermic-like modified radula tooth and a venom gland to attack and paralyze their prey before engulfing it. The tooth is sometimes likened to a dart or a harpoon. It is barbed and can be extended some distance out from the mouth of the snail, at the end of the proboscis. Cone snail venoms are mainly peptides. The venoms contain many different toxins that vary in their effects; some are extremely toxic. The sting of small cones is no worse than a bee sting, but the sting of a few of the larger species of tropical cone snails can be serious, occasionally even fatal to human beings. In recent years cone snail venom is showing great promise as a source of new, medically important substances (Jakubowski, 2005).

Fig.1.4.Conus snails

1.1.2. a. 3. Sea Urchin

Sea urchins are commonly kept in marine aquariums. Venomous species are not commonly sold for aquarium use.

Fig.1.5. *Echinometra mathaei*

The most common injuries result from tissue penetration by sharp spines, causing local tissue reactions and a burning sensation. The site of injury is often stained purple by a harmless dye secreted by many sea urchins. To treatment it must be administered antibiotics and analgestics, gave appropriate antitetanus agent (Slaughter *et al*., 2009).

Table 1.1.Treatment for marine envenomations (Utox updates, publication of the UTAH poison control center for health professionals, 2001).

Marine Organism	Detoxification	Treatment
Stingray; Lionfish; Scorpionfish; Catfish	Submerge injury in hot water for 30-90 min.	Irrigation with normal saline. Exploration and debridement. Administer antibiotics and analgesics as indicated. Give appropriate antitetanus agent. Observation for 3-4 hours to rule out systemic envenomation. Elevate extremity until edema has receded.
Stonefish	Same as outline for stingray	Same as outlined for stingray. Stonefish antivenin for severe systemic reactions.
Fire coral; Hydroids; Anemones	Irrigate with seawater (not fresh water). Topical 5% acetic acid (vinegar). Shave affected area.	Topical corticosteroid cream for dermatitis.
Portuguese man-of-war; Sea nettles	Same as for fire coral. Use forceps or gloves to remove tentacles.	Topical corticosteroid cream for dermatitis. All patients with systemic symptoms should be observed for 8 hours. Severe systemic symptoms mandate hospitalization with supportive care.
Box jellyfish	Same as for Portuguese man-of-war	Same as for Portuguese man-of-war. Give Chironex antivenin. Supportive care for hypotension and respiratory depression.
Sea Urchin	Hot water. Removal of any spines or pedicellariae.	Exploration and removal of any spines.

1.1. 2.b. Vertebrates

1.1. 2. b. 1. Sea snakes

Fig.1.6.Yellow-bellied sea snake, *Pelamis platurus*

Sea snakes are venomouselapidsnakes that inhabit marine environments for most or all of their lives. Though they evolved from terrestrial ancestors, most are extensively adapted to a fully aquatic life and are unable to even move on land, except for the genus Laticauda, which retain ancestral characteristics which allow limited land movement. They are found in warm coastal waters from the Indian Ocean to the Pacific. All have paddle-like tails and many have laterally compressed bodies that give them an eel-like appearance. Unlike fish, they do not have gills and must surface regularly to breathe. They are among the most completely aquatic of all air-breathing vertebrates. Among this group are species with some of the most potent venoms of all snakes. Some have gentle dispositions and bite only when provoked, but others are much more aggressive. Currently, 17 genera are described as sea snakes, comprising 62 species. As it is easier for a snake's tongue to fulfill its olfactory function under water, its action is short compared to that of terrestrial snake species. Most sea snakes are able to respire through their skin. This is unusual for reptiles, because their skin is thick and scaly, but experiments with the black-and-yellow sea snake, *Pelamis platurus* (a pelagic species), have shown that this species can satisfy about 20% of its oxygen requirements in this manner, which allows for prolonged dives. Sea snakes do not occur in the Atlantic Ocean and in the Red Sea, believed to be due to its increased salinity. Most sea snake species prey on fish, especially eels. One species prefers mollusks and crustaceanssuch as prawnswhile a few others feed only on fish eggs, which is unusual for a venomous

snake. Except for a single genus, all sea snakes are ovoviviparous; the young are born alive in the water where they live their entire life cycle. The one exception is the genus Laticauda, which is oviparous; its five species all lay their eggs on land (Campbell & Lamar, 2004).

1.1.2. b. 2. Sea snakes Venom

The majority of sea snakes are highly venomous like their cousins in the Elapidae family, however when bites occur, it is rare for much venom to be injected, so that envenomation symptoms usually seem non-existent or trivial. For example, *Pelamis platurus* has venom more potent than any other terrestrial snake species in Costa Rica, but despite its abundance in the waters off its western coast, few human fatalities have been reported. Nevertheless, all sea snakes should be handled with great caution (Campbell & Lamar, 2004).

Bites in which envenomation does occur are usually painless and may not even be noticed when contact is made. Teeth may remain in the wound. There is usually little or no swelling, and it is rare for any nearby lymph nodes to be affected. The most important symptoms are rhabdomyolysis (rapid breakdown of skeletal muscle tissue) and paralysis. Early symptoms include headache, a thick-feeling tongue, and thirst, sweating, and vomiting. Symptoms that can occur after 30 minutes to several hours post-bite include generalized itching, stiffness, and tenderness of muscles all over the body. Passive stretching of the muscles is also painful. This is followed later on by symptoms typical of other elapidenvenomations: a progressive flaccid paralysis, starting with paralysis of voluntary muscles. Paralysis of muscles involved in swallowing and respiration can be fatal. After 3–8 hours, myoglobin as a result of muscle breakdown may start to show up in the blood plasma, which can cause the urine to turn a dark reddish, brown, or black color, and eventually lead to acute renal failure. After 6 to 12 hours, severe hyperkalemia, also the result of muscle breakdown, can lead to cardiac arrest (Warrell, 2004).

1.1.2. b. 3. Venomous and poisonous fishes

There are approximately 1000 species of fishes which are known to be either poisonous or venomous. Although these terms "poisonous" and "venomous" have often been used synonymously in the literature, there is an important distinction between them.

Poisonous fishes are those fishes which cause a biotoxication upon ingestion of their tissues which are either partially or entirely toxic. It has further classified this group of fishes into: those which contain a poison within their musculature, viscera,

or skin (Ichthyosarcotoxic), within their gonads (Ichthyotoxic), or within their blood (Ichthyohemotoxic).It is added a fourth division to include those fishes which produce a toxin by glandular secretion, but which lack a true inflicting apparatus (Ichthyocrinotoxic). Poisonous fishes and their associated toxins have been studied in the past few decades, and the chemistry and pharmacology of these toxins have been extensively reviewed.

In some cases, the study of poisonous fishes has led to the discovery of valuable pharmacological and neurological tools, such as tetrodotoxin from the puffer fishes (family Tetrodontidae) and the porcupine fishes (family Diodontidae). Venomous fishes, on the other hand, are those fishes which are capable of producing a toxin in a highly developed secretory organ, gland, or specialized group of cells, and which can administer this venom with some form of spine or stinging apparatus. Venomous fishes and their toxins, however, have not been studied in much detail. It was estimated that out of the entire group of venomous fishes, of which there are over 200 species, less than five percent of these have been studied even in a cursory manner. The study of the biological activities of fish venoms is complicated by a number of factors which also help to explain the lack of basic knowledge in this field. The primary reason is that a characteristic common to all fish venoms is their extreme instability. Most fish venoms lose activity at room temperature and some are labile even at temperatures of 0°C. Activity can also be lost or significantly decreased even upon lyophilization. Fish venom research is further complicated by the fact that quantitative and qualitative differences in venom composition may exist intraspecifically as well as interspecifically. Fish venom can possibly vary within the individual animal at different times of the year or under varying environmental conditions. The toxins of venomous fishes differ considerably in their pharmacological and chemical properties from the toxins of the poisonous fishes, and especially from the toxins of other aquatic and terrestrial venomous animals. For example, there is no relationship between lionfish venom and pufferfish poison (Kapoor, 2010).

Although these inherent unstable properties of fish venoms have impeded much progress in this field, a number of chemical, pharmacological, and anatomical studies on a wide variety of venomous fishes were done in the late 1950's and early 1960's. Fish reported to sting humans include Lionfish, scorpionfish, stonefish, stingrays, and catfish. They are primarily composed of proteins and polysaccharides of various sizes. The toxic and painful components tend to be large, unstable proteins that are rapidly destroyed by heating. Therefore, the primary method of decontamination is soaking the appendage in hot water for 30-

90 minutes. Venoms can retain full potency for at least 24 to 48 hours after the death of the fish (Kapoor, 2010).

1) Scorpionfishes

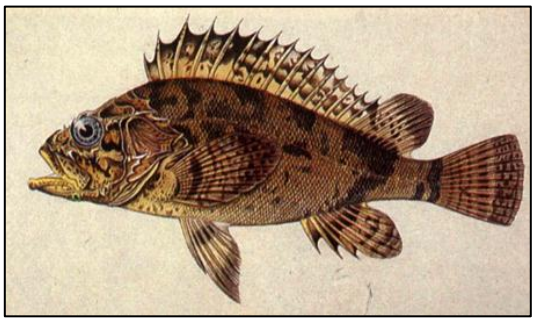


Fig.1.7. Scorpionfish

The family Scorpaenidae contains hundreds of species which are divided into three groups; zebrafish (or lionfish), scorpionfish and stonefish, based on the structure of their venomous organs.

There are approximately 80 species of fishes belonging to the suborder Scorpaenoidei (families Scorpaenidae and Synancejidae) which have been determined to be venomous. This group encompasses the true scorpionfishes (genus Scorpaena), the lionfishes or turkeyfishes (genus Pterois), and the stonefishes (genus Synanceja). As stated previously, studies on fish venoms have been hampered due to the instability of these venoms. However, certain chemical similarities of the venoms of the scorpionfishes (suborder Scorpaenoidei) have been reported, and data obtained on the venom of one species has been correlated with results from venom studies of other species (Carrijo *et al*., 2005).

2) Lionfish

Fig.1.8.Lionfish

Lionfish are the least venomous of all scorpionfish and are commonly kept in saltwater aquariums. They are capable of inflicting painful, but relatively mild wounds. Most injuries are self-limiting and can be treated at home. In comparison to the amount of research done with stonefish (genus Synanceja) and scorpionfish (genus Scorpaena) venoms described above, relatively little work has been done with lionfish venom.

Stings by lionfishes cause immediate intense and sometimes burning pain which quickly radiates from the site of injury to other parts of the body. Lionfish envenomations seem to cause more severe pain and are more dangerous than those from stingrays, scorpionfishes, and weaverfishes; however, they are not as dangerous as the stings from the stonefishes. Deaths have been reported, although not confirmed, from lionfish stings, and in some cases the non-fatal envenomations can -be very serious (Balasubashini, 2006).

3) Stonefish

Fig.1.9.Stonefish

Stonefish are among the most venomous fish in the world. Lee and colleagues (2004) reported aspirated the fluid from the dorsal fin spine sacs of the stonefish (*Synanceja horrida*).

Stonefish venom has been compared to cobra venom in potency. It can paralyze both skeletal and smooth muscle due to direct muscle toxicity. Stonefish are found in the Indo-Pacific region and not in U.S. coastal waters. Stonefishes possess the most lethal, painful, and dangerous venom of all of the venomous fishes, and envenomations from them usually requires imediate intensive care. Totally, Scorpionfish include the stonefish and the sculpin commonly found along the California coast. Envenomations are most likely to occur after a fish is caught and is handled improperly, or a swimmer touches or steps on one hiding among the rocks. Envenomations are moderate to severe, but are rarely lethal. Stings are immediately painful, peaking in 60 to 90 minutes if not treated. Pain typically persists for 6 to 12 hours, but may persist for days.

For treatment spines should be removed immediately. This is often a difficult task due to the brittleness of the spines. Soaking the wound in hot water may help expel some spines from the body. Vinegar has been used to dissolve superficial spines, but is not effective at dissolving the embedded spines. Spines will eventually be absorbed by the body, but increase the risk of infection. Special concern must be taken if spines have penetrated near a joint (Lee *et al*., 2004).

4) Catfish

Fig.1.10.Catfish

Most catfish found in the U.S. are freshwater fish and are capable of inflicting painful stings that resolve in a few hours. The Indo-Pacific region contains the most venomous species of catfish. Catfish stings are caused by the spines found in the dorsal and pectoral fins and not by the sensory whiskers. Stings are most likely to happen after a fish is caught and handled improperly.

General wound care principles apply to all fish stings. Wounds should be irrigated with the available water (seawater in many cases) to remove debris and venom. The affected area should then be soaked in hot water for 30-90 minutes to reduce pain and inactivate the venom. The injured person may have difficulty judging the temperature of the water, so care must be taken not to scald the skin. All patients should receive the appropriate anti-tetanus agent if needed. Large wounds may require debridement and possibly closure. The main concern at this point is removal of fragments remaining in the wound and prevention of infection. Infected wounds should be cultured for aerobes and anaerobes (Ololade& Oginni, 2009).

5) Pufferfish

Pufferfish are considered poisonous because they possess a potent neurotoxin, tetrodotoxin (TTX) and its analogs, which act on site 1 of the voltage-dependent sodium channels of excitable membranes, blocking sodium influx and, consequently, action potential. These toxins are acquired through the food chain or from symbiotic bacterial strains found on skin or in the digestive tract of these fish. In some localities, pufferfish species can be utilized for food. Food poisoning by human consumption of toxic puffers has occurred, especially in Japan and China where the flesh of these fish is considered a delicacy (Arakawa *et al*., 2010).

Fig.1.11. Pufferfish

6) Stingrays

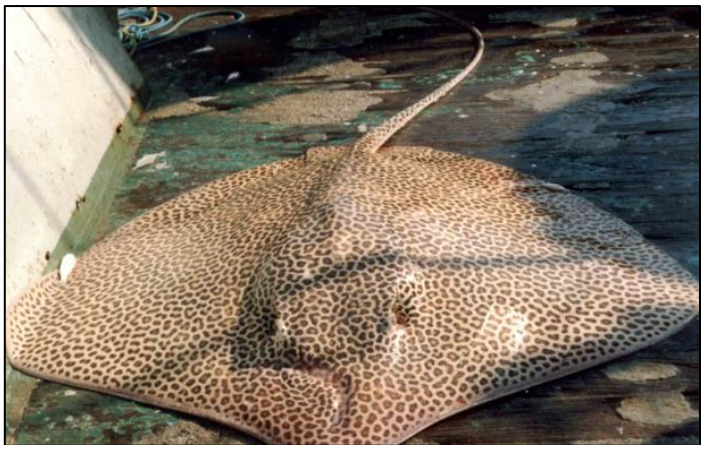


Fig.1.12.Stingray

In America, stingrays probably sting humans more frequently than any other fish. Stingrays are commonly found in tropical, subtropical, and warm temperate oceans. Eleven species are found in U.S. coastal waters. Stingray envenomations typically occur when a wader steps on a stingray resting on the sea floor. The stingray reflexively whips its tail upward and extends the stinger. This usually produces a laceration or puncture-wound in the foot or leg of the victim. The stinger is covered by a cartilaginous sheath that breaks apart and releases venom as it penetrates the skin of the victim. The sting results in immediate intense pain and variable amounts of bleeding. The pain peaks after 30-60 min and may last up to 48 hours. Venom,

mucous, pieces of the sheath, and even fragments of the spine can be released in the wound, and large laceration can be formed. After initial treatment, the extremity should be elevated until edema resolves (Rathinam *et al.*, 2011).

7) Toadfish

The entire family Batrachoididae is called toadfishes. They are benthic ambush predators, known for their ability to produce sound with their swim bladders. *Thalassophryne nattereri*, popularly known as niquim, is a cuneiforme and about15 cm long. It has a repugnant look and a special apparatus for venom injectionincluding spines located near to the opercular and dorsal. Accidents occurthrough contact with these spines causing perforation of the tegument andinjection of venom from the venom glands (Lopes-Ferreira *et al*., 2000).

Fig.1.13. toadfish

8) weeverfish

Weevers (or Weeverfish) are nine extant species of fish of family Trachinidae, order Perciformes. They are long (up to 37 cm), mainly brown and have venomous spines on their first dorsal fin and gills. During the day, weevers bury themselves in sand, just showing their eyes, and snatch prey as it comes past, which consists of shrimps and small fish. Weevers are unusual in not having a swim bladder as do most bony fish and as a result sink as soon as they stop actively swimming. With the exception of *Trachinus cornutus* from the south-east Pacific, all species in this family are restricted to the eastern Atlantic (including the Mediterranean). In fact, the word “weever” is believed to derive from the old French word “wivre”, meaning serpent or dragon, from the Latin 'vipera'. It is sometimes also known as

the viperfish, although it is not related to the viperfish proper (i.e., the stomiids of the genus Chauliodus)

(Frickhinger, 1996).

Fig.1.14.Weeverfish

2. Introduction

2.1. Scats (Scatophagus)

So far, it has been done a little research about Scats and we try to find some of toxic effects in this inquiry.

2.1.1. Nomenclature and distribution

The name "scatophagus" actually means "the dung eater" because of their feeding habits of eating just about anything. *Scatophagus argus argus*, commonly known as butterfish, argus fish, spade fish, spotted spade fish and spotted scat(Sivan, 2007), is widely distributed in brackish water and in distributed in the mudflats, mangroves swamps, harbors, upstream swamps, estuaries and marine habitats of the Indo-Pacific, South and South-East Asia, Persian Gulf, the Malay Archipelago, the Philippines and Australia .The name Scatophagidae is not preoccupied by Scathophagidae (dung flies, Diptera), and appeared as "subfamilia Scatophagiformes" in . The quality and taste of the fish ranks it as an edible fish and the beautifully spotted rhombic body ranks it as a fascinating aquarium fish (Parenti,2004).

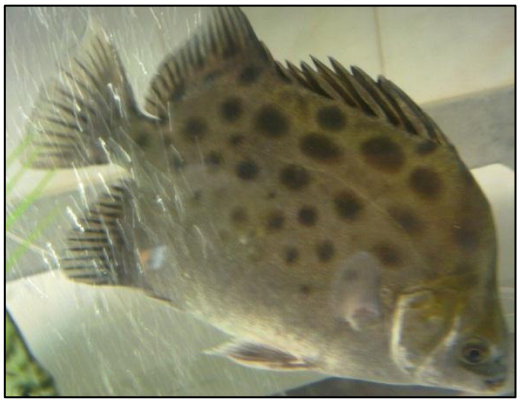


Fig.2.1.External feature of *Scatophagus argues argus* (photo by writer).

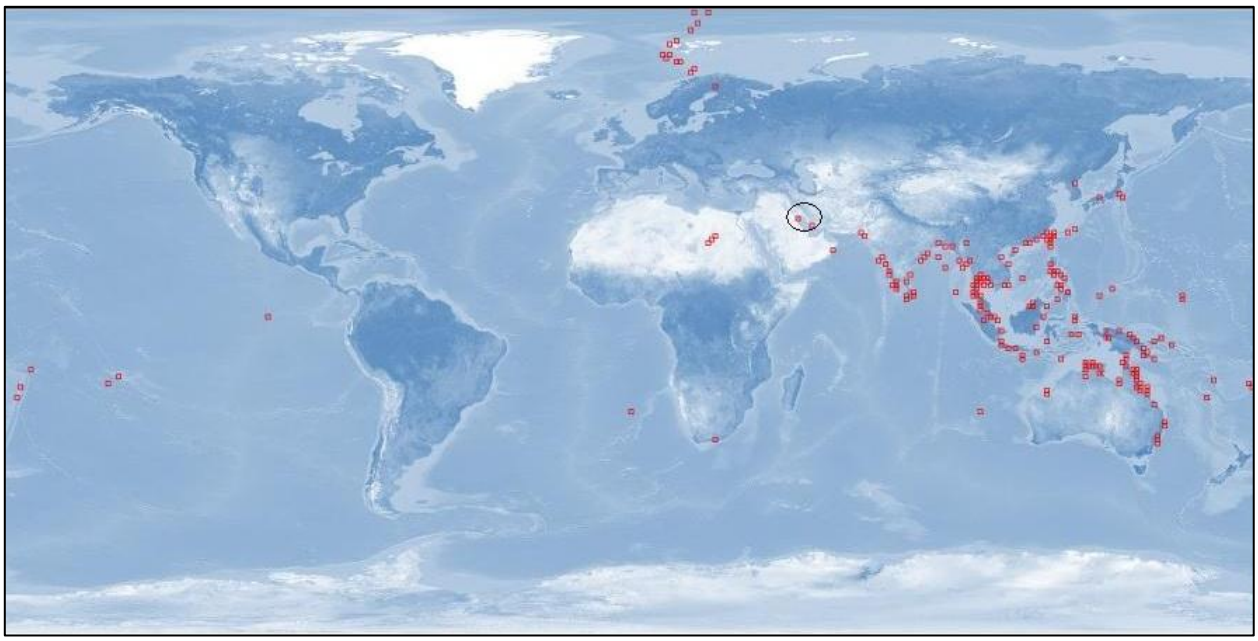


Fig.2.2. Distribution of *S.argus* in the world and in Persian Gulf (circle).

2.1.2.Morphology

Scats have compressed, deep bodies, like butterfly fishes (Chaetodontidae), covered by small ctenoid scales which extend onto the soft tissue at the bases of the median fins. Head scaly, without spines. Preopercle margin not serrated. Dorsal profile of head slightly concave to straight in juveniles, distinctly concave above the eyes in adults. Mouth non protrusible, armed with long, flattened teeth with tridentate distal ends, disposed in several broad bands; vomer and palatines edentate; maxilla covered by preorbital bone when mouth closed. Pelvic axillary process present. Dorsal fin deeply notched, with 11–12 spines and 16–17 (rarely 15 or 18) soft rays; first three spines progressing in length, with the fourth either of the same length as the third or slightly the longest in the series; first pterygiophore bearing the first two spines. Anal fin with 4 spines and 14–16 (rarely 17) soft rays. Pectoral fins short and rounded. Pelvic fins with 1 spine and 5 branched rays. Caudal fin rounded in juveniles, truncate to slightly double emarginated in adults, with 14 branched rays. Vertebrae 23 (10 + 13). Lateral line distinct, running about parallel to dorsal profile. Gill membranes united and forming a narrow fold across the isthmus. Swim bladder present and simple. Maximum size and Longevity of green scat can grow to about 35cm, sometimes even larger in the wild. They can live anywhere between 10 and 15 years old. Scatophagids have a larval development that has heavily pigmented pelvic fins, early developing small spiny scale precursors, and a heavily armored head with fused plates, blunt spines, and

projecting ridges capped with swollen granulated pads, all vanishing with growth .The family includes two genera, each containing two species. Two additional species, each belonging to a separate genus, are known from the fossil record (middle Eocene–Oligocene, northern Italy) provided a key to the extant and fossil genera (Parenti, 2004).

2.1. 3.Color and Varieties

Young fish usually dark, but there are a few different types of scats but only the red, green and silver regularly enter fresh water.

Red Scat: the red scat is probably the most popular of the scats because of its colors. They are a round fish with a tall body and have spiky dorsal fins that are said to have a small amount of toxins in them. They have vertical lines that run down the body that are a red to ruby color when they are younger as the mature they lose these lines and spots become more predominant. The red scat (*Scatophagus argus rubifrons*) might not be a distinct variety of scat, but merely a developmental stage of the common spotted scat.

Silver Scat: They are a round fish with a tall body and have spiky dorsal fins that are said to have a small amount of toxins in them. They have vertical lines that run half way down the body that is black the rest of the body is covered in spots. As the fish matures they will lose the stripes and they will be replaced with spots.

Green scat: The body of it is quadrangular and strongly compressed.The dorsal head profile is steep and the snout is rounded. Spines and rays of the dorsal fin are separated by a deep notch. Small ctenoid scales cover the body. The body is greenish-brown to silvery with many black spots. Juveniles are greenish –brown with either a few large, rounded blotches (approximately the size of the eye). Individuals greater than about 4 cm SL (standard length) have blackish spots (typically slightly smaller than eye). In large adults the spots can fade and appear only dorso-laterally. Adults can approximately reach 30 cm TL (total length)(Parenti, 2004).

Fig.2.3. Red scat

Fig.2.4. Silver scat

Fig.2.5. Green scat

2.1.4. Sexing and maturity

Males have concave curvature of the head above the eye, whereas in females the head ascends at a constant slope.

Females mature at about 7-9 monthes of age and 150 g, while males at a smaller size. In the Philippines spawning is triggered by mansoon rains that begin in June and July and bring rainfall, cooler temperatures increase river outflows and lower salinities (Parenti, 2004).

2.1.5. Classification

Table 2.1. There are some other scientific names for *Scatophagus argus*

Genus	**Species**
Chaetodon	*Chaetodon argus*
Chaetodon	*Chaetodon atromaculatus*
Chaetodon	*Chaetodon pairatalis*
Ephippus	*Ephippus argus*
Cacodoxus	*Cacodoxus argus*
Scatophagus	*Scatophagus aetatevarians*
Scatophagus	*Scatophagus bougainvillii*
Scatophagus	*Scatophagus maculatus*
Scatophagus	*Scatophagus ornatus*
Scatophagus	*Scatophagus purpurascens*
Scatophagus	*Scatophagus quadranus*
Scatophagus	*Scatophagus argus tromaculatus*
Scatophagus	*Scatophagus argus ocellata*
Scatophagus	*Scatophagus argus argus*

Table 2.2.Taxonomy of *S. augus argus* (Linnaeus, 1766)

Kingdom	***Animalia***
Phylum	***Chordata***
Subphylum	***Vertebrata***
Class	***Osteichthyes***
Subclass	***Actinopterygii***
Division	***Euteleostei***
Order	***Perciformes***
Family	***Scatophagidae***
Genus	***Scatophagus***
Species	***S.argus***
Subspecies	***S.argus argus***

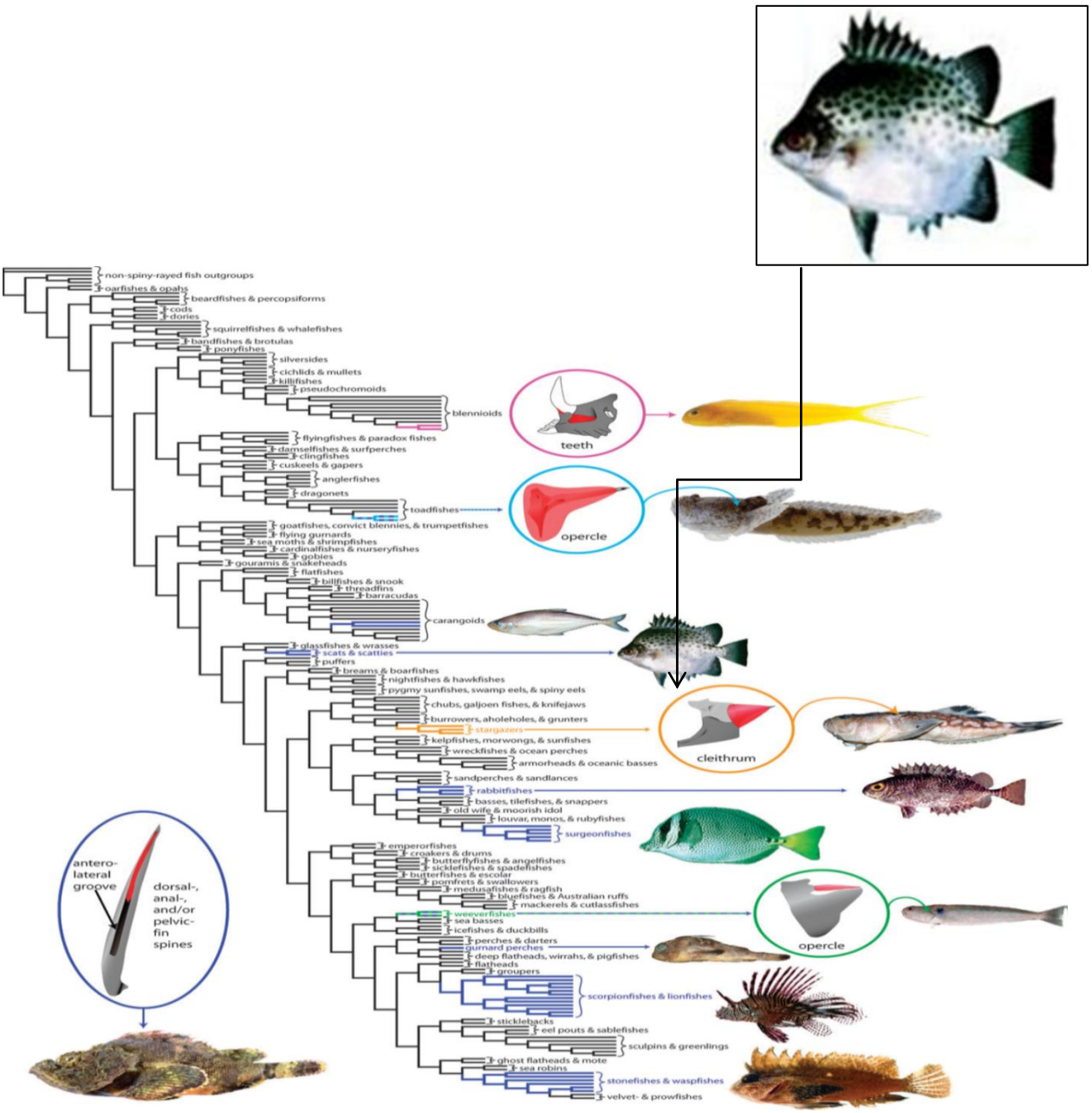


Figure2.6. Phylogenetic road map for venomous fishes (Smith &Wheeler, 2006).

2.1.6. Feeding

Spotted scat has a broad diet and eats detritus, filamentous algae, phytoplankton, aquatic saprophytes, zooplankton, benthic invertebrates and other zoobenthos. So scats are omnivores and will eat anything provided such as beef heart, dry foods, and vegetable matter such as spinach or algae. They also thrive on small

crustaceans and aquatic insects. Scats also readily eat frozen foods as well as pellets and flake (Sivan *et al*., 2007& Sivan,2009).

2.1.7. Ecology

2.1.7.1. Water Quality

Scats prefer hard, alkaline to brackish water conditions. Temperature is from 20°C to 30°C, pH is from 7.5 to 8.2 and general hardness is from 250 to 300 ppm or higher (Siven *et al*., 2007).

2.1.7.2. Compatibility

These fish are a very versatile fish that will mix with most species that inhabit similar water conditions. They are ideally suited to brackish community tankswith species such as; Archerfish, other Scats (silver scat)and Madagascar rainbows. It must be avoided keeping anything smaller than their mouth in the tank as they can be a predatory fish (Karmakar *et al*., 2004; Muhuri *et al*., 2004).

2.1.8. Envenomation

The wide distribution of *Scatophagus argus argus,* large catch and close proximity to humans (who are unaware that the scats are venomous) result in its envenomation.Fishermen are wounded while handling *S. argus*. Most patients are treated with an empirical procedure such as immersion of the wounded area in hot water. The puncture is painful and aches for many hours. Of the family Scatophagidae, *S. argus* is said to inflict more painful wounds than allied species. During envenomation, the spines are erect and the mechanical pressure on the spine tears or pushes down the integumentary sheath over the spine as venom passes into the wound (Karmakar *et al*., 2004; Muhuri *et al*., 2004).

2.1.8.1. Clinical Symptoms and signs

Scatophagus argus of the family Scatophagidae inflicts painful wounds in fishermen during handling. *S. argus* envenoming appears within 5–10 min as the clinical picture is characterized by excruciating and persistent local pain disproportionate to the size of injury, redness, swelling and a throbbing sensation that extends to the limbs, followed by dizziness.

The symptoms are variable, depending on the size of the fish and the quantity of poison injected. Therefore, envenomation represents a serious health hazard. As reported in FishBase (Froese&Pauly, 2003) a sting of fish *S.argus* produces

tremendous local pain, sever swelling, rise of body temperature, etc. (Karmakar *et al*., 2004; Muhuri *et al*., 2004).

2.1.8.2. The venom apparatus

The venom apparatus of *S. argus* is made up of elongated venom glands in grooves in the anterior portion of 11 dorsal spines, 2 pelvic spines and 4 anal spines possessed a pair of venom glands accommodated in paired anterolateral grooves in each fin spine. The venom glands of the larger specimens were shorter than those of the smaller fishes. Scales extended relatively further distally along the fin spines in larger specimens than in smaller specimens. The glands were aggregations of large gland cells in the thickened epidermis of the integumentary sheath which filled the spine grooves. The glands were not encapsulated in connective tissue sheaths. Elongate supporting cells occurred among the venom gland cells some of which possessed unusual rod-like bodies in their cytoplasm (Cameron & Endean, 1977).

Fig.2.7. Dorsal spines of *Scatophagus argus argus*

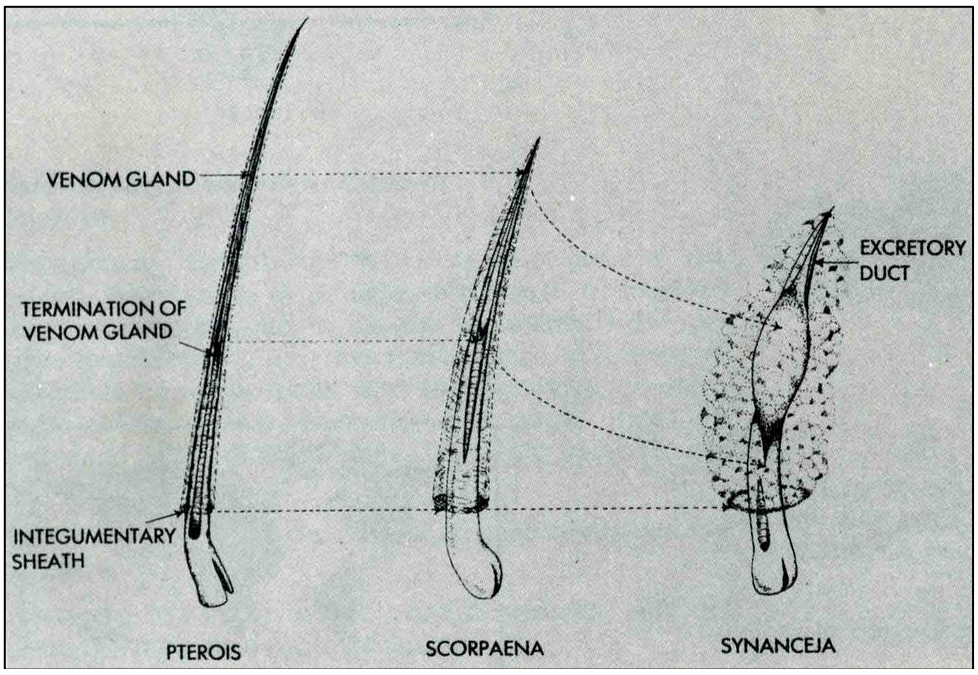


Fig.2.8. The external spine image of three venomous fishes.

2.1.8.3. Venom Preparation in venomous fishes

Studies on the chemical and biological properties of piscine venom have mainly focused on stonefish (Lee *et al*., 2004), lionfish (Balasubashini, 2006), scorpion fish (Carrijo *et al*., 2005) and toadfish (Pareja-Santos *et al*., 2009).

Totally five methods of venom extraction, which did not involve the killing of the fish, were studied. The rubber sheet method of Endean (1961) and Cameron and Endean (1966), the sponge method of Carlisle (1962), the hypodermic needle method of Saunders and Tokes (1961), and the aspiration and batch method of Schaeffer and colleagues (1971). In this study, the method of Sivan (2007) was used with some modification at first. Then a new method was established with some modification by Termine protocol that was used for demineralization of bones (1981).

2.1.8.4. Toxins isolated from piscine venoms

There are several toxins isolated from venomous fishes.

Table2.3.The toxins isolated from piscine venoms (Church *et al*.,2002).

Species	Toxin	Structural properties	Author(s)
Stingray (*U. halleri*)		100 kDa	Russell (1965)
Greater weeverfish (*T. draco*)	Dracotoxin	105 kDa polypeptide	Chhatwal and Dreyer (1992b)
Lesser weeverfish (*T. vipera*)	Trachinine	324 kDa. Composed of four identical subunits	Perriere et al. (1998)
Californian sculpin (*S. guttata*)		50–800 kDa	Schaeffer et al. (1971)
Lionfish (*P. volitans*)		Non-proteinaceous toxin. 327 Da	Nair et al. (1985)
Bullrout (*N. robusta*)	Nocitoxin	170 kDa	Hahn and O'Connor (2000)
Brazilian niquim (*T. nattereri*)		47 kDa	Lopes-Ferreira et al. (1998)
Indian catfish (*P. canius*)	Toxin-PC	15 kDa	Auddy and Gomes (1996)
Reef stonefish (*S. verrucosa*)	VTx	Four subunits 2α (83 kDa) and 2β (78 kDa)	Garnier et al. (1995)
Indian stonefish (*S. horrida*)	SNTX	Two subunits α = 699AA(79 388 Da), β = 702AA(79 290 Da)	Poh et al. (1991)
Estuarine stonefish (*S. trachynis*)	TLY	158 kDa	Colasante et al. (1996)

SNTX=vasodilation, TLY=heart contracture, VTx=hypotention, Dracotoxin=membrane depolarising activity,

2.1.8.5. Toxin affects mechanisms

Table 2.4.The toxins affect mechanisms (Sitprijaa & Suteparakb, 2008).

Table 1. Animal toxin effects on ion channels and pore formation

	Na channel	K channel	Ca channel	Cl channel	Pore formation
Jellyfish	↑		↑		+
Sea anemone	↑	↓	↑		+
μ conotoxin	↓				
δ conotoxin	↓				
ω conotoxin			↓		
Ciguatoxin	↑				
Tetrodotoxin	↓				
Saxitoxin	↓				
Gonyautoxin	↓				
Palytoxin	↑	↓			+
Maitotoxin			↑		
Stonustoxin			↑		
Annelid	↑				
Brevetoxin	↑				
Bratachotoxin	↑				

* ↑ = activation or slow inactivation; ↓ = inactivation

2.2. Techniques used in protein purification

2.2.1. Animal cells

Animal cells in culture (diameter 10 m) are surrounded by a thin (6.0 nm) plasma membrane which can be easily broken by the shear forces generated by tissue grinders, homogenizers or sonicators. In addition, the integrity of animal cell membranes can be broken by the inclusion of membrane destabilizing compounds (e.g. detergents and solvents) or by osmotic shock.

Animal cells from tissue (e.g. liver or lung), after being trimmed from surrounding fatty tissue, can be cut with scissors into small fragments and disrupted by the shear forces generated by homogenization. Small amounts of tissue can be fragmented in a tissue grinder or small handheld homogenizers. Larger amounts of tissue or animal tissue with a lot of fibrous extracellular material can be homogenized using a blender(Bonner, 2007).

2.2.2. Protein purification strategy and equipment

The Purifying a protein is essential first step in understanding its function (Berg *et al*., 2006). For this reason we try to purify the proteins and some scientific methods have been practiced in this research.

2.2.2.1. SDS – PAGE

Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis using polyacrylamide is a very popular method of analyzing protein mixtures because the technique is both reproducible and flexible. The polyacrylamide gels are formed by the polymerization of acrylamide monomers in the presence of the N, N-methylene bisacrylamide which acts as a cross-linking reagent. The reaction is free radical catalysis, and following initiation there is a period of polymer elongation and then termination. Two catalysts are required to initiate the polymerization tetramethylene diamine (TEMED) and ammonium persulfate (APS). TEMED catalyzes the decomposition of the persulfate ion to produce a free radical. Gels with different percentages of acrylamide can be cast to alter the fractionating properties of the gel. A 10% acrylamide gel will fractionate proteins in the mass range of 15000 to 200000, whereas a 5% acrylamide gel will fractionate proteins in the mass range 60000 to 350000. Smaller pores prevent entry of large proteins onto the gel. This is called molecular sieving and it should be remembered that proteins with a mass larger than the upper limit will not be able to gain entry into the gel. A gel with a low percentage of acrylamide (<5%), called a

stacking gel, is routinely cast on top of the main gel (resolving gel) with a higher percentage acrylamide designed to fractionate the sample. The purpose of the wide-pored stacking gel is not to fractionate the samples but to concentrate them, so that all the samples (irrespective of the volume applied) enter the resolving gel as a narrow band. This is achieved by using discontinuous buffers. A popular discontinuous buffer system uses Tris–HCl pH 8.8 as the resolving gel buffer, Tris–HCl pH 6.8 as the stacking gel buffer and Tris–glycine pH 8.3 as the electrode buffers. At the start of the experiment the glycinate ions (pH 8.3) in the upper buffer chamber and the chloride ions in the stacking gel (pH 6.8) are fully charged. When the glycinate ion enters the stacking gel (pH 6.8) it loses a lot of charge and is less electrophoretically mobile than the negatively charged protein complexes or the chloride ion. The protein complexes are sandwiched between the fast-moving chloride ion and the slower-moving glycinate ion (Timbrell, 2009).

2.2.2.2. Visualizing the proteins on polyacrylamide gels

The most commonly used protein stain, after SDS-PAGE, is Coomassie blue (sensitivity approximately 100 ng per protein band) in a methanol–acetic acid mixture (used to precipitate the proteins within the gel, preventing them from floating away before analysis). Other Coomassie-based protein gel stains are available to purchase (e.g. colloidal Coomassie) with increases in the levels of detection and ease of use. Silver staining increases the level of sensitivity approximately 100-fold, detecting down to 1 ng of protein in a band. Silver staining can be used instead of Coomassie blue (or after Coomassie blue staining) to increase the levels of detection. Fluorescent stains such as (Invitrogen) give a comparable level of sensitivity to silver. A rapid method of visualizing polypeptide bands in gels is to include 0.5% (v/v) trichloroethanol (TCE) in the acrylamide mixture prior to polymerization (Ladner *et al*., 2004). After electrophoresis the polypeptides can be visualized by exposure to UV light for 5 min, which catalyzes a reaction between tryptophan and TCE to produce a fluorescent product. Once visualized, the gel can use for other analytical techniques such as western blotting (Timbrell, 2009).

2.2.2.3. Chromatographic techniques

Biomolecules are purified using chromatography techniques that separate them according to differences in their specific property as shown in (Fig.2.9).

Tab.2.5. The properties of chromatographic techniques (Amersham, 2002).

Property	Technique
Size	Gel filtration (GF), also called size exclusion
Charge	Ion exchange chromatography (IEX)
Hydrophobicity	Hydrophobic interaction chromatography (HIC) Reversed phase chromatography (RPC)
Biorecognition (ligand specificity)	Affinity chromatography (AC)

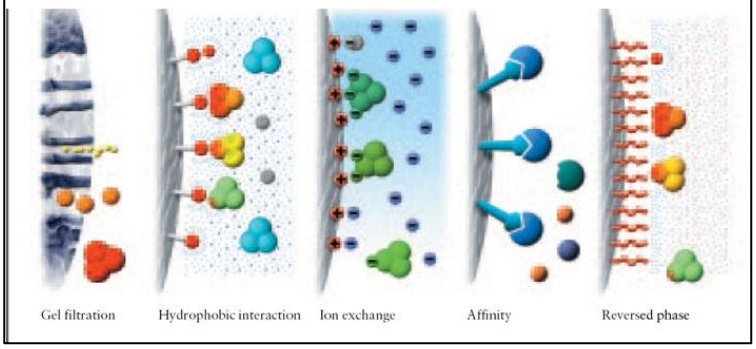


Fig.2.9. separation principles in chromatography purification (Amersham, 2002).

Techniques based on liquid chromatography (LC) are the most widely used non-bioassay methods for determination of PSP (paralytic shellfish poison) compounds. During the last decade considerable effort has been applied to the development of an automated LC method for routine analysis of PSP toxins(Amersham, 2002).

2.2.2.4. Reverse phase HPLC

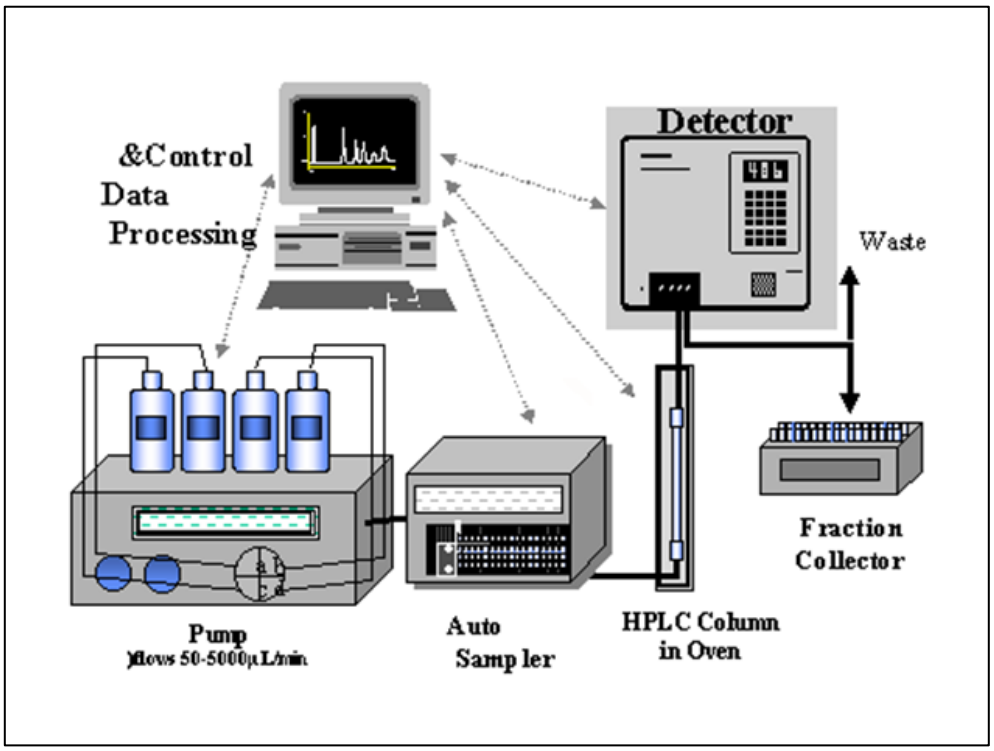


Fig.2.10.(RP-HPLC)

Reversed phase HPLC is a well-established technique in the isolation and analysis of proteins. Aliphatic groups (e.g. C8, C12 or C18) bonded to a silica resin form the stationary phase in the column. There are many resins specifically manufactured for use with proteins and peptides. At the start of the chromatographic run, the mobile phase is initially a polar solvent.

When a protein sample is applied to the RP resin, the hydrophobic regions in the protein's structure interact with the hydrophobic stationary phase. Gradually increasing the hydrophobicity of the solvent over a period of time will elute the bound proteins, with the most hydrophobic proteins eluting later in the chromatographic run. To increase the amount of bonded phase in an RP column, the particle size is normally 5 µm but can be as small as 2 µm. Elevated pressures, produced by piston pumps, are required to move the mobile phase through the small-diameter resins. The pumps can also be controlled to produce a solvent gradient. A detector capable of monitoring in the UV region is also required, as proteins absorb UV light at 280 nm (due to the presence of the amino acids tyrosine and tryptophan) and at 206 nm (due to the presence of the peptide bond). A fluorescence detector can be used (excitation wavelength 280 nm and emission

wavelength of 320 nm for tyrosine or 340 nm for tryptophan) which will increase the sensitivity, and a fraction collector after the detector can be used to collect the samples.

The solvents used in RP-HPLC (e.g. methanol and acetonitrile) are volatile and easily removed from the sample at the end of the chromatographic run. However, the tertiary structure of a protein may be altered as a result of the interaction with the stationary phase, and the protein may not fold back into its native conformation, resulting in loss of biological activity. The loss of biological activity may be minimized by reducing the run times and by switching to a less damaging solvent. The inclusion of inhibitors or cofactors can help to stabilize a protein's structure. Also, the addition of a concentrated buffer in the fraction collector tubes may help the protein to refold, as the protein will spend less time in solvent. The loss of biological activity may be a problem if RP-HPLC is to be used to purify the protein, but is not a problem when the technique is used to monitor the purity of protein fractions, or when a protein is being prepared for raising antibodies (Sheehan, 2009).

2.3. Determination of protein Concentration

2.3.1. Colorimetric Methods in General

The colorimetric reagent changes color upon reaction with a protein. There are two general classes: Cu redox chemistry based and Coomassie blue binding.

Protein denaturation in these methods is absolutely required in order to get color change. Denaturation in Biuret, Lowry, and BCA is due to high [NaOH] and denaturation in Bradford due to high [H3PO4]. Therefore all these methods are pretty caustic and suitable precautions should be taken. Bradford assay protocol is less time-consuming. On the other hand, detergents such as, deoxycholate or Triton X100 make trouble because they form coarse precipitates in the strong acidic reagent, and this method also gives false results if the sample is microheterogeneous, as observed in the case of some membrane protein preparations. The SDS interferes strongly at concentration above 0.2%. The blank is mostly high, but there is no influence on the measurement. The absorption is measured at 590 nm after 5 min (Bonner, 2007).

2.3.1.1. Bradford method

A simple procedure for the determination of protein concentration in solutions is the Bradford protein assay which was described first by Bradford.

An estimation of protein concentration is essential to be done rapidly and accurately in many fields of protein study. The Bradford assay has become the preferred method for quantifying protein in many laboratories. This technique is simpler, faster, and more sensitive than the Lowry method. Furthermore, when comparedwith the Lowry method, it is subject to less interference by common reagents and non-protein components of biological samples. The Bradford assay relies on the binding of the dye Coomassie Blue G-250 to protein.

Detailed studies indicate that the free dye can exist in three different ionic forms for which the pKa values are 1.15, 1.82, and 12.4. Of the three charged forms of the dye that predominate in the acidic assay reagent solution, the more cationic red and green forms have absorbance maxima at 470 nm and 650 nm, respectively. In contrast, the more anionic blue form of the dye, which binds to protein, has an absorbance maximum at 590 nm.

Thus, the quantity of protein can be estimated by determining the amount of dye in the blue ionic form. This is usually achieved by measuring the absorbance of the solution at 595 nm (Bradford *et al*., 1976).

2.3.1.2. BCA method

The BCA assay primarily relies on two reactions. Firstly, the peptide bonds in protein reduce Cu^{2+} ions from the cupric sulfate to Cu^{+} (a temperature dependent reaction). The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Next, two molecules of bicinchoninic acid chelate with each Cu^{+} ion, forming a purple-colored product that strongly absorbs light at a wavelength of 562 nm(Smith, 1985).

The bicinchoninic acid Cu^{+} complex is influenced in protein samples by the presence of cysteine/cystine, tyrosine, and tryptophan side chains. At higher temperatures (37 to 60 °C), peptide bonds assist in the formation of the reaction product. Incubating the BCA assay at higher temperatures is recommended as a way to increase assay sensitivity while minimizing the variances caused by unequal amino acid composition. The amount of protein present in a solution can be quantified by measuring the absorption spectra and comparing with protein solutions with known concentrations (Smith, 1985).

2.3.1.3. UV method

Ability of protein (or a prosthetic group)utilizes to absorb light in a specific region of UV-visible spectrum (280 nm). Proteins are not denatured by these methods.

Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm. Secondary, tertiary, and quaternary structure all affect absorbance, therefore factors such as pH, ionic strength, etc. can alter the absorbance spectrum(Stoscheck, 1990).

2.4. Enzymatic activities of venom

2.4.1. Loss of protein during a purification schedule

Whatever level of purity is required it is important during the purification procedure to minimize the number of steps to maximize the yield. At every stage in the purification process, activity will be lost(Stoscheck, 1990).

2.4.2. Hemolytic activity

2.4.2.1. Erythrocytes and Hemolysis

The red cell membrane of the fishes consists of: Proteins~50%, Lipids ~ 40%, Carbohydrates~10%. Lipids are: Phosphatidylcholine (Lecithin), Phosphatidyl ethanolamine, Sphingomyelinand Phosphatidyl serine. The choline phospholipids-Phosphatidyl choline and sphingomyelin are mainly present in the extracellular layer. In some symptoms (ex. envenomation), piercing of the membrane and exit of hemoglobin from red blood cells or hemolysis will been occurred (Stankovic, 2006). As an initial approach to characterizing this complex venom, we focused on the hemolytic activity for which there is a standardized assay.

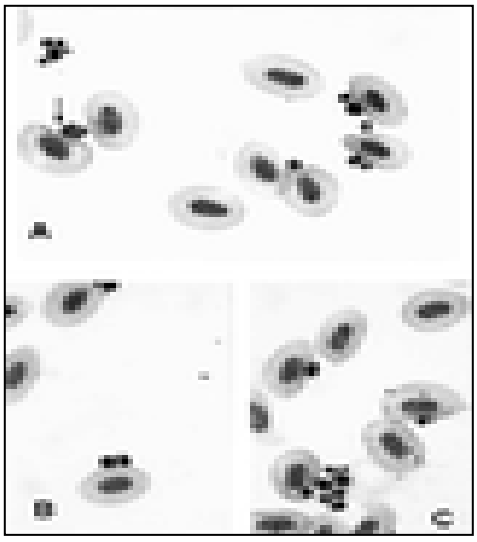


Fig.2.11. The red blood cells of the fish (with nucleuses)

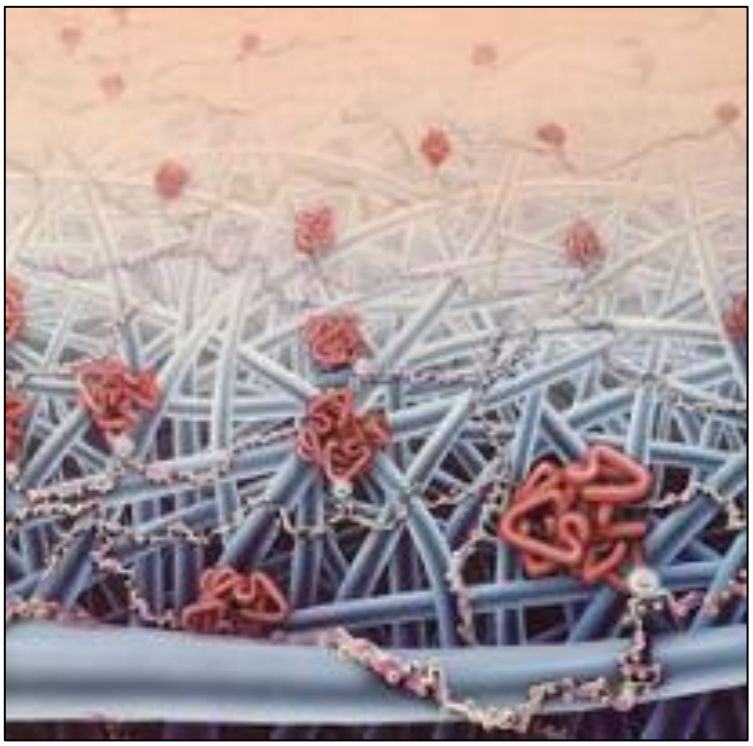


Fig.2.12. The existence of hemoglobin from red blood cells

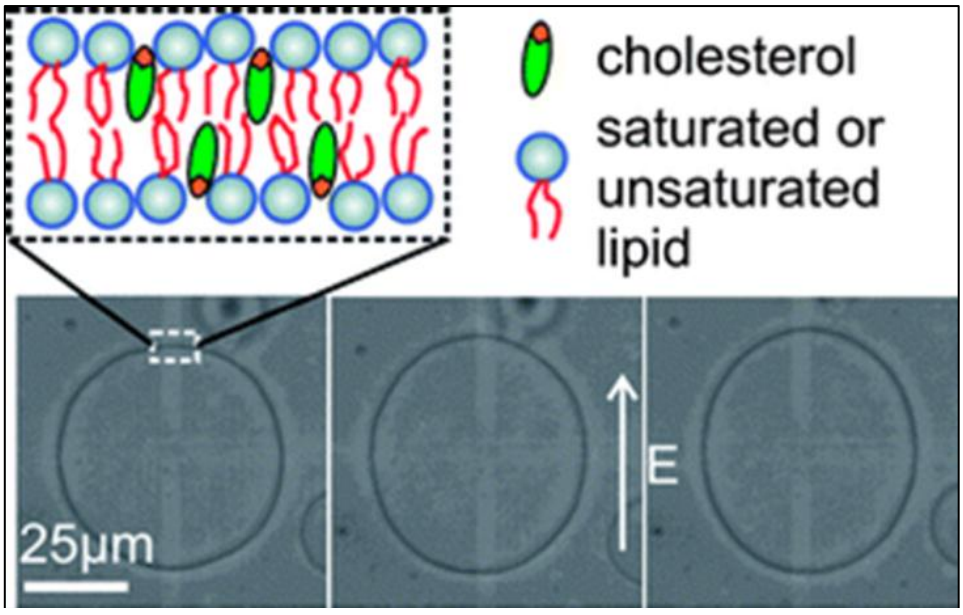


Fig. 2.13. The memberane of the erythrocyte

2.4.2.2. Hemolytic activity of the piscine venoms

Table 2.6.The hemolytic activity of the piscine venoms (Church, 2002).

Haemolytic activity of piscine venoms (✓✓✓: high haemolytic activity, ✓✓: medium activity, ✓: low activity, ×: no evidence of haemolytic activity)

	Rabbit	Rat	Human	Mouse	Cow	Sheep	PLA_2?
S. horrida/SNTX	✓✓✓	✓✓	×	×			×
S. trachynis/TLY	✓✓✓	✓✓	×	×	×	×	×
S. verrucosa/VTx	✓✓✓						
T. draco/dracotoxin	✓✓✓	✓✓	×	✓	✓		
P. volitans	✓✓✓						×
S. guttata						✓	
N. robusta			✓				×
T. natteieri				✓			×
P. lineatus		✓					
Toxin-PC	✓✓✓	✓✓		✓	✓		×
A. thallasinus			✓		✓	✓	

Erythrocyte membrane injury induced by PLA2 can cause intravascular hemolysis (Sitprijaa& Suteparakb, 2008).

2.4.2.3. The hemolysis mode mechanism

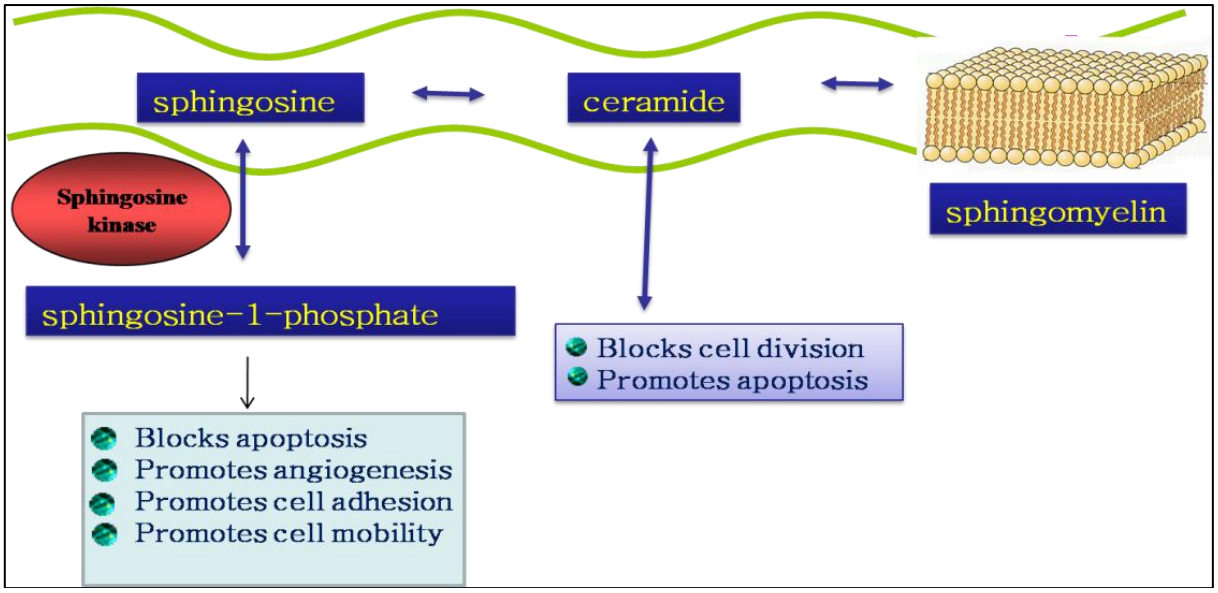


Figure2.14.The mechanism of hemolysis

2.4.2.4. Hemolytic toxins extracted from other animals

There are some hemolytic toxins extracted from other animals such as honey bee, spider, stonefish, sea snake, and scorpion. . Intravascular hemolysis is commonly observed in snake and arthropod envenoming. Some coelenterate toxins can also cause hemolysis (Sitprijaa& Suteparakb, 2008).

Table 2.7. Some hemolytic toxins extracted from other animals.

Species	**Number of amino acids**
Apis melifera زنبور عسل	**70 aa**
Loxosceles laeta عنکبوت	**304 aa**
Synanceja horrida (Stonustoxin subunit alpha) ماهی	**703 aa**
Deinagkistrodon acutus (Chinese moccasin),Phospholipase A2 homolog acutohaemolysinمار	**138 aa**
Hemiscorpius lepturus (heminecrolysin)(Shahbazzadeh *et al*.,2010) ,عقرب	**?**

2.4.2.5. Protolythic activity

Proteolysis is the breakdown of proteins into smaller polypeptides or amino acids. This generally occurs by the hydrolysis of the peptide bond, and is most commonly achieved by cellular enzymes called proteases.

Certain types of venom, such as those produced by venomous snakes, can also cause proteolysis. These venoms are, in fact, complex digestive fluids that begin their work outside of the body (Barrett *et al*., 2003).

2.4.2.6. Phospholipase activity

Phospholipase C (PLC) is a class of enzymes that cleave phospholipids just before the phosphate group (see fig.2.16). It is most commonly taken to be synonymous with the human forms of this enzyme, which play an important role in eukaryotic cell physiology, in particular signal transduction pathways. Thirteen kinds of mammalian phospholipase C are classified into six isotypes according to structure (Walter & Boron, 2003; Alberts *et al*., 2002).

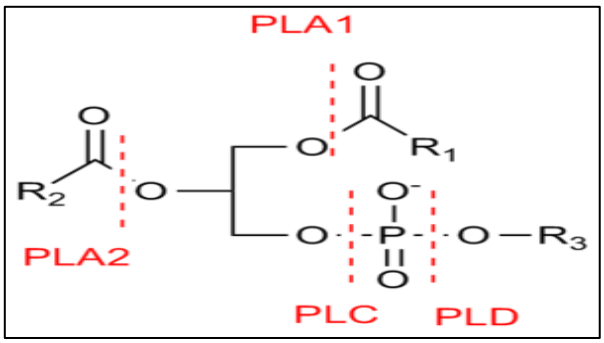


Fig.2.15.Cleavage sites of phospholipases on lecithin. Phospholipase C enzymes cut just before the phosphate attached to the R3 moiety (Walter & Boron, 2003).

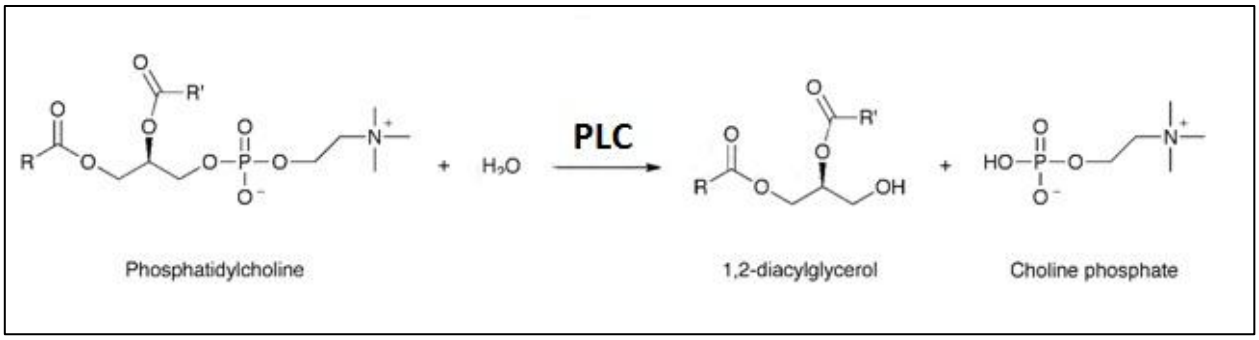


Fig. 2.16. The interaction of PLC on the phosphatidyl choline

2.4.2.7. Paw edema

Paw edema is a widely used test to determine the anti-inflammatory activity, and it has been fully characterized in the past. Mouse paw edema has also been increasingly used to test new anti-inflammatory drugs as well as to study the mechanisms involved in inflammation (Sivan, 2010).

2.4.2.8. Dermonecrolysis

Necrosis is a form of cell injury that results in the premature death of cells in living tissue. Necrosis is caused by factors external to the cell or tissue, such as infection, toxins, or trauma that result unregulated digestion of cell components. Dermonecrotic toxin is a toxin produced by an animal that causes extensive local necrosis on intradermal inoculation (Pereira *et al*., 2007).

2.4.2.9. Blood coagulation

Coagulation is a complex process by which the blood forms clots to block and then heal a lesion/wound/cut and stop the bleeding. It is a crucial part of homeostasis - stopping blood loss from damaged blood vessels.

Upon vessel injury, platelets adhere to macromolecules in the sub endothelial tissues and then aggregate to form the primary haemostatic plug. The platelets stimulate local activation of plasma coagulation factors, leading to generation of a fibrin clot that reinforces the platelet aggregate. Later, as wound healing occurs, the platelet aggregate and fibrin clot are broken down. Mechanisms that restrict formation of platelet aggregates and fibrin clots to sites of injury are necessary to maintain the fluidity of the blood (Lillicrap *et al*., 2009). Snake venom enzymes, through their effects on the blood coagulation cascade, platelets and vascular endothelium, result in hemorrhagic diathesis and disseminated intravascular coagulation. Bee stings and spider bite can also cause disseminated intravascular coagulation (Sitprijaa& Suteparakb, 2008).

3. Material and methods

3.1.Sampling

3.1.1.Sample collection

Eighty-two specimens *Scatophagus argus argus*, with total length of 5-25 cm (both sexes) were collected by trap from coastal waters of Boushehr (south of Iran) periodically from March 2011 to November 2012.The samples were transported from catching area to Pasteur Institute, lively by plane. The specimens were kept alive in an aquarium (25°C) in the Lab. Total lengths of the specimens were measured.

3.1.2. Ethical issues

Several ethical issues considered in our study including rapid anesthetizing, disinfection of injured tissue, and fast recovery of specimens as well as minimizing the complications. In this inquiry, clove powder (*Caryophillus aromaticus*), the specimens were incubated by 0.125 g/L clove for 2 fish,selected as anesthetizing agent. Some specimens were anesthetized in a little different manner by clove liquid(250g clove seed was boiled in700mL water for 12 fish).

3.2. Venom extraction and preparation

3.2.1. Acetone extraction

It was done in a modified extraction manner of Sivan (2007). The specimens were incubated in 0.125 g/L clove (*Caryophillus aromaticus*) powder for 3-4 minutes in a sterile container. After confirmation of general anesthesia, the rough dorsal (11 number) and anal (3 number) spines were cut just below the base .

The specimens recovered completely in 10 minutes. The surface layers and derma of spines were trimmed and cleaned completely. The spines cut into small pieces using a hand–held mortar made of cast iron with liquid Nitrogen. The fragments powdered in porcelain with liquid Nitrogen. The pieces homogenized at 3000×rpm in 0.9% NaCl for 5-10 min on ice and centrifuged at 4000×rpm for 15 min at 4°C. The pellet discarded and the supernatant filtered against 5KDa filter (Millipore) at 4°C and then lyophilized with a freeze dryer (Christ, Alpha 1-2 LD Plus–Germany).Lyophilized powder (0.5 g) dissolved in 100µl distilled water and centrifuged at 5000 ×rpm for 5 minutes at 4°C. Supernatant precipitated with acetone (20%) and centrifuged at 5000× rpm for 5 minutes at 4°C (in earlier stages of this study the supernatant was used without acetone addition). Precipitated

protein was quantified by Bradford method (Bradford *et al*., 1976) using a micro plate spectrophotometer (Biotek, EPOCH – USA).

3.2.3. Urea extraction (new method)

For increasing the concentration of proteins of scat spine, Urea method was used by previously published method with some modifications (Termine *et al*., 1981). This new method consists of three stages (solubilizing, refolding and dialysis).

3.2.3.1. Solubilizing

The fragments of spines (prepared in liquid nitrogen) were solubilized in

Solubilizing buffer Containing:

1-Urea (8 M)

2- NaCl(0.2 M)

3- Tris-Cl(50 mM)

4- EDTA (2mM)adjusts the pH to 8.5 if necessary.

3.2.3.2. Refolding

The extracted proteins must be refolded by refolding buffer containing:

1- Tris (50mM)
2- Nacl(9.6mM)
3- KCl(o.4mM)
4- EDTA (1mM)
5- Triton (0.5%)
6- Urea (2mM)
7- DTT (1mM), adjusts the pH to 8.

Two stages must be done in cold room on the stirrer.

3.2.3.3. Dialysis

Removing of salts and the other materials except proteins was done by dialysis (cut off 10) in PBS (0.0001 X) for 24 h on the stirrer in cold room.

3.2.3.4. Removing of lipid particles

It was obtained by Filtering in 0.2 µm(Takara,Millipore).

Takara,Millipore, syringe filters are a universal solution for numerous filtration applications. Takara filters can be used with most organic solvents and aqueous materials, and they are suitable for sample volumes up to 100 ml.

3.2.3.5. Freeze drying

The sample was lyophilizedbythe freeze dryer (Christ, Alpha 1-2 LD Plus– Germany).

3.3.Determination of protein Concentration

3.3.1. Bradford Assay

It was fulfilled in Bradford method (1979) by spectrophotometer (Chrom-tech, CT-5000). The Bradford solution was prepared (see appendix 4). Two µl from sample was injected in 1ml of solution and the absorption was read in 595nm.

3.3.2. UV method

The concentration of protein was determined in 280nm using a micro plate. Proteins in solution absorb ultraviolet light with absorbance maxima at 280nm. In addition to standard liquid handling supplies a spectrophotometer with UV lamp spectrophotometer (Biotek, Epoch – U.S.A.) and quartz cuvette are required (Stoscheck, 1990).

It was carried out steps 1-4 (280 nm only) and all steps if nucleic acid contamination is likely:

1. Warming up the UV lamp (about 20 min)
2. Adjusting wavelength to 280 nm
3. Calibrating to zero absorbance with buffer solution only
4. Measuring the absorbance of the protein solution
5. Adjusting wavelength to 260 nm
6. Calibrating to zero absorbance with buffer solution only
7. Measuring the absorbance of the protein solution

3.3.3. BCA method

This method was done in Elisa plate and spectrophotometer (Bioteck, U.S.A.) by BCA solutions and was read in562nm(Smith, 1985).See appendix 5.

1. Preparing working solution containing 980 µl “A” solution and 20µl “B” solution.
2. Adding 10 micro liters sample to each 200 micro liters working solution and mixed. Then incubated30 min at 37° C.
3. Reading the absorbance of the samples at 562 nm.

3.4. Venom activities

3.4.1. Hemolytic activity

Hemolytic activity was performed as described before with some modification (Shahbazzadeh *etal*., 2007). Fresh human blood from healthy volunteer was drawn by venous puncture into heparinized tubes. Plasma and Buffy coat were removed by centrifugation for 5 min at 3000×rpm at 10°C, and the erythrocytes were washed three times with phosphate-buffered saline (pH 7.4), containing 2 mM CaCl2. A suspension was made at 1% packed cells in PBS. From this suspension, aliquots containing 200 µl each were incubated with serial concentrations of venom prepared in a microplate from 60 µg in 100µl PBS. After incubation for 1 h at 37°C, the samples were centrifuged for 5 min at 3000 rpm, and the absorbance of the supernatant was determined at 540 nm. The degree of hemolysis was determined by comparing the amount of hemoglobin released in the supernatant with 0% hemolysis in PBS and 100% hemolysis in 1% triton X100. The percentage of hemolysis was calculated as follows: hemolysis (%) = 100 ($OD_{test} - OD_{saline}$)/ ($OD_{triton} - OD_{saline}$).

3.4.2. Phospholipase activity

The colorimetric assay was used by phenol red, a pH indicator, as previously published (Sivan*et al*., 2007) with some modifications. Briefly, 4 mM of solid egg lecithin (phosphatydilcholine, Sigma-Aldrich, Cat. No. P3556) dissolved in 1 ml ethanol at 45°C, then Triton X100 (860 µl), 0.1 M NaCl, 5.5 mM Phenol red, 1 M CaCl2 and H2O up to 16.6 ml, were added consecutively. The color inverted to neutral red by drop adding of 2 M NaOH. Scat crude venom serially diluted in two fold manner started from 0.46 to 60 µg with deionized H2O. Then, substrate solution (100 µl) was added to each microtube and incubated at 45°C for 15 min. Deionized water was used as negative control and phospholipase A2 from scorpion venom used as acid producer control. Optical density was read at 550 nm.

3.4.3.Proteolytic activity

Proteolytic activity was determined according to the method of (Sivan *et al.*, 2007) with some modification. One milliliter of 0.5% casein was incubated for 2 h at 37°C with 400 µl of test solutions containing 20µg of venom in the presence of 0.008 M calcium chloride at pH 8.8. Trichloroacetic acid (5%) was added to the solution to stop the reaction. The hydrolyzed peptides contained in the supernatants were quantified according to Bradford method (Bradford *et al*., 1976).

3.4.4.Edematic activity

The ability to induce edema was studied in mice (male, 20 g). Different concentrations of *S. argus* venom (5 and 10 µg), prepared in sterile 0.9% NaCl

(W/V), and 50 µl was injected into the sub plantar region of the left hind paw. The right hind paw received the equal volume of sterile saline alone and served as the control. The diameters of both paws were measured using caliper at 0.5, 2, 4, 6, 24 and 48 h after venom administration and the percentage of edema was calculated (Sivan,2007).

3.4.5. The effect on blood coagulation

Fresh human blood was collected from corresponding author directly into a test tube containing 8% sodium citrate solution in a proportion of 1:19 (v/v). It was mixed immediately by gentle agitation. The plasma was then separated by centrifugation at4000× RPM for 5 min. The separated plasma was pooled together and kept at 4°C for subsequent clotting assay. Plasma (50 µl) was incubated at 37°C and 0.2ml calcium chloride (1%) was added into the test tube and mixed. Crude venom (50µl) was then added to the mixture and clot formation checked within 5 minutes (Sivan, 2007).

3.4.6. Dermonecrotic activity

The experiment was performed on rabbit derma (male, 3Kg). Different concentration of scat venom (5, 7.5 and 10 µg/µl) were administrated subcutaneously. The venom of scorpion *Hemiscorpius lepturus* (5µg) used as positive control and normal saline (50µl) injected as negative control (Borchani *et al*., 2011).

3.5. Protein purification

3.5.1.SDS- PAGE

Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out according to standard method (Laemmli, 1970). The venom samples were loaded onto a 12% polyacrylamide gel and stained with Comassie brilliant blue. Theapparent molecular masses of the purifiedproteins were estimated by SDS-PAGE, by comparison witha mixture of protein molecular markers (10–250 kDa, Maxell).

3.5.2. RP-HPLC

The crude venom (0.5 g)was dissolved in distilled water (100 µL), and insoluble material discarded by centrifugation at 13000×rpm for 10 min at 4°C.

The venom fractions were separated using a HPLC instrument (knauer-Germany)50 µl of the prepared venom (5µg/µl) manually injected to C18 column

(5µm. 100Å - 250 × 4.6mm) and eluted in a linear gradient of acetonitrile containing 0.05% TFA (solution C) and 0.05% TFA in water (solution D) at flow rate 1ml/min. Protein fractions detected at 214nm and 280nm, and were collected manually. Isolated fractions were lyophilized by a freeze dryer (Christ, Alpha 1-2 LD plus–Germany). The purity of isolated proteins was subsequently checked by SDS-PAGE.

It was tried to perform high-quality dissociation of fractions with regard to enhancement of purification thus several gradients were applied successively but the proteins were not separated completely and we obliged to change it (table 3.1 -3.14).

3.5.2.1. Method 1

At first a method in 70 minutes was done (table 3.1)

Table 3.1

Time	Flow rate	ACN %	TFA%
0	1	0	100
5	1	60	40
60	1	90	10
65	1	90	10
70	1	0	100

3.5.2.2. Method 2

Table 3.2

Time	Flow rate	ACN %	TFA%
0	1	0	100
5	1	10	90
15	1	60	40
65	1	90	10
70	1	90	10
75	1	0	100

3.5.2.3. Method 3

Table 3.3

Time	Flow rate	ACN %	TFA%
0	1	0	100
25	0.5	0	100
35	1	10	90
75	0.5	0	100

3.5.2.4. Method 4

Table 3.4

Time	Flow rate	ACN %	TFA%
0	1	0	100
30	0.5	0	0
60	0.5	90	10
65	0.5	0	100

3.5.2.5. Method 5

Table 3.5

Time	Flow rate	ACN %	TFA%
0	1	0	100
25	0.5	0	100
35	0.5	10	90
75	0.5	0	100

3.5.2.6. Method 6

Table 3.6

Time	Flow rate	ACN %	TFA%
0	1	90	10
60	0.5	90	10
65	0.5	0	100

3.5.2.7. Method 7

Table 3.7

Time	Flow rate	ACN %	TFA%
0	1	0	100
5	1	5	95
65	1	60	40
75	1	90	10

3.5.2.8. Method 8

Table 3.8

Time	Flow rate	ACN %	TFA%
0	1	5	95
5	1	5	95
65	1	65	35
75	1	90	10
80	1	5	95

3.5.2.9. Method9

Table 3.9

Time	Flow rate	ACN %	TFA%
0	0.8	0	100
60	0.8	30	70
120	0.8	90	10
130	0.8	90	10
132	0.8	0	100
140	0.8	0	100

3.5.2.10. Method 10

Table 3.10

Time	Flow rate	ACN %	TFA%
0	1	0	100
5	1	0	100
60	1	60	40
65	1	90	10
70	1	0	100

3.5.2.11. Method 11

Table 3.11

Time	Flow rate	ACN %	TFA%
0	1	0	100
5	1	0	100
20	1	15	85
60	1	35	75
65	1	90	10
70	1	0	100

3.5.2.12. Method 12

Table 3.12

Time	Flow rate	ACN %	TFA%
0	1	0	100
5	1	0	100
60	1	40	60
70	1	90	10
75	1	90	10

3.5.2.13. Method 13

Table 3.13

Time	Flow rate	ACN %	TFA%
0	1	0	100
5	1	0	100
60	1	60	40
65	1	90	10
70	1	90	10

3.5.2.14. Method 14

Table 3.14

Time	Flow rate	ACN %	TFA %
0	1	0	100
5	1	0	100
60	1	60	40
70	1	90	10
75	1	90	10

3.5.2.15. Method 15

Eventually HPLC in a new gradient (table 3.15) applied and the following method was used for isolation of scat venom components: The elution time was 80 min and the flow rate was 1ml/min.The first5 min was for equilibration then in 55 min ACN runup to 60% because of probable hydrophobicity of proteins. In a step gradient it

eluted to 90% ACN in 5 min and in a linear gradient it arose to 95% in 5 min. At last it received to 0% and in turn TFA to100% to equilibrating the column.

Table 3.15

Time	Flow rate	ACN %	TFA %
5	1	0	100
60	1	60	40
65	1	90	10
70	1	90	10
75	1	95	5
80	1	0	100

3.5.3. Hemolytic assay - Microscopic method

To direct inspection of hemolysis by toxic fraction, the test performed under a microscope. Fresh human blood from healthy volunteer was drawn by venous puncture in heparinized tubes. Plasma and Buffy coat were removed by centrifugation for 5 min at 3000 rpm at 25°C, and the erythrocytes were washed three times with phosphate-buffered saline (pH 7.4). A suspension of erythrocytes was made at 0.0001% dilution in PBS.From this suspension, 10 µl RBC placed on a Neubauer slide as negative control. A suspension containing 5 µl RBC, 2 µl of each fraction and 3 µl PBS used for the assay.The results were observed with a microscope (Bell, photonic) at 40× magnification and documented using a digital camera (Canon G12 – Japan). Hemolytic event was done in direct visual test.

4. Results

4.1. Sampling

Eighty two specimens of *Scatophagus argus argus* (Fig.4.1) with total length of 5-25 cm (both sexes) were collected by trap (Fig.4.2) from coastal waters of Boushehr (South of Iran) (Fig.4.3) periodically from March 2011 to November 2012. The specimens were kept alive in an aquarium (25°C) in the Lab.

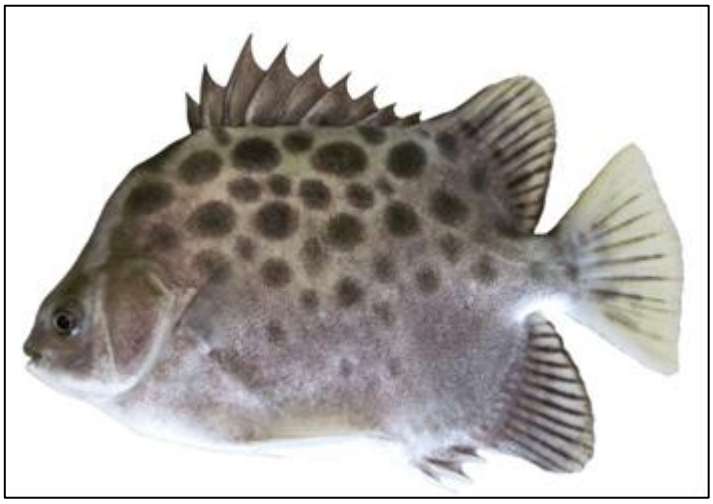


Fig. 4.1.*Scatophagus argus argus* isolated from coastal waters of Boushehr before cutting spines (photo by writer).

Fig.4.2. Trap (locally called “gargoor”)

Fig.4.3. Study areas in Boushehr city, Boushehr province, south of Iran (29° N, 51° E).

4.2. Ethically processes (following up the viability and compatibility)

The specimens that were collected in summer (on May), leaved in a pool at Pasteur Institute of Iran after operation. The environmental conditions were considered carefully for a month including temperature, pH and types of phytoplankton too. Compatibility with other aquatics such as golden fish was studied too.

The average temperature and pH of the pool recorded in a month was 31°C and 7.45, respectively. Microscopic analysis revealed phytoplanktons (green algae from desmids and chlamydomonas) (Fig. 4.4).

During the study period, it was observed that scats lived compatibly with other species (Cyprinidae such as *Carrasius auratus*). They generally got along well with them. They were peaceful schooling fish and a mixed school of scats and golden fish that kept in the pond too, were observed. The scats were more curious and outgoing than them. They had very hearty appetites and would greedily eat

anything that came their way. In the pool all of them swam in an aggregation together with other fish and feed on the bottom of the pond.

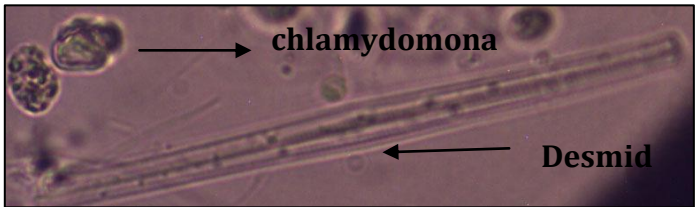


Fig.4.4. Some phytoplanktons in the pool water (Pasteur Institute of Iran).

4.3. Vertical section of spine

Total length of an examined fish was 5cm. The length and diameter of spine was 11mm and 4mm respectively. Two collecting ducts were observed alongside the spine. Blood vessels within bonny central axis were observed too. A presentation of the vertical section of spine demonstrated in Fig. 4.5.

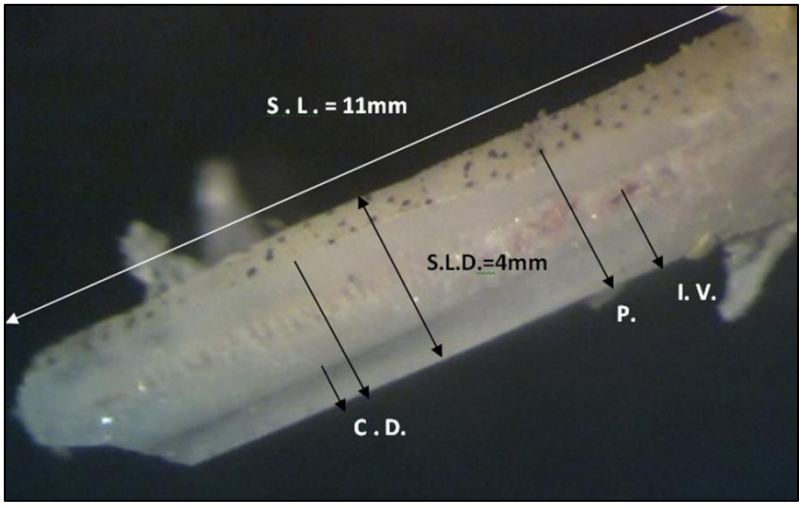


Fig. 4.5. S.L. (Spine length=11mm), S.L.D. (spine large diameter= 4mm), C.D. (two collecting ducts that collect the venom produced by venomous cells), I.V. (Internal bony blood vessel), P. (pigments in scat derm),(zoom lens=50).

4.4. Venom extraction and preparation

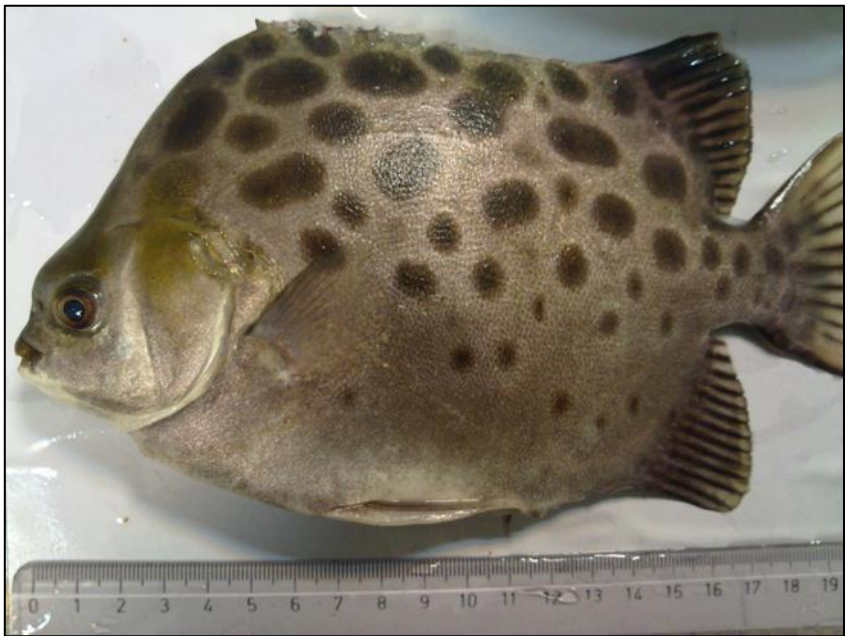


Fig.4.6. *Scatophagus argus argus* after cutting the spines.

Fig.4.7. Cleaned spines, before cutting to small pieces.

We obtained from 0.5g lyophilized powder of the crude venom, 0.5 µg/µl protein in normal saline method, and 2 µg/µl protein in acetone extraction nevertheless in urea extraction 25.7 µg/µl protein was acquired.

Fig.4.8. The trimmed spines were fragmented in liquid nitrogen with pestle and mortar

4.5. SDS-PAGE

In previous method the concentration was 0.5µg/µl and SDS-PAGE revealed 12 separated proteins (Fig. 4.9).

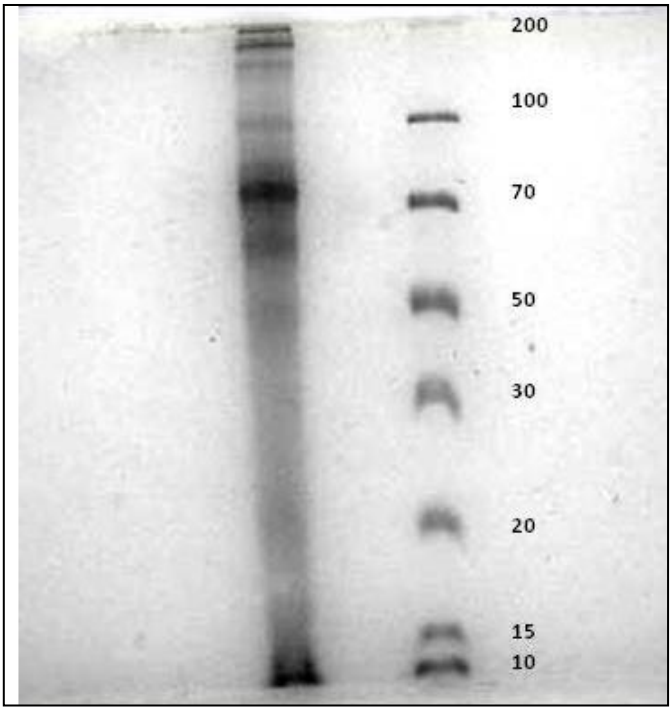


Fig.4.9. Electrophoretic profile of *S. argus argus* venom. The venom was analyzed by SDS PAGE using 12% polyacrylamide gel and stained with Comassie brilliant blue. Numbers at right corresponds to molecular markers. The apparent molecular masses of the purified proteins were estimated by comparison with a mixture of protein molecular markers (10–250 KDa, Fermentase).

In modified protocol (precipitation by acetone) we got only weighted proteins (Fig. 4.10). At final protocol (with urea), 12 separated proteins obtained with the thick concentration of 25.7 µg/µl (in UV method) (Fig. 4.11).

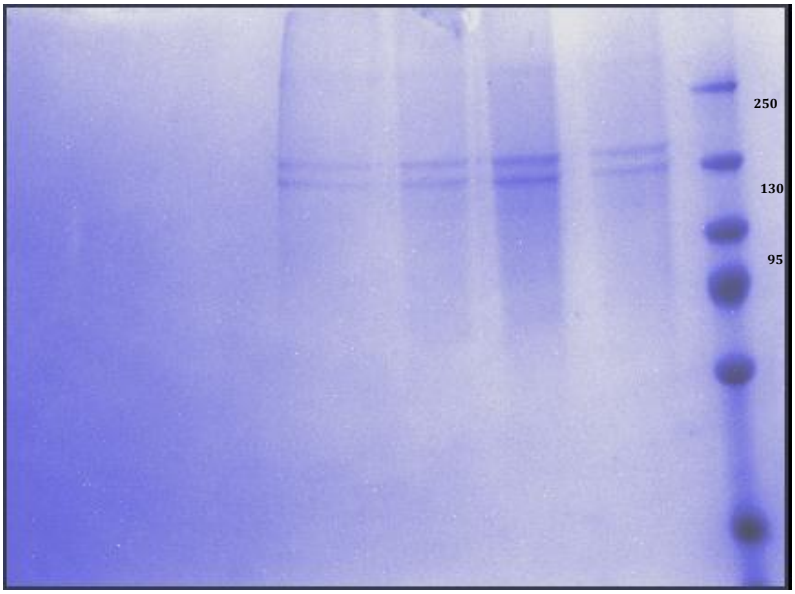


Fig.4.10. Electrophoretic profile of *S. argus argus* venom. The venom was analyzed by SDS PAGE using 12% polyacrylamide gel and stained with Comassie brilliant blue. In acetone method the pretty weighted proteins were sedimented by acetone. Numbers at right corresponds to molecular markers (maxell, KDa).

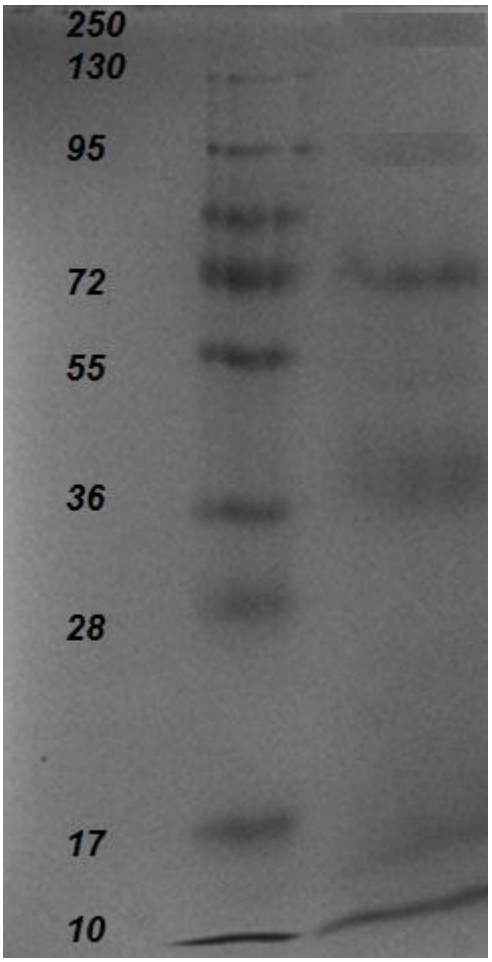


Fig.4.11. Electrophoretic profile of *S. argus a*rgus venom (urea extraction). The venom was analyzed by SDS PAGE using 12% polyacrylamide gel and stained with Comassie brilliant blue. The numbers at right corresponds to molecular markers. The apparent molecular masses of the purified proteins were estimated by comparison with a mixture of protein molecular markers (10–250 KDa, maxell).

4.6. Hemolytic activity

There were prepared some tissues to hemolytic test to realizing if the derm has toxic effect or only spine. In this respect the hemolytic test was done on three separated tissues: the spine, the derm, and the spine and derm (Fig. 4.12), so it was observed that only spine revealed hemolytic activity.

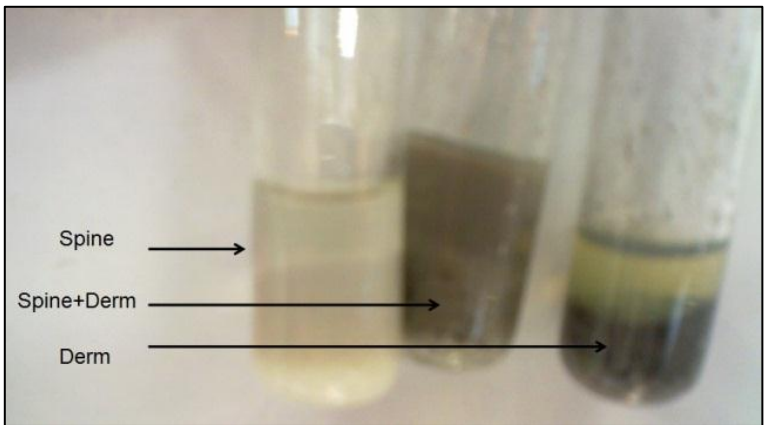


Fig.4.12. Prepared tissues (spine, derm, spine and derm) for hemolytic test.

S. argus venom exhibited hemolytic activity on 1% human washed erythrocytes. The amount of 1.7 µg crude venom produced 100% hemolysis of human erythrocytes (Fig. 4.13, 4.14).

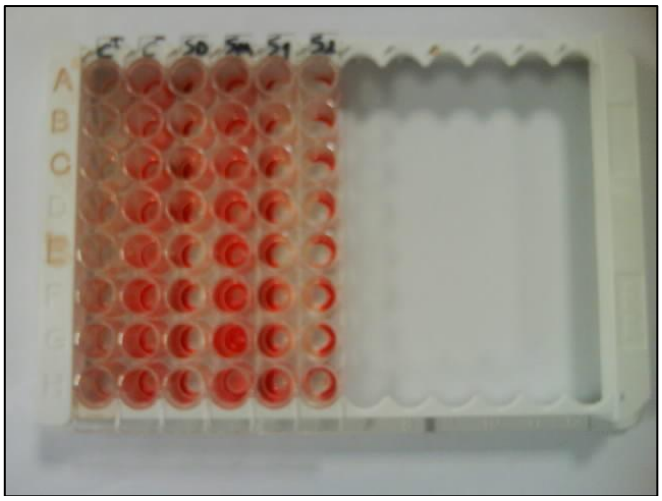


Fig. 4.13. Hemolytic test in an ELISA microplate

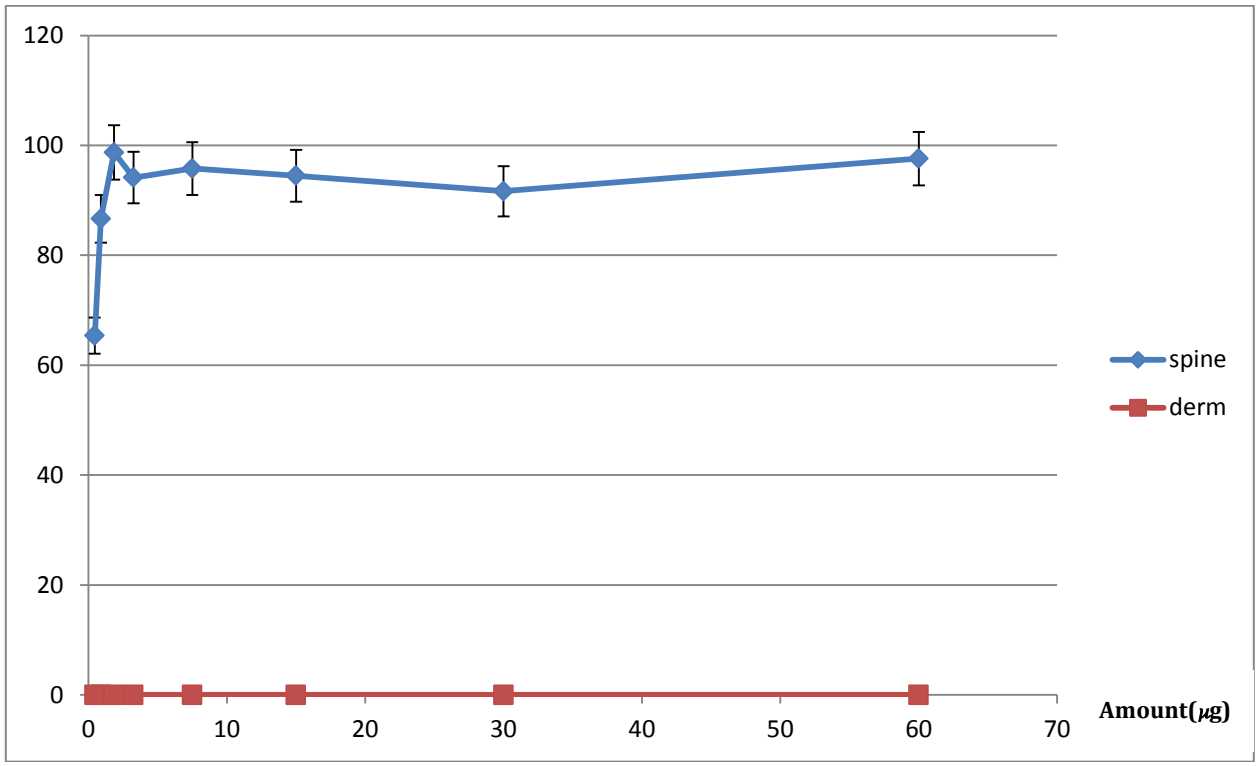


Fig.4.14. Hemolytic activity of *S. argus* venom on human erythrocytes. Human erythrocytes were incubated with increasing doses of crude venom for 1 h. Release of hemoglobin was determined by measuring the absorbance at 540 nm. PBS was used as negative control, and 100% cell lysis was achieved by Triton X100 (1%) that used as positive control. There is no seen any hemolytic activity in the derm.

4.7. Phospholipase activity

Phospholipase C (PLC) activity was detected in *S. argus* venom. The spectrophotometric method was employed by using egg lecithin as substrate and phenol red as a sensitive dye for pH indication in a microtube and a micro plate. *S. argus* venom produced alkaloid pH that can be absorbed at OD550 nm, which the density of color is dose dependent. As shown in Fig. 4.15, PLC activity of *S. argus* venom was recorded in minimum concentration of 3.125 µg.

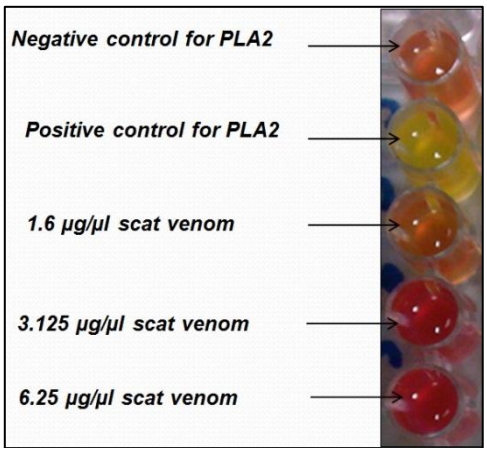


Fig.4.15. Phospholipase C test in the micro plate

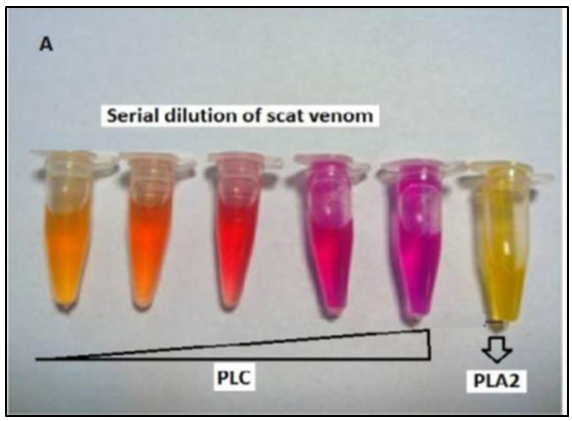


Fig. 4.16. PLC test in the micro tube. The yellow color in the right microtube is the result of phospholipase A2 activity from snake venom, based on the fact that the enzymatic reaction of scorpion venom (*Hemiscorpius leptorus*) acidifies the medium by liberating fatty acids from phospholipids and thus changes the absorption spectrum of the dye. On the left, the activity of phospholipase C in scat venom is depicted. Serial dilution of the venom exposed to lecithin containing medium (0-50 µg). PLC (lecithinase) activity of scat venom was detected in spectrophotometer method based on the pH change due to the negative charge of phosphate radical linked to phosphorylcholine which induces the solution to be reacted as an alkali (red color).

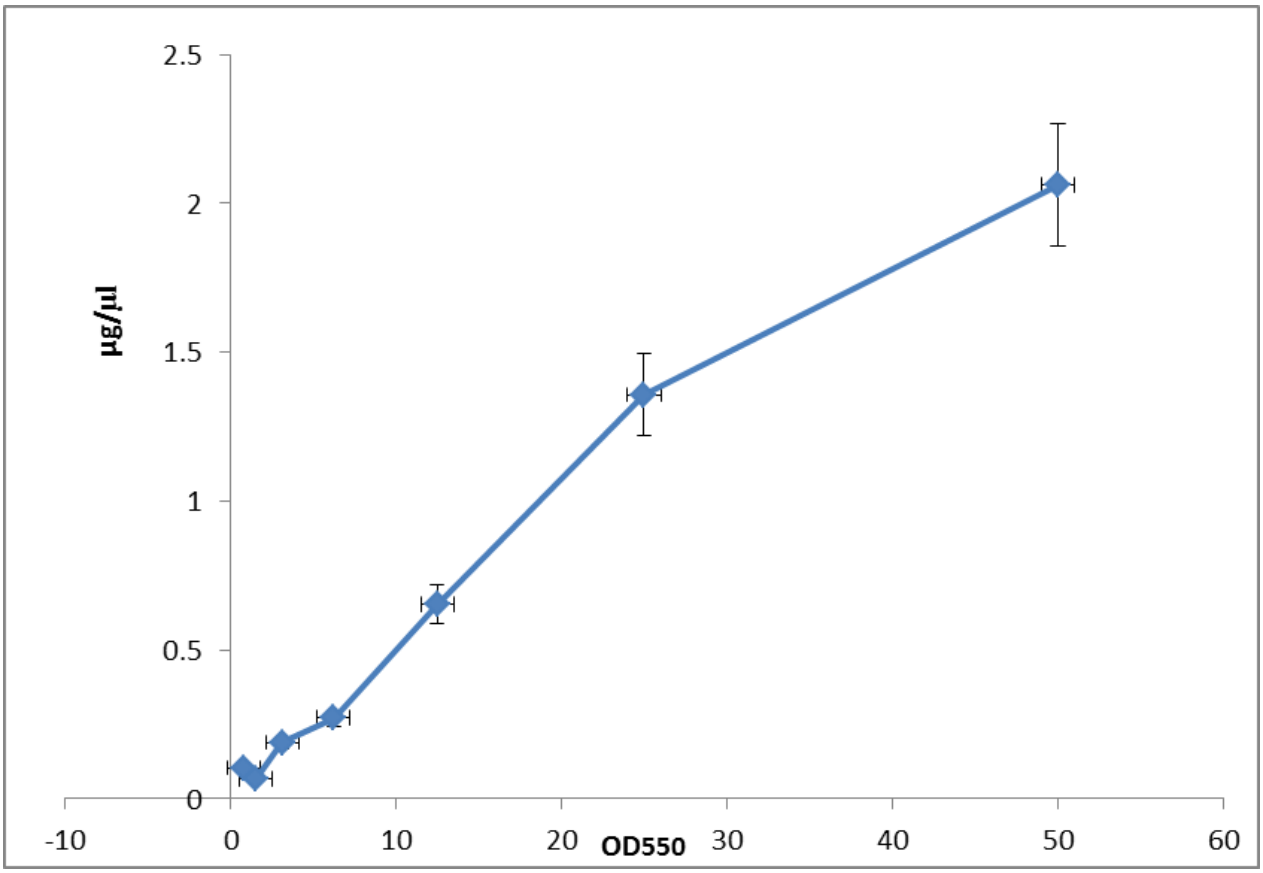


Fig.4.17. Determination of Phospholipase C activity of *S. argus argus* venom by using colorimetric assay in a medium containing lecithin as substrate. The degree of red color correlated with the absorbance at OD550. Phospholipase C activity of *S. argus argus* venom was obtained with the minimum concentration (3.125µg).

4.8. Coagulation test

No clotting was observed when blood plasma was incubated with different doses of venom, nor did it cause any lysis of the clot formed.

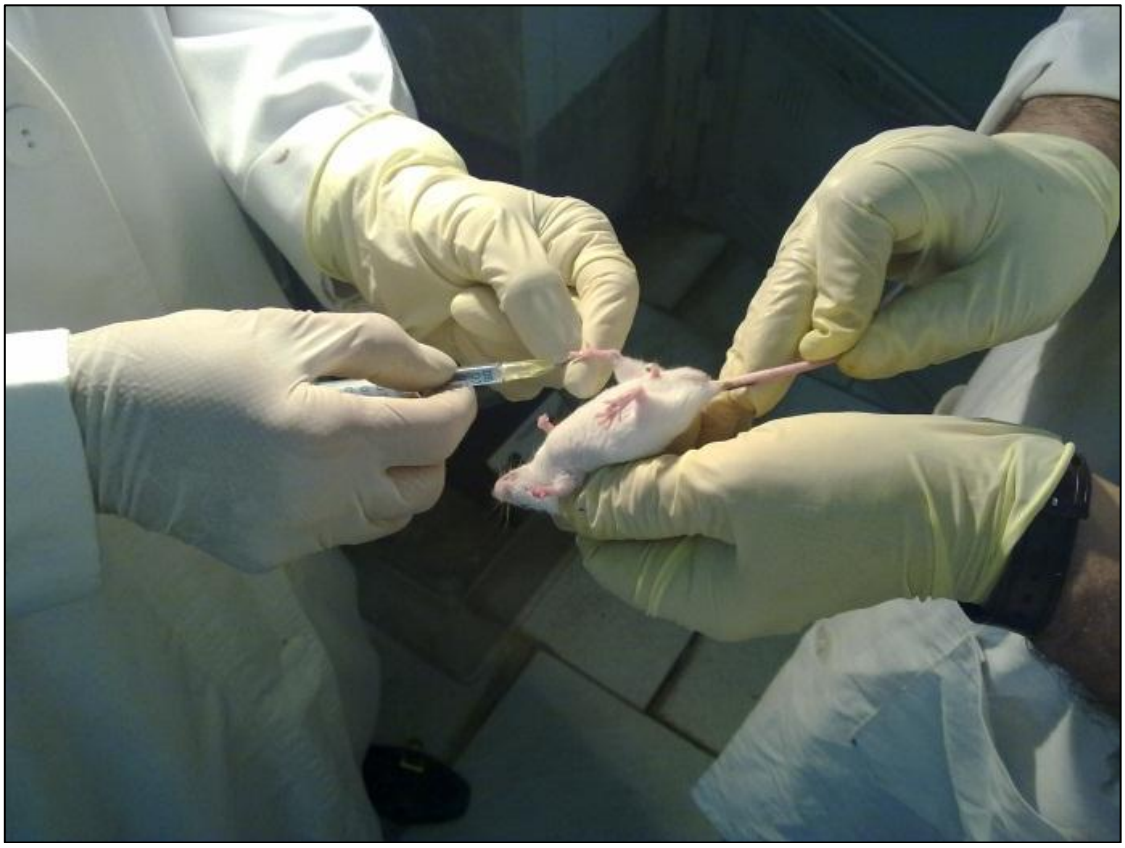
4.9. Proteolytic activity

The venom had proteolytic activity on 0.5% casein. The crude venom could enhance the absorbance from 0.290 to 0.379 indicated the release of protein and peptide fragments from casein. Crude venom (20 µg) released 813 µg proteins from 5 mg casein.

4.10. Edematic assay

Crude venom induced edematic effect on mice paw at dose dependent response (Fig. 4.20). Maximal response was observed from 1 to 6 h, when compared with

the control. Edema percent increased during 30 minutes and reduced gradually over 48 hours.



4.18. The venom injection to mice paws

4.19. The measuring of edema using the calipper

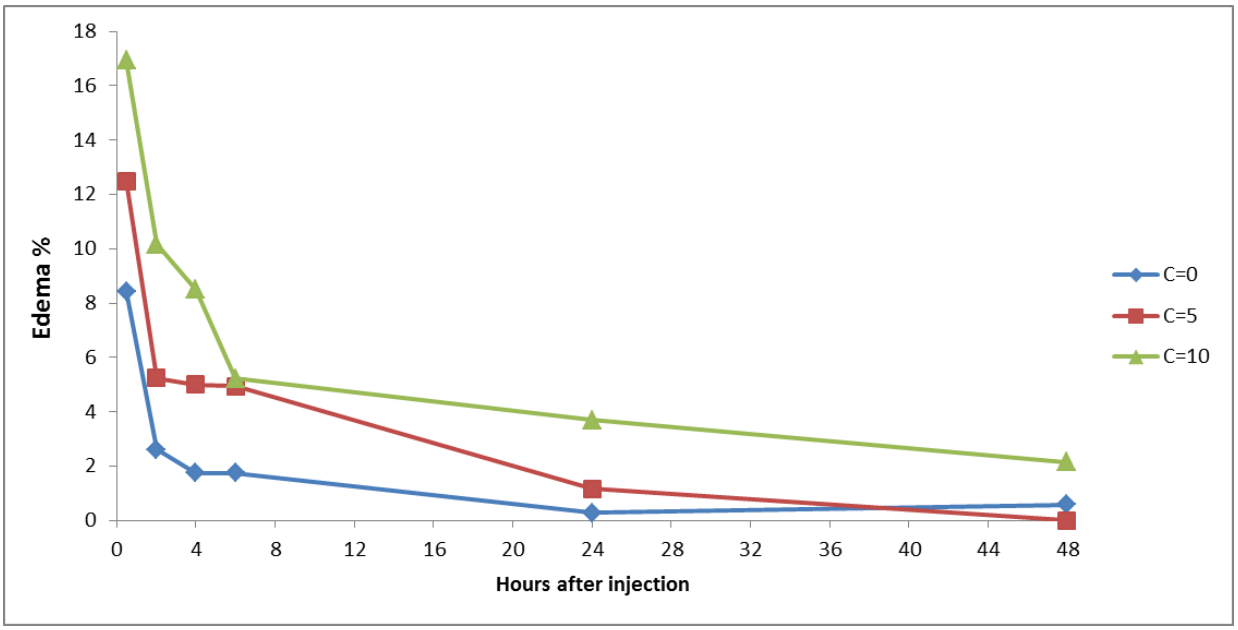
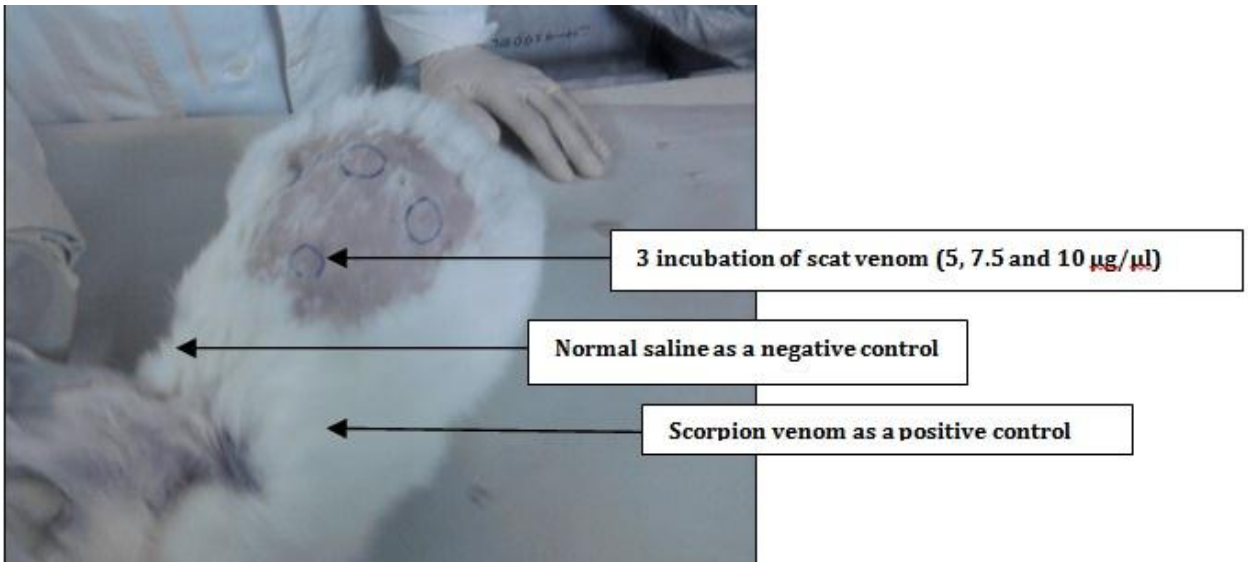


Fig.4.20. Two concentrations of *S. argus argus* crude venom (5 and 10 µg) prepared in sterile 0.9% NaCl (W/V), and 50 µL from each one was injected into the sub plantar region of the left hind paw. The right hind paw received the equal volume of sterile saline alone and served as the control. The edematic change of both paws was measured using caliper at 0.5, 2, 4, 6, 24 and 48 h after injection of the venom, and the percentage of edema was calculated.

4.11. Dermonecrotic activity

Dermonecrotic activity did not record after 24h. The venom of *Hemiscorpius lepturusus* was used for producing necrosis as a positive control (Fig.4.21, 4.22).



4.21. The injection of scat venom in the derm of rabbit. The venom of scorpion, *Hemiscorpius lepturus* used for producing necrosis as a positive control and normal saline as a negative control.

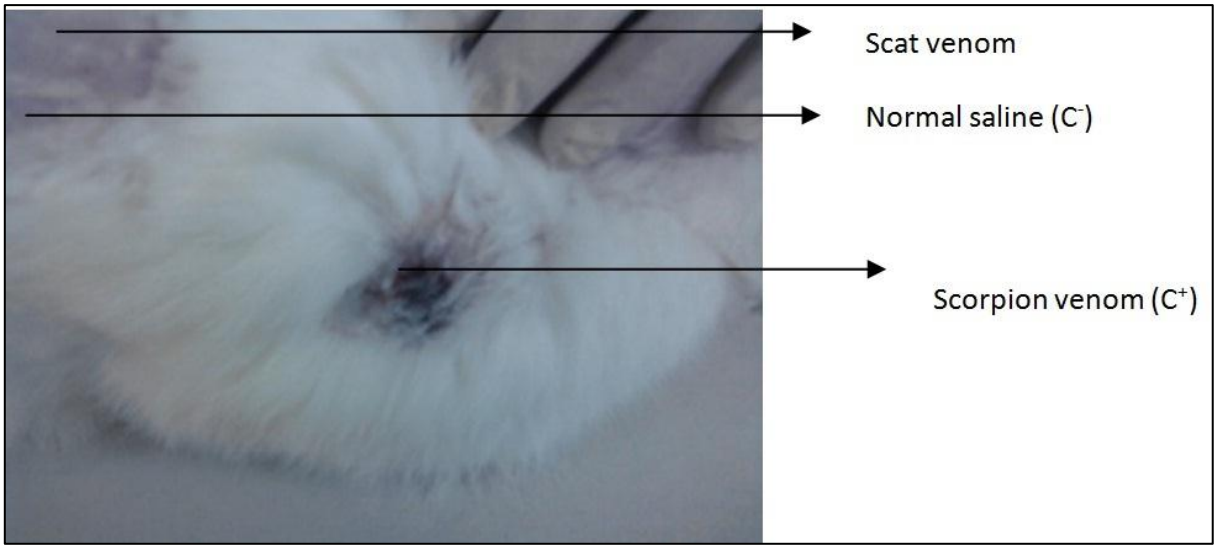


Fig. 4.22. Study of dermonecretic effect on the derm of rabbit. Positive control was scorpion venom and negative control was normal saline. After 48h incubation, it was not observed any necrosis on the rabbit skin because of scat venom.

4.12. RP-HPLC

4.12.1. Protein purification by RP-HPLC

HPLC chromatography of *Scatophagus argus argus* venom on C18 column in a new gradient results in 30 fractions. Absorbance was read at 280 and 214 nm. Maximum optical density of the fractions was 1500. The aliquots of each fraction were used for hemolytic activity. For isolation details see (Fig. 4.23-4.24).

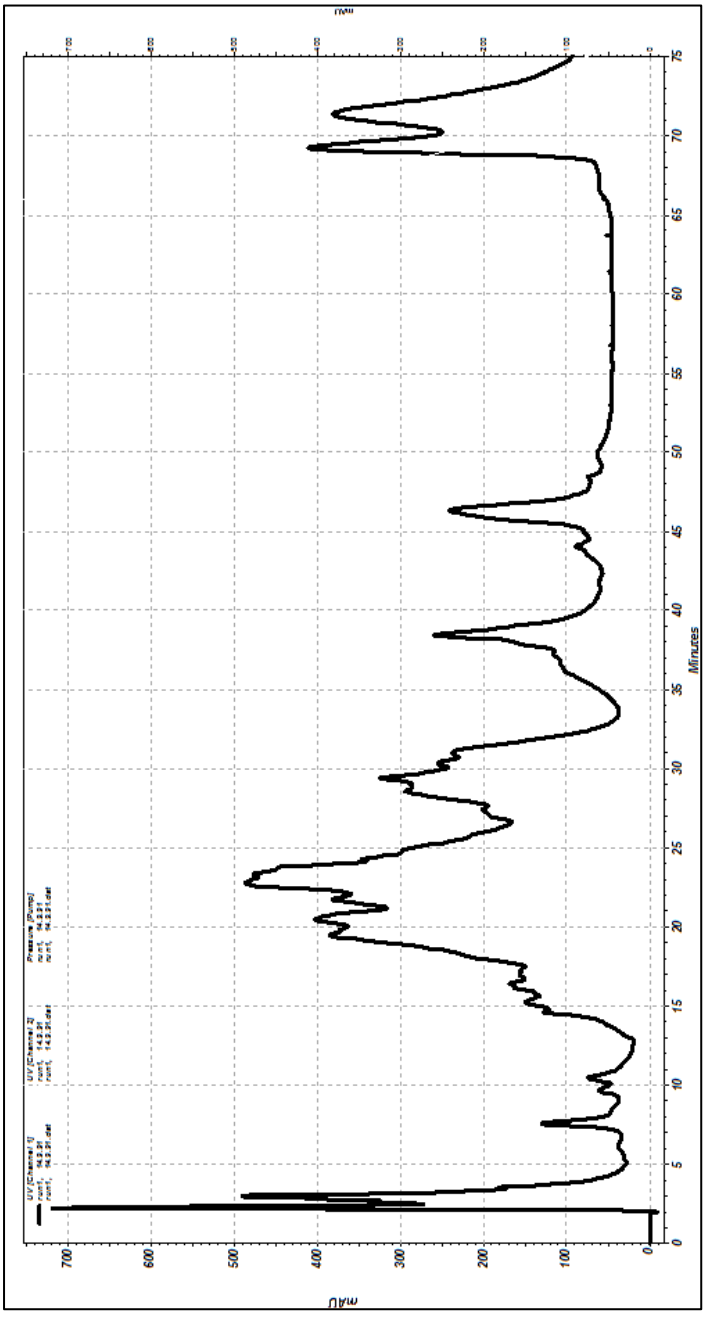


Fig.4.23. HPLC chromatogram of scat crude venom (in this method the proteins were detached of column completely).

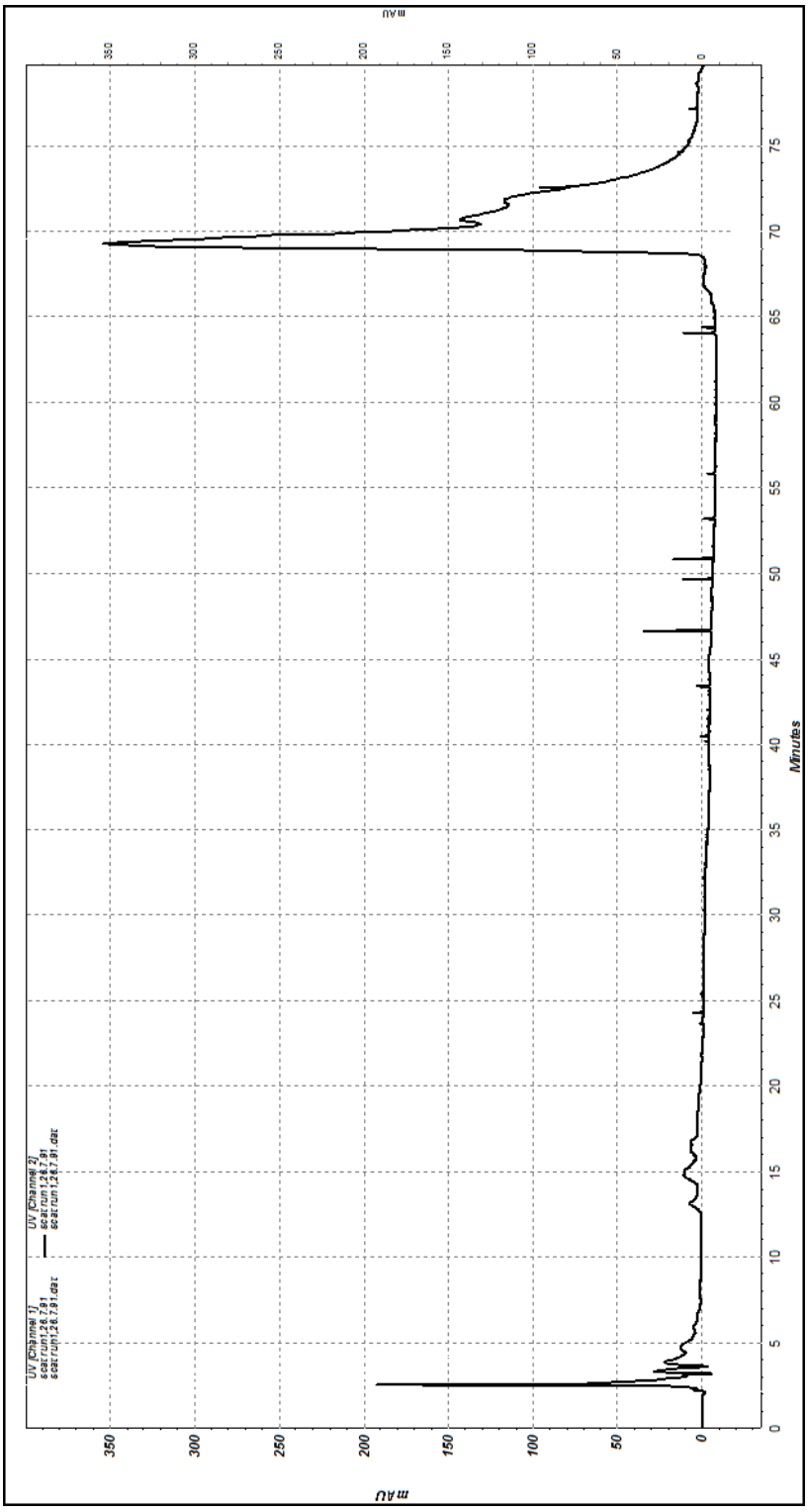


Fig.4.24. HPLC chromatogram of scat crude venom (in this method the proteins were detached of column completely).

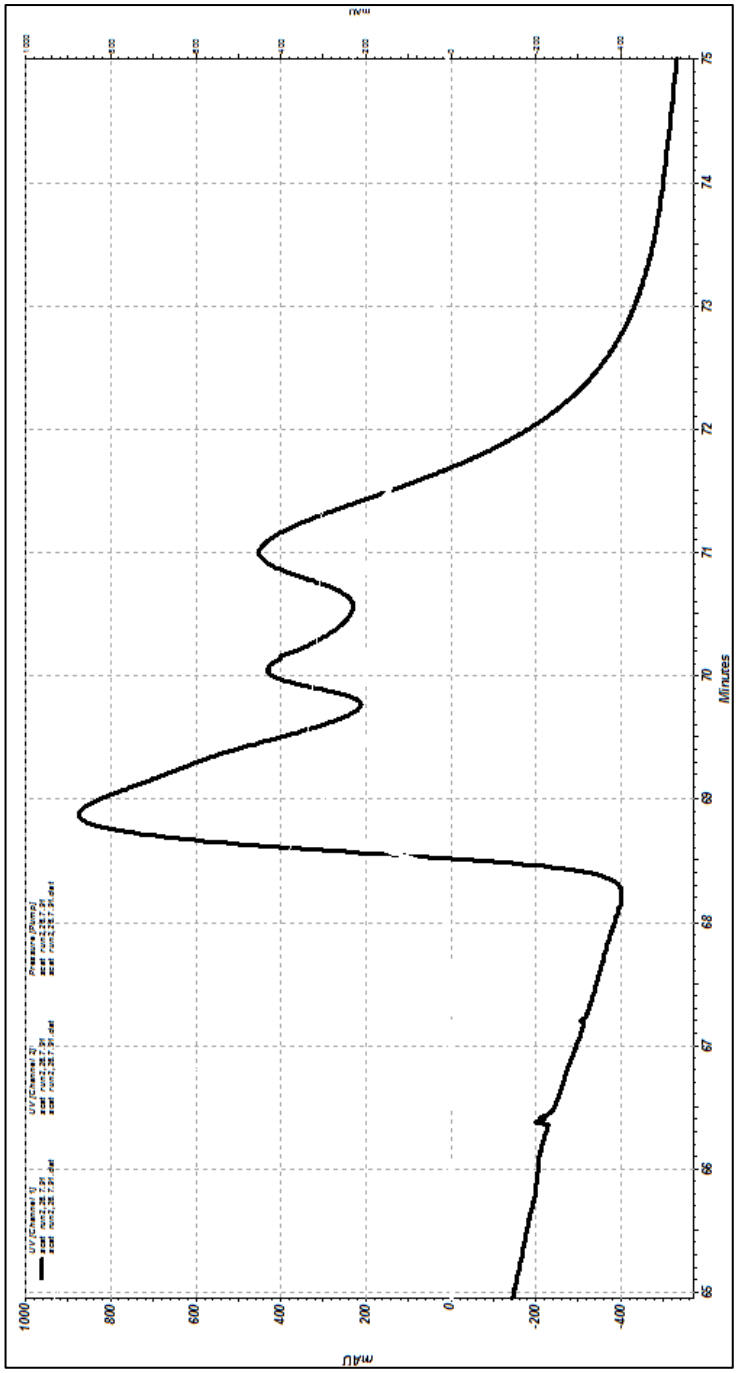


Fig.4.25. HPLC chromatogram of scat crude venom (in this method the proteins were detached of column completely). There are 3 isomers which magnitudes in this chromatogram.

4.12.2. The percentage area of the hemolytic protein

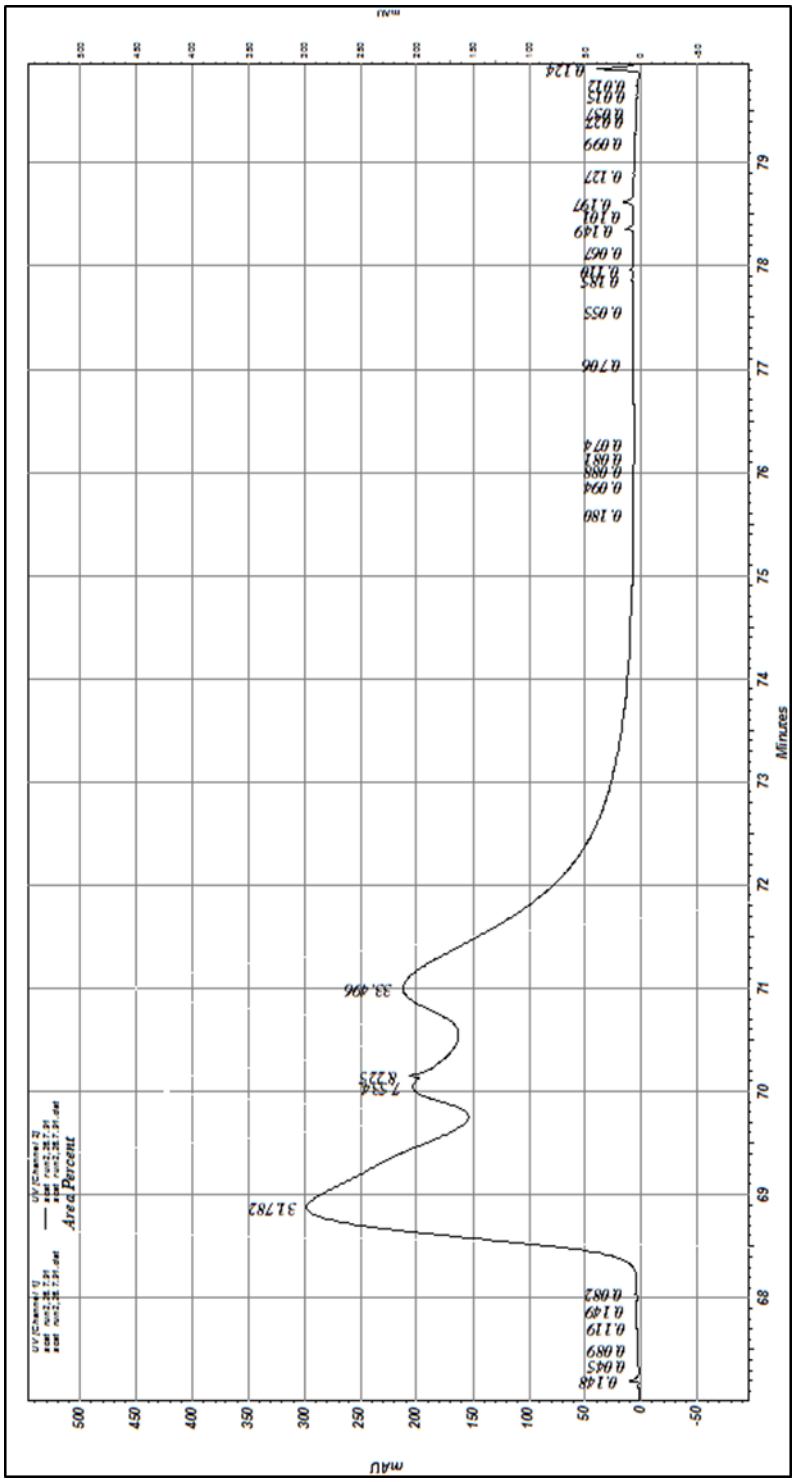


Fig.4.26. The percentage area of the hemolytic protein was about 32% of total proteins in scat venom.

4.12.3. The hemolytic fraction

It was performed a microscopic method as hemolytic test and the immediate interaction between the erythrocytes and hemolytic fraction was observed with a microscope at 400× magnification and documented using a digital camera.

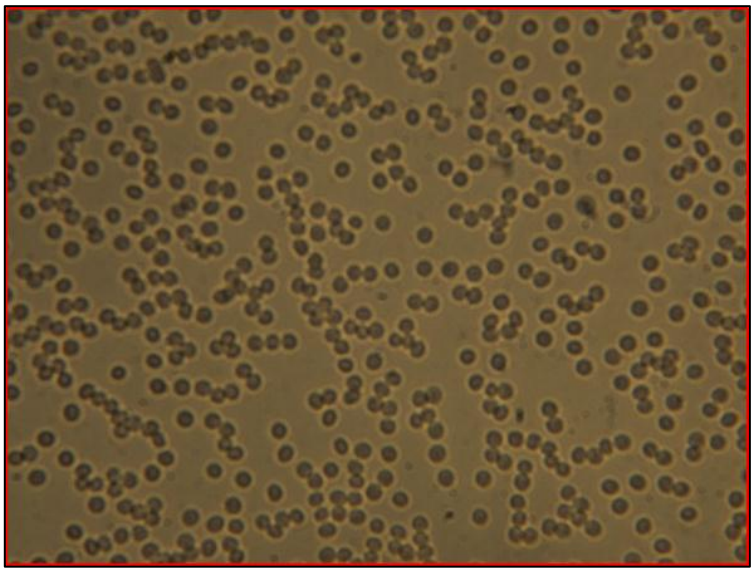


Fig.4.27. The intacted erythrocytes before venom injection.

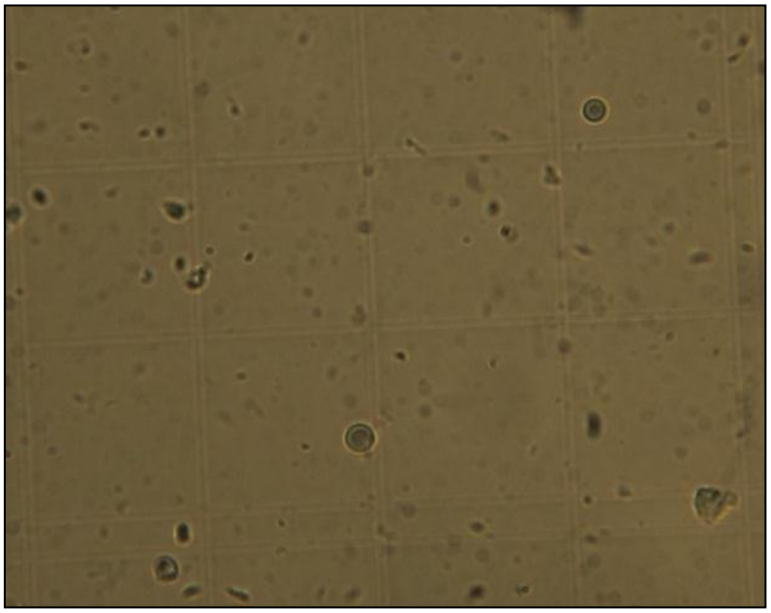


Fig.4.28. Erythrocytes after scat venom incubation. The results were observed with a microscope (Bell, photonic) at 400× magnification and documented using a digital camera (Canon G12 – Japan).

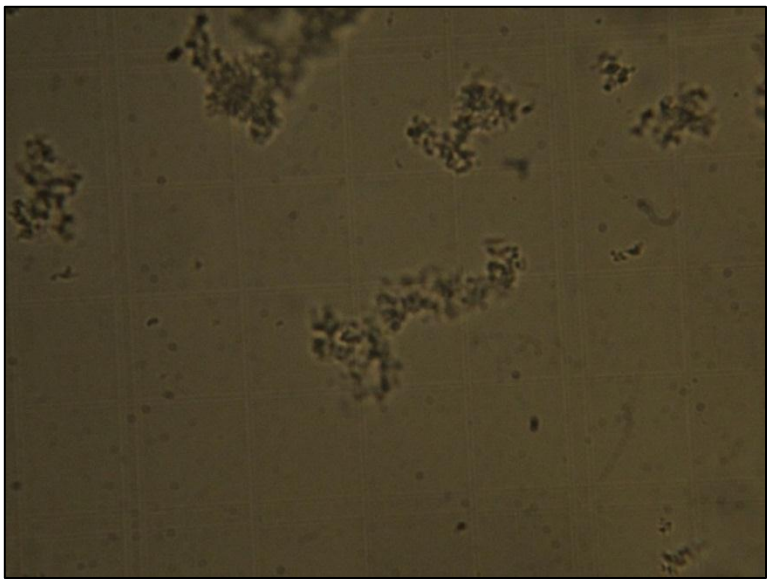


Fig.4.29. Aggregated erythrocytes after Ca+ incubation. It was showed that scat venom did not depend to Ca^{+}.

4.12.4. The weight of hemolytic protein extracted from green scat

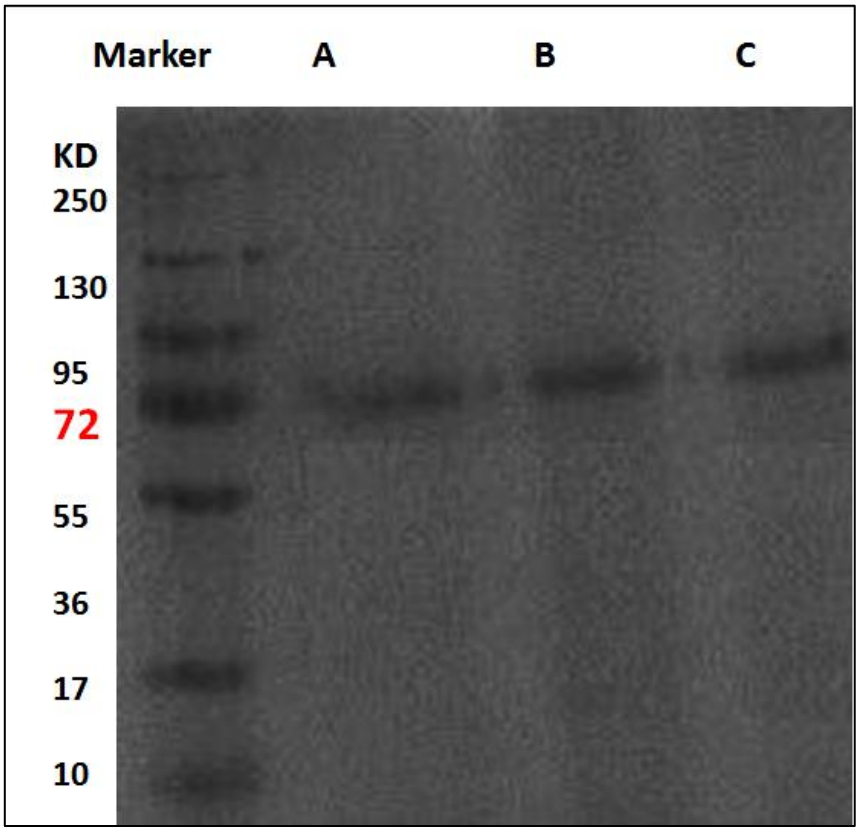


Fig.4.30. Electrophoretic profile of *S. argus argus* venom. The venom was analyzed by SDS-PAGE using 12% polyacrylamide gel and stained with Comassie brilliant blue. Numbers at left corresponds to molecular markers. The apparent molecular masses of the purified proteins were estimated by comparison with a mixture of protein molecular markers (10–250 KDa, Maxell).Three proteins were detached at A=68, B=69 and C=70 minutes from the column were isomers and all revealed 72 KDa.

5. Discussion

Discoveries of toxins and venoms especially from marine resources are racing ahead because of their extremely complex and unique action on various mammalian physiological systems. Venom secretion is part of an organism's defense and predatory mechanisms whose specificity has been extended over a million years of evolution. Scat as a venomous fish has been less considered regarding its toxicity so far. According to data gathered from local medical resources in Iranian southern coastal waters, green scat envenomation induce local paralysis, acute pain, and less significant signs. The patients are treated by immersion of the wounded area in hot water with vinegar (personal observation).

There are some chemical materials used for fish anesthetizing such as MS 222 (Donald & Andrew, 2009), Benzocaine, 2- phenoxyethanol and tricaine methane sulfonate (Donald& Andrew, 2009; Ghanawi *et al*., 2011) and clove oil was used in fish anesthetizing (Ghanawi, *et al*., 2011).

The natural material that used in this study (clove powder= 0.125g/L) was appropriate because of its biosafety for these animals and avoiding possible disadvantages. Clove powder as a natural source has several benefits including rapid desensitizing and disinfection of injured tissue with minimum complications as well as fast recovery of specimens. They anesthetized soon in about 3-4 minutes and recovered in about 10 minutes without any side effects. Cutting the spines just below the base avoid major lesion that led to fast regeneration of dermal tissue. From the point of ethical issues, the examined method for simultaneous anesthetizing and disinfection, cutting the spines, and release of scats in an optimum environment led to surviving them after the operation.

Despite a contemplation regarding too simple venom extraction from the spines of a venomous fish, scat is an exception and hard challenge. There are three different methods for extracting venom from venomous fish basically.

In this study because of anatomical feature of spine, we could not exploit current extraction methods. It could not use rubber sheet method (Endean, 1961) because the spines were very hard, inflexible and impenetrable structure like Needle method (Saunders & Tokes, 1961). A homemade device made for collection of a little amount of liquid venom. After vertical section of spines, no liquid venom was seen in the internal lateral ducts. Therefore, this device was not applicable in this case as well as sponge method as described previously (Carlisle, 1962).

In earlier years, Sivan *et al.,* did some studies on scat in a different extraction manner. They cut the spines with derm and basal tissues 3-5 mm from their base. It is supposed that this method of extraction made impure venom containing derm and basal tissue proteins led to variety of results.

In spite of Sivan 2007, method (decapitation before cutting the spines), the specimens stayed alive in our study. Viability of specimens after removing the spines depend on many factors including the conditions of transportation, the cutting manner, natural source of analgesic material, recovery conditions, and optimum environmental conditions in the pool.

In this study we tried to develop a novel method to improving extraction efficiency regarding quantity and quality. In the acetone extraction the proteins were precipitated with acetone 20% but only high molecular weight were observed in SDS-PAGE. For this reason the acetone protocol was not performed and a new method of extraction with urea was done. The obtained proteins were solubilized in a buffer containing 8 M Urea, and dialysed after refolding.

In contrast to lipoid nature of puffer fish venom and carbohydrate nature of zooantids venom (symbiosis to corals), the SDS-PAGE results confirmed that green scat venom had proteinaceous nature and composed of 12 separate proteins or peptides in the range of 10-250 KDa. Our results agree with Sivan *et al*., (2010) regarding to natural character, although Indian green scat had ranged between 6.5 to 68 KDa.

Biological activity of *S. argus* venom was determined with hemolytic activity on washed human erythrocytes, at the first step. Almost all piscine venoms possess this activity (Sivan *et al*., 2007). The crude venom possessed hemolytic activity in 1% human erythrocytes with an EC50 and EC100, approximately at 0.13 and 1.7 µg and in total the lysis of erythrocytes was not dose dependent. According to Sivan *et al*., (2007), 2.0 mg of *S. argus* venom produced 50% hemolysis of 2% of erythrocytes suspension and 6.4 mg produced 100% hemolysis. The crude venom used for hemolysis test in Sivan study was a homogenate including spines, derm and basal tissues. Based on our experiments hemolytic activity was sensitive to heat. We found that the hemolytic fraction of the venom was independent to Ca^{+} and this ion caused aggregation of the erythrocytes (direct microscopic visual inspection).

Previous investigation on the scat venom, from the spine extraction a haemoragic protein (SA-HT) has been isolated with molecular weight of 18.1 KD. The toxin

produces severe hemorrhage on stomach wall, but is devoid of coutaneous haemorage. SA-HT significantly increases plasma plasmin, serum MDA level and decreases SOD level indicating the possible involvement of cyclooxygenase and lipooxygenase pathway (Karmakar *et al*., 2004; Muhuri *et al*., 2004).

In other study, stone fish as one of the most venomous fish, has been studied before (Lee *et al*., 2004). Among the toxic effects of stonefish venom identified to date, the components causing hemolysis and some other toxic effects and eventual death are known to have a molecular size of about 150KDa(Kreger, 1991) whereas hemolytic protein of scat has molecular size of about 72KDa in our study.

The hemolytic activity of crude venom extract from the greater weeverfish *Trachinus draco* was also sensitive to heat and proteolytic treatment. Rabbit erythrocytes were most sensitive to venom followed by rat erythrocytes. Mouse and cattle erythrocytes were only slightly sensitive, whereas human, chicken and guinea pig erythrocytes were totally resistant (Chhatwal& Dreyer, 1992).

Hemolysis is the most extensively studied activity with scorpionfish venom (Kreger *et al*., 1991) and seems to be an important factor in the venom toxicity of the marine sources. Stonefish venom has been reported to been selectively hemolytic for rabbit erythrocytes and produced very little or no effect on erythrocytes from other sources such as sheep, cow, human etc. (Kreger *et al*, 1991). Hemolytic activities of venoms from stingray, weeverfish, scorpionfish and zebrafish also showed selectively and different potencies for erythrocytes from different sources. A recent study on bullrout venom also showed hemolysis of human erythrocytes (Hahn & O'Connor, 2000).

The *Thalassophryne nattereri* (toadfish) venom also showed strong hemolytic activity, 3.12 mg causing 100% hemolysis of the erythrocytes present in 50 ml of a 3% suspension of these cells. A low level of proteolytic activity (5.47 U/mg) and no detectable phospholipase activity was found (assayed as described by Garnier *et al.,* (1995). *T. nattereri* venom is devoid of PLA2 activity which is usually correlated to edema, hemolysis and necrosis. This suggests that the induced edema, necrosis and hemolysis may follow different mechanisms, yet to be understood.

There are some reports on phospholipase A2 (PLA2) activity in fish venoms including: *Pterios volitans*, *Synanceja trachynis*, *Gymnapistes armoratus*, *T.* nattereri and *Nothesthes robusta* (Hahn & O'Connor, 2000; Sivan, 2007). It is the first report on phospholipase C (PLC) activity in fish venom in our research. In the present study we were able to detect PLC in the crude venom of *S. argus*.

Liberation of phosphorylcholine from lecithin due to the enzymatic activity of phospholipase C, as a lecithinase, induced alkaline pH and led to alteration of absorption spectrum of phenol red to strong red color that can be adsorbed at 550 nm (Harrison *et al*., 2005).

The *S. argus* venom did not make any coagulation when blood plasma was incubated with different doses of venom. It was corroborated Sivan results (2007).

Proteolytic activity was observed in scat venom. According to calculated ratio from the results, the amount of 1 µg venom could degrade 40.65 µg casein. Our findings are comparable well with proteolytic activity in *T. maculosa* (2.0–4.4 U/mg) and stingrays, *P. cf. scobina* and *P. gr. orbignyi* (Sosa-Rosales *et al*., 2005; Magalhaes *et al*., 2006). Mild proteolytic activity has been observed in bullrout, (*N. robusta)* venom (Hahn and O'Connor, 2000).

The molecular mass of the scorpionfish gelatinase estimated by SDS-PAGE was around 80KDa under reducing conditions and 72KDa under non-reducing conditions (Carrijo *et al*., 2005). Some Loxosceles spider venom proteases are suggested to act on basement membranes causing disruption of these structures as well as cytotoxicity on endothelial cells led to the loss of vessel integrity (Silveira, 2002). This proteolytic activity has been recently reported for the venom of the toadfish *Thalassophrine nattereri* that exerts a strong cytolytic effect on platelets and endothelial cells and causes a consequent increase in vascular permeability and hemostatic disturbances (Lopes-Ferreira *et al*., 2002).

The inflammatory response induced by *S. argus* venom in the mice hind paw was studied by measuring paw edema. Our finding showed that the edematic activity remained over 48 h after injection and was dose dependent. Studies on stonefish, toadfish and stingray venoms are known to induce intense and sustained edematic response in mice (Lima *et al*., 2003; Magalhaes *et al.*, 2006).

In marine venom fractionation which was carried out alternatively in the past, the most venomous fishes were considered; such as stonefish, scorpionfish, weeverfish, toadfish, pufferfish and so on.

In general, biology researchers use the chromatography techniques for protein purification. Reversed phase HPLC has a non-polar stationary phase, silica and an aqueous, moderately polar mobile phase. The polarity of acetonitrile is (5.8), it was used as mobile phase in the majority of the investigations. One common stationary phase is which has been surface-modified with a straight chain alkyl group (C18).

With such stationary phase, retention time is longer for molecules which are less polar, while polar molecules elute more readily (early in the analysis).

The purification of the scorpionfish (*Scorpaena plumieri*) venom was followed by SDS PAGE-gelatin. The freshly obtained fish venom extract (24 mg) was initially fractionated on a Sephacryl S-200 column. The gelatinolytic activity was predominantly associated with ascendant part of the second peak (in the Sephacryl column chromatogram); this material corresponded to approximately 34.1% of the protein in the crude venom extract. The material in this peak was further separated on a DEAE-cellulose column developed at pH 7.6 with a stepwise gradient of 0–0.4 M NaCl. The protease activity was associated with the fractions eluted with 0.1 and 0.2 M NaCl. This fraction contained around 11.5% of the protein from the initial material and was used for further purification. This material was further purified by reverse-phase HPLC using ananalytical C4 column, employing a linear gradient of acetonitrile (ACN). A symmetrical protein peak was eluted at a concentration of 40% ACN, and represented about 2% of the total venom protein. This material had gelatinolytic activity and migrated as a single band on an SDS-PAGE gel under reducing or non-reducing conditions, indicating the purified protein was homogeneous (Carrijo *et al*, 2005).

The active crude fish venom of *P. volitans* was applied to sephadex G-50column, equilibrated with 0.1 M phosphate buffer (potassium dihydrogen phosphate and dipotassium hydrogen phosphate pH 7.4). Again the purity was checked by the reverse phase HPLC using C18 column. A single peak with the retention time of 25 min was obtained. Purification was carried out in three batches under same experimental condition (Balasubashini *et al*., 2006).

Stonefish venom was purified using column chromatographies. The purified toxin was found to be an unstable protein that was susceptible to heat and freeze-thawing. This protein showed to have a molecular size of 46KDa. Some disorders such as hemolysis, myocardial injury and necrosis, as well as hyaluronidase activity were reported (Lee *et al*., 2004).

Purification and properties of stonefish venom, *Synanceja verrucosa* was done by Sephadex G-75 and Sephacryl S-200 column chromatographies were carried out isocratically. CM-Sephadex C-50, DEAE-Sephadex A-50 and Butyl Toyopearl column chromatographies were performed by the linear gradient method at 4°C. The fractions were collected by a fraction collector, and the eluates were monitored at 280 nm by a REC-2 recorder. Crude venom from *Hypodytes rubripinnis* and *S. verrucosa* (scorpionfish) were applied to RP-HPLC column C8. Elution was carried

out using 0.1% aqueous TFA and 0.08 % TFA in ACN 0-80 % gradient in 30 min. The flow rate was maintained at 1 ml/min and absorb was monitored at 230 nm (Ambreena, 2002).

The Stonefish venom was initially analyzed using exclusion HPLC, produced three well resolved peaks, but only the one corresponding to the smaller molecular weight contained the biological activity (the Peak marked with retention time 22.7 min). T22.7 was rechromatographed with a reversed phase chlorobutylsilane column. Only one peak contained all the identifiable biological activity. The elution time of the peak was 47.2 min, corresponds to an eluate containing 67.4% CH3CN (Sosa-Rosales *et al*., 2005).

Stonustoxin (SNTX) has been purified to homogeneity by a two-step procedure, using Sephacryl S-200 HR gel permeation and DEAE-Bio-Gel chromatography. High-performance size-exclusion liquid chromatography of SNTX showed a single protein peak with a molecular weight of 148 KDa. As determined by sodium dodecyl sulphate– polyacrylamide gel electrophoresis, SNTX is comprised of two subunits, and, with molecular weights of 71 and 79 KDa, respectively (HoonEng, 2002).

The venom of stonefish (*Synanceia verrucosa*) verrucotoxin was isolated by DEAE and hydroxyapatite chromatography, followed by FPLC gel filtration on Superdex 200 HR 10/30. It was found to be a glycoprotein with a mol. wt of 322,000 +_ 2000, comprising four subunits, 2a (83,000) and 28 (78,000) (Garnier *et al*., 1995).

Fractionation of *T. nattereri* fish venom by either gel filtration or cation exchange chromatography resulted in a few distinct peaks but in both situations the biological activities were located in only one of the peaks which corresponded to basic proteins with approximately Mw=47KDa (Lopes-ferreira,2005).

Tetrodotoxin and its co-existing derivative (the venom extracted from puffer fish),anhydro-tetrodotoxin, were detected by mixing 4 N NaOH with the eluate at a 1:1 ratio through the reaction coil. Fluorescent detection was observed at 505 nm with 381 nm excitation. HPLC analysis were diluted with water and injected intraperitoneally into a group of three mice. HPLC analysis on randomly collected ballonfishes and cultured tiger puffers showed that TTX or its derivative, anhydro-TTX, was not present either in the liver, ovary, and muscle tissues of cultured *Takifugu rubripes rubripes*, or in the muscle sample of wild *Diodon holocanthus*. However, the ovary of *D. holocanthus* showed the presence of both TTX and

anhydro-TTX. It was also determined that the TTX content in the ovaries of balloonfishes was 0.5 μg /g (Chih-Yu Chen& Hong-Nong Chou, 1998).

In puffer fish to achieve the high sensitivity and specificity in TTX monitoring and to avoid the excessive killing of mice, (Yotsu *et al*.,1989) constructed a similar fluorometric TTX analyzer, by combining high performance liquid chromatography (HPLC) and a post column reaction with a hot NaOH solution, to detect tetrodotoxins and its derivatives. Such chemical methodology has resulted in the discovery of TTX-producing bacteria (Yasumoto *et al*., 1986) and the identification of novel TTX analogs from puffers (Yotsu *et al*., 1989).

The size separation of box jellyfish (*Carybdea alata*) venom achieved by Sephadex G-200. Anion exchange chromatography based on quaternary ammonium-bound resin (High Q) provided a high recovery of the hemolytic units present in crude venom. Comparing the hemolytic activity of serial dilutions of crude venom with hemolytically active fractions from G-200 and from anion exchange, the best recovery of hemolytic activity was obtained by using anion exchange chromatography. The resulting final protocol (methods) allowed rapid and selective recovery of most of the hemolytic activity in two peaks eluted from a High exchange chromatography column (Chung *et al*., 2001).

The crude toxin of the starfish *Stellaster equestris* was fractionated stepwise using diethylaminoethyl (DEAE) cellulose column chromatography. The crude toxin and all the adsorbed fractions exhibited potent hemolytic activity on chicken, goat and human blood. However, one group human erythrocytes were resistant to lysis by all fractions and another group by most of the fractions. Paw edema in mice was caused by the crude toxin and all fractions. Pheniramine maleate and piroxicam blocked the toxicity when administered earlier than, or along with, the crude or fractionated toxins but not when administered after the envenomation (Kanagarajan *et al*., 2008).

In our study, HPLC of scat crude venom revealed 30 fractions with maximum optical density of 1500mAU in 80 minutes. From which the protein with 68 retention time (moderately later than other proteins) is less polar (with hemolytic activity).

We found two other fractions with retention time of 69, 70 minutes with a molecular weight of about 72 KDa did not show hemolytic activity that can be assumed as the isomers of hemolytic protein of 68 min.

The elution time of the peak (68 min) corresponds to an eluate at 90% CH3CN. It was observed that hemolysis activity was too fast and immediate (maximum time of action was 30 sec). The percentage of hemolytic fraction showed that the hemolytic protein was 32 percent of total proteins of crude scat venom.

It shows that all of hemolytic proteins have the higher hydrophobisity than other molecules such as scorpion venom (*Hemiscorpius leptourus*) (Borchani *et al*., 2011), furthermore our findings agree with this subject and hemolytic protein in scat venom isolated in 68 retention time (the late of run time).

Enzymes are an important and common component of the venom of many animals including bees, snakes, spiders and scorpions, with several functions probably involved in the toxic action. Many proteases from various snake venoms, especially crotalid venoms, have been isolated and well-studied. They are responsible for some pathological activities triggered by these venoms. In this respect, the venom of fishes is no different, containing various enzymatic activities. There are two subgroups of metalloproteinases: exopeptidases or metalloexopeptidases, endopeptidases or metalloendopeptidases.

Well known metalloendopeptidases include matrix metalloproteinases

(Hu& Beeton, 2010).

Matrix metalloproteinases (MMPs) are zinc endopeptidases composed which belong to a subfamily of the metzincin superfamily. They play important roles in many pathophysiological events including development, organogenesis, angiogenesis, tissue remodeling and destruction, and cancer cell proliferation and progression by degradation of extracellular matrix (ECM) and non-ECM proteins and interaction with various molecules (Imai& Okada, 2008).Other family members of matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases. Collectively, they are capable of degrading all kinds of extracellular matrix proteins, but also can process a number of bioactive molecules. They are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands. MMPs are also thought to play a major role on cell behaviors such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defense. They were first described in vertebrates including humans, but have since been found in invertebrates and plants. They are distinguished from other endopeptidases by their dependence on metal ions as cofactors, their ability to degrade extracellular matrix (Van Lint & Libert, 2007). Matrix metalloproteinases (MMPs) degrade proteins by cleavage of peptide bonds.

MMPs play a critical role in cell invasion, cartilage degradation, tissue remodeling, wound healing, and embryogenesis. They therefore participate in both normal processes and in the pathogenesis of many diseases, such as rheumatoid arthritis, cancer, or chronic obstructive pulmonary disease (Hu& Beeton, 2010).

Aminopeptidases are the enzymes that catalyze the cleavage of amino acids from the amino terminus of protein or peptide substrates. They are widely distributed throughout the animal and plant kingdoms and are found in many subcellular organelles, in cytoplasm, and as membrane components. Aminopeptidases are used in essential cellular functions. Many, but not all, of these peptidases are zinc metalloenzymes some aminopeptidases are monomeric, and others are assemblies of relatively high mass (50 KDa) subunits (Hu& Beeton, 2010).

As we showed in this investigation the hemolytic fraction of scat venom was 72KDa and in this range it should be matrixmetalloprotoases or metalloproteinases.

Accordingly if hemolytic fraction in our study is metalloexoproteinase it is capable of degrading all kinds of extracellular matrix proteins and cleaves of peptide bonds besides can release of apoptotic ligands and thought to play a major role on cell behaviors such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defense so it can play an incredible role as an anti-cancer drug in the future.

Furthermore as we know the PLC is a zinc metallophospholipase requiring zinc for activation. First, the toxin binds to a binding site on the cell surface. The C-terminal C2-like PLAT domain binds calcium and allows the toxin to bind to the phospholipid head-groups on the cell surface. The C-terminal domain enters the phospholipid bilayer. The N-terminal domain has phospholipase activity. This property allows hydrolysis of phospholipids such as phosphatidyl choline, mimicking endogenous phospholipase C. The hydrolysis of phosphatidyl choline produces diacylglycerol which activates a variety of second messenger pathway sand several intercellular adhesion molecules. These actions combine to cause edema due to increased vascular permeability (Vollmar *et al*., 2011).

In this respect, it was suggest that the hemolytic protein of scat venom as a PLC is a zinc metallophospholipase. In conclusion our studies revealed that *S. argus* venom isolated from Persian Gulf contains hemolytic, edematic, PLC and proteolytic activities. The entity of these activities in our experimental animals almost resembled those of other fish venoms previously studied. The present study suggests that phospholipase C may be the major toxin for hemolysis in scat venom.

It was preferred that this fraction is candidate for TPA because of efficacious of hemolytic activity. It was observed that the 68^{th} fraction (hemolytic) with molecular weight 72KDa acts on the erythrocytes right away and blasts them (visual inspection).

6. Future prospects

Venomous animals have evolved a vast array of peptide toxins for prey capture and defense. These peptides are directed against a wide variety of pharmacological targets, making them an invaluable source of ligands for studying the properties of these targets in different experimental paradigms.

As a consequence of their high selectivity, venom peptides have proved articularly useful for in vitro and in vivo proof-of-concept studies. However, for therapeutic applications, a number of issues associated with safety, pharmacokinetics and delivery need to be addressed. Optimization of peptide delivery to peripheral and central targets will help to determine whether or not these peptides can be considered candidates for drug development. Peptides that block channels by altering the gating mechanism might have potential to become selective potassium-channel inhibitors, whereas pore blocking toxins could be designed to be selective inhibitors of subtypes of sodium channels. It remains to be determined how many of the peptides that are present in venoms can find a clinical utility.

Probable metalloprotease in scat venom maybe play anti-cancer responsibility. It is suggested to do more works on these aspects in the future. More over the immediately effect of hemolysis activity of scat venom on human erythrocytes proposed this protein for TPA drugs and blood vessels disclosing. As mentioned above, animal venoms may be seen as vast combinatory initial libraries of bioactive molecules and as such, represent an enormous opportunity for drug discovery.

Although marine envenomations are less than terrestrials in Iran, health care professionals should be prepared to treat delayed presentations of fish and coelenterate stings, and acute presentations of the fish stings.

Appendix 1:

Unsuccessful venom extraction techniques

Three methods of venom extraction, which did not involve the killing of the animal, but merely "milking" it of its venom, were attempted without success. The methods used were modifications of various live animal venom extraction methods, used successfully by other researchers with species of venomous fishes other than the scat.

A) Rubber Sheet Method

The rubber sheet method, for live animal venom extraction, was developed by Endean (1961) who studied the venom of the stonefish, *Synanceja trachynis*. His technique involved pulling a rectangular rubber sheet (2 mm thick) down over each individual dorsal spine. The rubber sheet was easily pierced by the rather strong, sharp dorsal spines of this species, and the sheet was pushed towards the proximal end of each spine. Stonefish venom was "violently expelled" from the twin venom ducts after the surrounding integumentary sheath had first constricted and then stretched the venom glands. Approximately 0.1 ml of crude venom was obtained in this manner, from each spine, which was then easily pooled and collected. Endean found that this species of stonefish could regenerate its venom glandular contents in three week time, demonstrating that some venomous fishes could indeed be "milked" of their venom without harm. Cameron and Endean (1966) also used this technique successfully with the scorpionfish (bullrout), *Notesthes robusta*. A modification of this method was attempted with the lionfish in a study. Rubber sheets similar to those used by Endean, dental dam material, prophylactic rubber material, and parafilm were all used in an attempt to extract the lionfish venom. None of these materials, however, were able to be gently pierce by the extremely thin, and somewhat fragile, dorsal spines of the lionfish without causing substantial damage to the animals. Considerable contamination from various adjacent cellular constituents was also present in the resultant extracts. Additionally, the venom gland morphology of the lionfish is considerably different from that of the stonefish. Lionfishes do not possess venom ducts as do the stonefish, which may help to explain why this method of venom expulsion was not successful.

B) Hypodermic Needle Method

This method of venom extraction, developed by Saunders and Tokes (1961), involved puncturing the bilateral venom sacs (two per spine) of the stonefish,

Synanceja horrida. After puncturing the sacs, the free-flowing venom could be easily collected into vials. Saunders and Tokes were able to obtain approximately 0.2 ml of crude venom per fish, and regeneration of the sacs was possible.

This method was also attempted with the lionfish, but resulted in extreme cellular contamination, and no toxic activity in the crude extracts. This method also severely damaged the animals.

C) Sponge Method

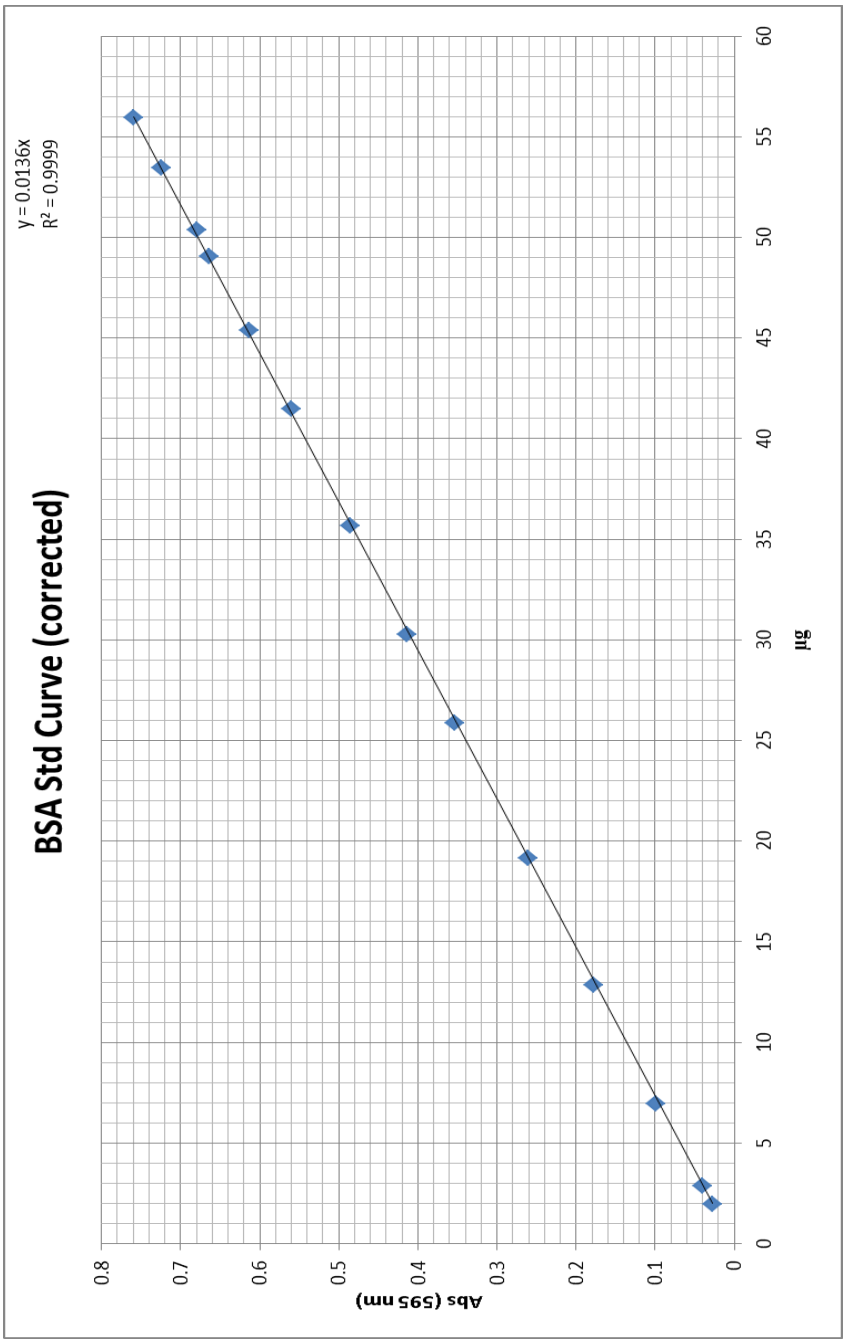
The sponge method of Carlisle (1962) involved placing a weeverfish (*Trachynus vipera)* in a small tank of seawater, and then provoking it with a small piece (6×6×6 mm) of polyurethane sponge, held in forceps, until the animal elicited its defense response and stabbed the sponge. After the stabbing, the sponge was held against the fish for 15 seconds, and then removed and washed three times with 1.0 ml volumes of distilled water.

Carlisle found this method to give an adequate yield in the weeverfish, and venom gland regeneration was apparent in as little as three days.

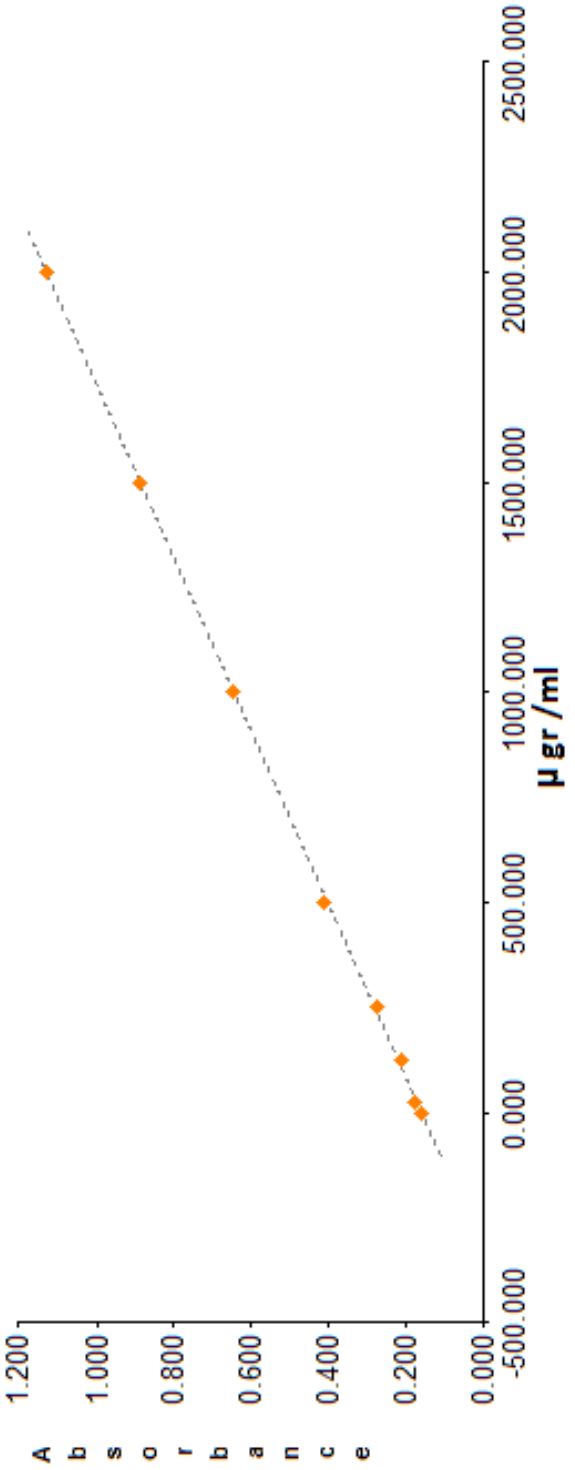
This method of venom extraction was also attempted with the lionfish, unsuccessfully. Again, due to the morphology of the lionfish venom apparatus, and the diffuse nature of the venom glands, this method resulted in a high degree of contamination from extraneous tissues, dilution, and the resultant extract possessed no activity.

It was after these multiple unsuccessful attempts that the present method of venom extraction, via direct venom apparatus removal and homogenization, was chosen for lionfish venom extraction.

Appendix 2: BSA standard curve (Bradford method)



Appendix 3: Standard curve (BCA method)



Appendix 4: Bradford solution

Preparation of 5X Bradford Reagent

For the preparation of the Bradford Dye (concentration 5X) we need the following chemicals in the described concentrations:

1) Coomassie Brilliant Blue G250	0.05% (w/v)
2) Ethanol	25 % (v/v)
3) Phosphoric acid	42.5 % (v/v)

Make up the final desired volume with MilliQ and filter the solution through Whatman Filter Paper No. 2. Store the reagent in an "Amber Bottle" and keep it in 4^{o} C (preferably, as such the reagent is also stable at RT). An aliquot for use can be kept at RT.

Precaution:

Make sure that the reagent is not exposed to light for too long. So you can keep the aliquot in the culture vial wrapped in aluminum foil.

Make sure that you measure the OD of the protein complex as soon as possible, as the protein complex coagulates as the time pass by.

Appendix 5: BCA solution

A stock BCA solution contains the following ingredients in a highly alkaline solution with a pH 11.25:

Bicinchoninic acid

Sodium carbonate

Sodium bicarbonate

Sodium tartrate

Cupric sulfate pentahydrate

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چکیده

اسکات خالدار *Scatophagus argus argus* یک ماهی آکواریومی متعلق به خانواده Scatophagidae است که می تواند زخم های دردناک و فلج موضعی در اندام آسیب دیده دراثر تماس با خارهایش ایجاد کند. خارهای سخت پشتی و شکمی این ماهی شامل سلول هایی هستند که زهر تولید می کنند و فعالیت های سمی دارند.

بر طبق اطلاعات منتشر نشده جمع آوری شده از بیمارستان های محلی در مناطق ساحلی جنوب ایران، اسکات خالدار بعنوان یک ماهی سمی گزارش شده است . مسمومیت عوارض کلینیکی مانند درد موضعی ، فلج نسبی ، بی نظمی ضربان قلب و خارش ایجاد می کند. در مطالعه حاضر82 عدد اسکات سبز (خالدار) از آبهای ساحلی خلیج فارس جمع آوری شد. SDS_PAGE از زهرخام 12 باند مختلف در اندازه 10-250 کیلودالتون تشخیص داد.

1/7 میکروگرم از زهر خام در خون 1٪ (گلبول قرمز) انسان ٪100 همولیز ایجاد نمود(LC). زهر خام 813 میکرو گرم پروتئین از کازئین ٪ 0/5 جدا کرد. فعالیت فسفولیپاز C در غلظت 3/125 میکروگرم زهر خام ثبت گردید. یافته های ما نشان داد که فعالیت ادم بیشتر از 48 ساعت بعد از تزریق باقی ماند. خالص سازی زهر خام با HPLC انجام شد و 30 پیک در طی 80 دقیقه بدست آمد ولی تنها پیک جدا شده در دقیقه 68 فعالیت همولیتیک نشان دادکه در استونیتریل ٪ 90 جدا شد. پروتئین همولیتیک شامل٪ 32 از مجموع پروتئین های زهرخام بود و وزن مولکولی آن 72 کیلودالتون نشان داده شد. نتایج آشکار ساخت که زهر خام استخراج شده از اسکات سبز ایرانی دارای خواص آنزیمی و سمی متفاوت است.

واژه های کلیدی:

Scatophagus argus argus،همولیز ،HPLC ، فعالیت همولیتیک،LC ، فسفولیپاز C.

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عنوان:

مطالعات سم شناسی(ونومیکس) و زیستی (بیولوژیک) زهر ماهی اسکات خالدار خلیج فارس، *Scatophagus argus argus* استخراج و خالص سازی پروتئین همولیتیک آن

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